The Effects of 3TSR and Combinational Metronomic Chemotherapy on Epithelial Ovarian Cancer

by

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ABSTRACT

THE EFFECTS OF 3TSR AND COMBINATIONAL METRONOMIC CHEMOTHERAPY ON EPITHELIAL OVARIAN CANCER

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Epithelial ovarian cancer (EOC) is the most lethal gynecological cancer in women. The purpose of this study was to identify the effects of 3TSR and combinational metronomic chemotherapy as a novel treatment for advanced stage disease. We hypothesized that due to its ability to normalize tumor vasculature and increase tissue perfusion, 3TSR will increase tissue uptake of chemotherapy drugs and synergize with metronomic chemotherapy delivery to induce disease regression. Using an orthotopic syngeneic mouse model of EOC, 3TSR and metronomic chemotherapy decreased primary tumor size and reduced secondary abdominal disease, resulting in a significant survival advantage. In addition, 3TSR and combinational metronomic chemotherapy reduced blood vessel density in primary tumors and up regulated the expression of apoptotic factors in tumor tissue. The results from this study suggest that 3TSR in combination with metronomic chemotherapy has significant effects on tumor burden and may be a possible clinical treatment for EOC.
ACKNOWLEDGEMENTS

As John F. Kennedy once said, “We must find time to stop and thank the people who make a difference in our lives.” Therefore, I would like to take this opportunity to acknowledge the people who have guided and supported me throughout my research.

First and foremost, I would like to thank my supervisor, Dr. Jim Petrik, for giving me the opportunity to join his lab and pursue an exciting research project. Your patience, enthusiasm and leadership played a large role in my success and I am grateful for your mentorship.

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Finally, I would like to acknowledge all of the mice that participated in my study. Without their sacrifice, my research would not have been possible.
DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in the body of this thesis was performed by me.

Dr. James Greenaway assisted in the preparation of ID8 cells and Dr. Jim Petrik performed tumor-initiating surgeries in the orthotopic syngeneic mouse model. Jacqueline Dynes, Dr. James Greenaway, Dr. Jim Petrik and Adam Agate assisted with some intraperitoneal injections. Dr. Jim Petrik assisted with tissue collections at the 90 day time point. Helen Coates sectioned the formalin-fixed, paraffin-embedded primary tumors collected at 90 days for immunohistochemical analysis. Dr. Jim Petrik performed and analyzed the immunohistochemical staining of CD31 and executed statistical analysis for the Kaplan-Meier survival curve.

ID8 cells were a kind gift from Drs. Katherine Roby and Paul Terranova at the University of Kansas. 3TSR was a kind gift from Dr. Jack Lawler of the Beth Israel Deaconess Medical Center at Harvard Medical School in Boston, Massachusetts.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ABAM</td>
<td>Antibiotic-antimycotic</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Ang</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma gene-2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD31</td>
<td>Cluster of differentiation 31</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>CD47</td>
<td>Cluster of differentiation 47</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagles medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EOC</td>
<td>Epithelial ovarian cancer</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ETOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FasL</td>
<td>Ligand of the fas death receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Flk-1</td>
<td>Fetal liver kinase 1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrocloric acid</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo daltons</td>
</tr>
<tr>
<td>LLC</td>
<td>Lewis lung carcinoma</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/Erk Kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Metronomic chemotherapy</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MTD</td>
<td>Max-tolerated dose</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NaV</td>
<td>Sodium orthovanadate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PI3 Kinase</td>
<td>Phosphatidyl inositol kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfony fluoride</td>
</tr>
<tr>
<td>rAAV</td>
<td>Recombinant adeno-associated virus</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse osmosis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF</td>
<td>Transformin growth factor</td>
</tr>
<tr>
<td>TIE</td>
<td>TEK receptor tyrosine kinase</td>
</tr>
<tr>
<td>TSP-1</td>
<td>Thrombospondin-1</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP nick end labeling</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular endothelial growth factor receptor 2</td>
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</table>
INTRODUCTION

Ovarian cancer is the most lethal gynecological cancer in women and accounts for approximately 5 percent of cancer deaths throughout Canada (Canadian Cancer Statistics, 2013). Ninety percent of ovarian cancer cases are classified as epithelial ovarian cancer (EOC) (Harries and Gore, 2002). Due to the late onset of vague symptoms and a lack of reliable screening techniques, EOC is not generally detected until it has progressed into stage III or IV. When detected early, the five-year survival rate is quite high, however, when detected in the more advanced stages five-year survival rates drops to less than 50 percent (Harries and Gore, 2002). Until a reliable biomarker is discovered, more effective treatments are needed in order to improve the survival and overall quality of life of women suffering with late stage disease.

When EOC is detected, standard treatment typically begins with surgical debulking. This cytoreductive surgery removes the large primary tumor as well as visible and accessible macroscopic secondary lesions throughout the abdominal cavity (Ledermann et al., 2013). Following surgery, patients are commonly treated with adjuvant combinational chemotherapy. Front-line chemotherapy regimens generally include carboplatin, a platinum-based cytotoxic agent, and paclitaxel, a microtubule inhibitor, administered intravenously (IV) once every three weeks for approximately six cycles (Ledermann et al., 2013). Unfortunately, even with optimal debulking surgery and chemotherapy treatment, approximately 70 percent of women will relapse within the first three years (Ledermann et al., 2013). Upon relapse, EOC generally rebounds in a chemoresistant state, which results in a very poor prognosis of less than 12 months (Ledermann et al., 2013). Clearly there is a desperate need for more efficient EOC treatments.

Angiogenesis is the formation of new blood vessels from pre-existing vasculature and is a necessary process for the growth and metastasis of ovarian tumors. Inhibiting this process
through anti-angiogenic therapy offers a promising alternative to standard EOC treatments. There are a variety of anti-angiogenic agents currently under review. However, one particular anti-angiogenic molecule holds promise. Thrombospondin-1 (TSP-1) is an endogenously produced anti-angiogenic molecule and has demonstrated the potential to induce regression of advanced stage ovarian tumors in mice (Greenaway et al., 2009). Thrombospondin-1, however, is a very large molecule, which makes its use as a therapeutic agent in its native state difficult. Previous studies have demonstrated that the majority of the anti-angiogenic effects of TSP-1 are mediated by the type-1 repeat region (Iruela-Arispe et al., 1999, Tolsma et al., 1993). By isolating the three type-1 repeats, one can sequester the majority of TSP-1’s anti-angiogenic effects in a smaller, more clinically relevant peptide known as 3TSR. 3TSR induces death of vascular endothelial cells from immature, dysfunctional tumor vessels, which results in vascular normalization, enhanced tumor perfusion and reduced tumor hypoxia (Petrik, unpublished). We hypothesized that this normalized vasculature and enhanced tissue perfusion can be exploited to more efficiently deliver drugs to the tumor. Therefore, 3TSR used in combination with chemotherapy may provide a unique opportunity to treat advanced stage EOC.
Ovarian Cancer

Ovarian cancer is the most lethal gynecological malignancy in women and is responsible for 4.7 percent of all cancer deaths in Canada (Canadian Cancer Statistics, 2013). In 2013, ovarian cancer caused an estimated 1,700 deaths Canada-wide, approximately 670 of which occurred in Ontario alone (Canadian Cancer Statistics, 2013). There are three different types of ovarian cancer: germ cell, stromal and epithelial. Ninety percent of ovarian cancer cases are classified as epithelial ovarian cancer (EOC) (Harries and Gore, 2002). Due to late detection of the disease, exact origin of EOC has not been specified, however, there are currently many different theories.

The incessant ovulation hypothesis was published in 1971 (Fathalla, 1971). Fathalla observed a high rate of metastatic ovarian cancer in hens that were forced to continuously lay eggs without any cessations in the ovulatory cycle (Fathalla, 1971). It was proposed that the release of eggs during ovulation caused damage to the ovarian surface epithelium, thereby leading to the formation of inclusion cysts and subsequently the origination of EOC (Fathalla, 1971). This theory is further supported in humans. The risk of developing EOC greatly decreases in women who undergo fewer ovulatory cycles by either utilizing oral contraceptives or undergoing multiple pregnancies (Soegaard et al., 2007). In 2003, however, a new theory was proposed. Ovarian inclusion cysts were found to contain both serous and ciliated cells, which happen to be the two cell types that make up the Fallopian tube (Piek et al., 2003). Therefore, Piek and associates hypothesized that EOC does not originate from the ovarian surface epithelium, but from the Fallopian tube (Piek et al., 2003). Discovering an accurate mechanism of origin is important in order to improve future disease prevention.
Despite a lack of clarity on the exact mechanism of initiation, EOC primarily originates in the ovaries which are oval-shaped organs that are located on the lateral pelvic walls (Heintz et al., 2006). At stage I, the disease is confined to the ovaries (Prat, 2013). Treatment at this stage is less invasive and generally more successful, compared to treatments initiated at later stages of disease. In fact, women who are diagnosed at stage I have a five-year survival rate of approximately 90 percent and most do not have to undergo adjuvant chemotherapy (Vergote et al., 2001). By stage II, the disease has spread from the ovary, but remains within the pelvic cavity (Prat, 2013). Stage II has the lowest prevalence and makes up less than 10 percent of diagnosed ovarian cancer cases (Prat, 2013). The majority of patients are diagnosed at stage III, where the disease has metastasized throughout the abdominal cavity. Common sites of metastasis include the omentum, peritoneal surface of the liver and spleen, as well as the diaphragm and intestinal surfaces (Prat, 2013). Two-thirds of women diagnosed at stage III develop ascites, which is an accumulation of fluid in the peritoneal cavity (Prat, 2013). It is this abdominal involvement and abdomen distention that commonly leads women to seek medical assistance and a diagnosis is made at this stage. Finally, approximately 12 percent of EOC cases are diagnosed at stage IV where the disease has metastasized beyond the abdominal cavity to distant sites of the body (Heintz et al., 2006; Prat, 2013). A complete description of the International Federation of Gynecology and Obstetrics ovarian cancer staging is presented in Table 1 (Heintz et al., 2006).
Table 1: Federation of Gynecology and Obstetrics staging of EOC

<table>
<thead>
<tr>
<th>Stage</th>
<th>Disease</th>
<th>Details</th>
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<tr>
<td>I</td>
<td>I</td>
<td>Disease confined to ovaries</td>
</tr>
<tr>
<td>IA</td>
<td>Disease limited to one ovary and ovarian capsule is intact, therefore, no tumor on ovarian surface and no malignant cells present in ascites</td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td>Disease present in both ovaries, but both ovarian capsules are intact</td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td>Disease present in both ovaries and one of the following has occurred: ruptured ovarian capsule, tumor present on ovarian surface or malignant cells present in ascites</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Disease has spread from ovaries to the pelvic cavity</td>
<td></td>
</tr>
<tr>
<td>IIA</td>
<td>Disease has metastasized to uterus or fallopian tubes</td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td>Disease is present on other pelvic tissue</td>
<td></td>
</tr>
<tr>
<td>IIC</td>
<td>Disease spread throughout pelvic cavity and malignant cells are present in ascites</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Disease has spread from ovaries and pelvic cavity to abdominal cavity and near-by lymph nodes</td>
<td></td>
</tr>
<tr>
<td>IIIA</td>
<td>Microscopic metastases present in peritoneal cavity</td>
<td></td>
</tr>
<tr>
<td>IIIB</td>
<td>Macroscopic metastases of 2cm or less are found within the peritoneal cavity</td>
<td></td>
</tr>
<tr>
<td>IIIC</td>
<td>Macroscopic metastases of 2cm found within peritoneal cavity and disease has spread to regional lymph nodes</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Disease involves both ovaries and has spread beyond the abdominal cavity to distant sites of the body.</td>
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Adapted from (Heintz et al., 2006)

Ovarian cancer is typically classified based on the cellular compartment from which neoplasia initiates. The ovary is made up of three different types of cells: epithelial cells, germ cells and stromal cells. Therefore, there are three major categories of ovarian cancer depending on the cell of origin. Epithelial ovarian tumors are most common, occurring in approximately 90 percent of ovarian cancers, whereas germ cell and stromal cell account for 10 percent (Harris and Gore, 2002). EOC can be further divided into at least five classifications: high-grade serous, low-grade serous, endometrioid, clear-cell and mucinous carcinomas (Lee et al., 2003).

Approximately 75 percent of EOC cases are classified as serous tumors and are categorized by differentiated cells expressing fallopian tube cellular characteristics (Lee et al.,
Furthermore, serous tumors are graded on a 2-tier system, dividing them into either high-grade or low-grade serous carcinomas (Vang et al., 2009). In general, low-grade nuclei and scarce mitotic figures represent low-grade serous carcinomas, while high-grade nuclei and abundant mitotic figures signify high-grade serous carcinomas (Vang et al., 2009). Due to these characteristics, low-grade serous tumors are believed to have a better treatment outcome than high-grade tumors (Vang et al., 2009).

Endometrioid, clear-cell and mucinous carcinomas account for approximately 10 percent, 10 percent and 3 percent of EOC cases respectively (Lee et al., 2003). Endometrioid ovarian tumors closely resemble endometrial cancers. The expression of predominantly clear or hobnail cell types characterize clear-cell tumors (Lee et al., 2003). Finally, mucinous ovarian carcinomas contain goblet cells randomly distributed throughout the tumor. This means that some cells express intracytoplasmic mucin in an otherwise non-mucinous epithelium (Lee et al., 2003). According to the International Federation of Gynecology and Obstetrics, the various ovarian cancer histotypes respond differently to specific treatments, therefore, accurate classification impacts treatment efficacy (Prat, 2013).

Due to vague, non-specific symptoms and a lack of reliable screening techniques, over 70 percent of ovarian cancer cases are diagnosed in stage III or stage IV (Goff et al., 2007). At these advanced stages, the five-year survival rate can be as low as 20 percent, thereby giving ovarian cancer its name, the silent killer (Goff et al., 2007). Until there are more efficient biomarkers and screening techniques available to the public, EOC patients are in desperate need of more effective treatments for advanced stage disease.

**Angiogenesis**
Angiogenesis is the formation of new blood vessels from pre-existing vasculature (Shojaei, 2012). It is a stable process that occurs in normal physiological activities such as wound healing, female menstrual cycles and embryological development (Shojaei, 2012, Risau and Flamme, 1995, Engerman et al., 1967). In addition, angiogenesis is utilized in many pathological processes, such as tumor growth and metastasis. Currently, there are two major types of angiogenesis: intussusceptive angiogenesis and sprouting angiogenesis. Because intussusceptive angiogenesis does not necessarily rely on cellular proliferation, it is a fast process occurring in a matter of hours (Burri et al., 2004). Intussusceptive growth plays a large role in the expansion of existing capillary beds, such as those in developing organs (Caduff et al., 1986, Burri et al., 2004). In contrast, sprouting angiogenesis tends to have invasive properties, thereby allowing capillaries to migrate through areas lacking in vasculature (Burri et al., 2004). This mechanism of angiogenesis relies heavily on the proliferation of endothelial cells and is, therefore, a relatively slow process in comparison to intussusceptive (Burri et al., 2004).

In 1986, while studying the microvasculature of developing rats, Caduff and associates observed the formation of blood vessels “in-itself”, otherwise known as intussusceptive growth (Caduff et al., 1986). In order for intussusceptive angiogenesis to occur, columns of intravascular tissue, or transcapillary pillars, must form within the capillaries (Burri and Tarek, 1990). Burri and associates observed four different morphologies of the transcapillary pillars and, therefore, concluded that they represented four different stages of pillar development (Burri and Tarek, 1990). The first step of transcapillary pillar formation is the creation of an interendothelial transluminal bridge (Burri et al., 2004). This is where endothelial cells from opposite sides of a capillary come into contact, producing a bridge that is approximately one micrometer in diameter (Burri et al., 2004). During stage II, the bilayer is perforated and in stage
III interstitial tissue and pericytes invade into the bridge (Djonov et al., 2003). By stage III, the diameter of the transcapillary pillars has increased to approximately 2.5 micrometers due to invading tissue (Djonov et al., 2003). Finally, stage IV occurs when the pillars of interstitial tissue increase in thickness, ultimately splitting one capillary into two (Djonov et al., 2003).

Intussusceptive angiogenesis is believed to play an important role in the growth and expansion of capillary beds (Djonov et al., 2003). One of the major factors that initiate intussusceptive growth is shear stress (Djonov et al., 2003). For example, in a chicken embryonic vascular bed, changes in hemodynamics, such as an increase in local blood flow and therefore shear stress, triggers transcapillary pillar formation in a matter of minutes (Djonov et al., 2002). Because shear stress is dependent on vessel diameter, formation of a pillar acts as a mechanism of decreasing stress by cutting vessel diameter in half (Djonov et al., 2003). Intussusceptive angiogenesis requires an established vasculature system with blood flow, therefore, it is difficult to study this method in vitro.

Sprouting angiogenesis is the most extensively studied angiogenic type and occurs in pathophysiological processes, such as tumor vessel formation. In order for sprouting angiogenesis to occur, blood vessels must first become dilated and permeable. Nitric oxide is an endogenous promoter of vasodilation. In addition, vascular endothelial growth factor (VEGF) is widely known to induce vascular permeability. Src family kinases may play an important role in the promotion of VEGF-induced vascular permeability (Eliceiri et al., 1999). Once the vessels are dilated and permeable, the endothelial cells must become destabilized and a path must be cleared for cellular migration.

Angiopoietin-1 (Ang1) is an endogenous inhibitor of angiogenesis. It binds to the TIE-2 receptor located on endothelial cells and stabilizes the vasculature (Suri et al., 1996).
Angiopoietin-2 (Ang2), however, is an Ang1 antagonist and therefore prevents Ang1’s stabilizing effects (Maisonpierre et al., 1997). Ang2 is highly expressed in areas undergoing vascular remodeling, suggesting that it plays an important role in the destabilization of endothelial cells during angiogenesis (Maisonpierre et al., 1997). In addition, matrix metalloproteinases (MMPs) have many pro-angiogenic effects. MMPs are most widely known for their activity in degrading the surrounding extracellular matrix (ECM) in order to allow for migration and invasion of endothelial cells. However, they partake in many other activities as well. For example, MMP-7 is a promoter of endothelial cell proliferation (Huo et al., 2002). Moreover, some MMPs cleave vascular endothelial cadherin, thereby destabilizing endothelial cell-to-cell adhesion (Herren et al., 1998). Once MMPs have cleared the way for cellular migration, endothelial cells undergo proliferation and journey towards the source of pro-angiogenic factors.

According to current literature, VEGF is the most potent regulator of angiogenesis (Kieran et al., 2012). VEGF is a homodimeric glycoprotein that was purified from pituitary follicular cells in 1989 (Ferrara and Henzel, 1989). Curiously, this same structure was identified five years earlier under the name Vascular Permeability Factor (Senger et al., 1983). Many isoforms of VEGF bind to the tyrosine kinase receptor known as VEGFR2 or Flk-1. VEGFR2 is expressed primarily on vascular endothelial cells and, therefore, VEGF predominantly affects blood vessels. Once VEGF binds to its receptor, VEGFR2 dimerizes and the tyrosine kinase receptor becomes autophosphorylated. This results in a signaling cascade that promotes angiogenesis.

VEGF promotes endothelial cell proliferation, survival and migration through many different signaling pathways. Two pathways are believed to be responsible for VEGF induced
cellular proliferation and these are the Ras-Raf-MEK-ERK and mitogen activated protein kinase (MAPK) pathways (Kroll and Waltenberger, 1997, Byrne et al., 2005). The phosphatidylinositol kinase (PI3-Kinase) pathway is thought to be responsible for endothelial cell survival (Gerber et al., 1998). Finally, focal adhesion kinase, PI3 Kinase/Akt and p38/MAPK pathways are all implicated in cellular migration (Qi and Claesson-Welsh, 2001, Rousseau et al., 1997). VEGF is also known to enhance vessel permeability and it is thought to be this enhanced “leakiness” that results in fluid extravasation and ascites accumulation in the abdomen in ovarian cancer patients (Hu et al., 2005, Hu et al., 2005).

Fibroblast growth factor (FGF) is another mediator of angiogenesis (Friesel and Maciag, 1995). Although there are approximately nine different isoforms in the FGF family, FGF-1 and FGF-2 are speculated to stimulate neovascularization (Friesel and Maciag, 1995). FGF-1 is classified as a pro-angiogenic molecule due to the fact that its secretion results in the proliferation of arterial intima and the initiation of angiogenesis in vivo (Nabel et al., 1993). FGF-2, on the other hand, is speculated to be a powerful mitogenic factor in vascular smooth muscle (Linder et al., 1991).

Platelet-derived growth factor (PDGF) is another pro-angiogenic factor involved in sprouting angiogenesis (Friesel and Maciag, 1995). PDGF receptors are found on activated endothelial cells, suggesting that PDGF plays a role in angiogenesis (Risau et al., 1992). In addition, PDGF has been shown to stimulate endothelial cell proliferation as well as induce the formation of endothelial tubes (Battegay et al., 1994).

Once the endothelial cells have migrated and arranged into a cord, the lumen begins to form. Many isoforms of VEGF have been linked to luminal formation. Specifically, VEGF$_{121}$ and VEGF$_{165}$ have been found to enhance both lumen formation and increase vessel length
(Carmeliet, 2000). Finally, pericytes are recruited to the newly formed vessel, resulting in a mature capillary.

**Angiogenesis in Cancer**

The relationship between blood vessels and tumor growth has been examined dating as far back as Franz Boll in 1876 (Boll, 1876). Boll, a German researcher, believed that vasculature plays a vital role in cancer (Boll, 1876). Since Boll’s hypothesis in 1876, theories in this field have grown and developed throughout the ages to become one of the hottest topics in cancer research today.

In 1945, Algire and associates studied the difference between blood vessel formation in skin laceration wound healing versus solid tumor growth (Algire et al., 1945). According to his study, capillary formation began approximately four to five days after injury in physiologically normal tissue. Moreover, these capillaries ultimately made up 25 percent of tissue volume in the wound (Algire et al., 1945). On the other hand, tumor capillaries formed more quickly and began to sprout on day three. In addition, blood vessels made up approximately 50 percent of the tumor volume (Algire et al., 1945). This discovery documented that during tumor formation, angiogenesis occurs more rapidly and to a greater extent than during physiologically normal processes, such as wound healing.

In 1971, Judah Folkman published a theory that revolutionized the industry of cancer research and created a strong foundation for future angiogenic studies. Folkman passionately believed that tumor growth depends on angiogenesis and hypothesized that tumors must recruit their own blood supply in order to grow beyond two to three millimeters cubed in size (Folkman, 1971). Oxygen can only diffuse approximately 150 micrometers from a capillary (Brown and Giaccia, 1998). Therefore, a tumor cell must be located within this radius in order to receive
enough oxygen and nutrients to survive (Brown and Giaccia, 1998). Cells that are located outside of this radius will lack sufficient oxygen and become hypoxic, eventually undergoing apoptosis or necrosis. Because of this, solid tumor size is limited by its access to blood supply and thus by the amount of oxygen that can diffuse from pre-existing capillaries. Angiogenesis allows tumors to meet their growing metabolic needs by recruiting their own blood supply through the sprouting of new capillaries from pre-existing ones.

In order for tumors to recruit their own blood supply, they must first flip the angiogenic switch in favor of blood vessel formation. Under normal conditions, there is a precise balance between pro-angiogenic and anti-angiogenic factors so that endothelial cells remain senescent and blood vessel formation is highly controlled. Tumors that become more aggressive must undergo angiogenesis and, therefore, must tilt the angiogenic switch in their favor by increasing the production of pro-angiogenic factors and decreasing the production of anti-angiogenic factors (Baeriswyl and Christofori, 2009).

Angiogenesis is promoted in solid tumors predominantly through the overexpression of VEGF. Tumor cells up regulate the expression of VEGF through various mechanisms. Firstly, VEGF expression is a direct result of tissue hypoxia. When a solid tumor is under hypoxic conditions, hypoxia inducible factor (HIF)-1α will heterodimerize with HIF-1β and translocate into the nucleus to act as a VEGF transcription factor (Ho and Kuo, 2007). Secondly, malfunctioning oncogenes or tumor suppressor genes impact VEGF expression. For instance, the ras oncogene is believed to target a DNA segment that promotes VEGF expression (Ho and Kuo, 2007). Finally, VEGF is up regulated by inflammatory cytokines and various transcription factors (Ho and Kuo, 2007).
In addition to amplified VEGF availability in the tumor microenvironment, there is also an increase in VEGFR2 expression. Tumor endothelial cells differ from those of physiologically normal tissue and express VEGFR2 at much higher concentrations (Plate et al., 1994). Therefore, not only is there an increase in ligand availability, but there is also an up regulation of receptors, resulting in a powerful pro-angiogenic tumor environment.

**Anti-angiogenic Therapy**

In 1971, Judah Folkman presented his theory on the role of angiogenesis in tumor progression. In addition, Folkman hypothesized that inhibiting a tumor’s ability to undergo angiogenesis will, in turn, inhibit tumor growth and metastasis (Folkman, 1971). Folkman, thereby, coined the term “anti-angiogenic therapy” (Folkman, 1971). Initially, anti-angiogenic therapy was declared to be a “magic bullet” in the treatment of solid tumors (Quesada et al., 2010). The idea was simple: cut off the tumor’s blood supply and kill the tumor. Unfortunately, researchers have yet to find that “magic bullet”. Current anti-angiogenic therapies have increased cancer patient survival by a matter of months, however, new anti-angiogenic agents are making huge strides in cancer research laboratories across the globe (Quesada et al., 2010).

Two major issues limit the success of current non-surgical treatments for malignant solid tumors. Firstly, abnormal organization of the tumor vasculature poses as a barrier for intravenous chemotherapeutic drugs. Blood vessels produced by tumor angiogenesis have a tendency of being very large, torturous and leaky with frequent blind-ends and atria-ventricular shunts (Hirano and Matsui, 1975). These abnormalities decrease the efficiency of blood flow to the tumor, thereby decreasing the delivery and efficacy of chemotherapeutics. Secondly, cancer cells frequently acquire drug-resistance through various mechanisms. Anti-angiogenic therapy is believed to help overcome both of these issues by not only normalizing tumor vasculature and
enhancing tissue perfusion, but also increasing the efficacy of treatment against drug-resistant disease (Jain, 2005, Browder et al., 2000).

Physiologically normal blood vessels in adults are generally very stable and are classified as mature due to the presence of smooth-muscle pericytes that envelope the exterior of the vessel. Pericytes provide a protective effect and induce vessel stabilization through cell-cell contact and by inducing expression of survival factors within the endothelial cells (Barlow et al., 2013, Franco et al., 2011). In contrast, tumor vasculature tends to be fast growing and immature, typically without pericyte coverage, making tumor blood vessels an ideal target for therapy. Anti-angiogenic therapy can act through many mechanisms, such as inhibiting the production of pro-angiogenic factors, neutralizing these factors, inhibiting the receptors of these factors or even inducing endothelial cell death. In addition, the highly proliferative nature of tumor endothelial cells leaves them susceptible to the lethal effects of anti-mitotic chemotherapeutic drugs. As a whole, anti-angiogenic therapies can be divided into three different categories: small-molecule inhibitors, monoclonal antibodies and endogenous therapies.

Small-Molecule Inhibitors

There are many tyrosine kinase receptors located on endothelial cells that play critical roles in angiogenesis, such as VEGF, FGF and PDGF receptors. Small-molecule inhibitors have the ability to translocate through the plasma membrane, which allows them to bind to the cytoplasmic domain of such receptors (Imai and Takaoka, 2006). This interaction disrupts kinase activity, thereby inhibiting downstream effects. Two examples of small-molecule inhibitors include sorafenib and pazopanib.

Sorafenib acts as an antagonist of VEGF and PDGF receptors (Wilhelm et al., 2004). In addition, sorafenib can inhibit cellular proliferation by targeting the Raf-MEK-ERK pathway,
thereby possibly inhibiting the downstream effects of VEGF (Wilhelm et al., 2004). The combination of tyrosine kinase receptor inhibition and anti-proliferative effects on endothelial cells could contribute to sorafenib’s anti-angiogenic effects. More recently, however, sorafenib has been linked to decreased production and accumulation of HIF-1α (Liu et al., 2012). This decrease in HIF-1α can result in decreased VEGF expression, thereby enhancing the anti-angiogenic properties of sorafenib.

Sorafenib, more commonly known as Nexavar, is FDA approved for the treatment of hepatic and renal cancer (Pazdur, 2010). Sorafenib used in combination with cytotoxic agents, results in a greater antitumor effect. For example, when hepatoblastomas are treated with sorafenib and cisplatin, a platinum-based chemotherapeutic agent, cell viability is significantly decreased (Eicher et al., 2012). Sorafenib has also been shown to act synergistically with other anti-angiogenic therapies. For instance, sorafenib and withaferin A act in a synergistic fashion when used in cases of thyroid cancer, resulting in a significant increase in cellular apoptosis (Cohen et al., 2012). Such discoveries suggest that when used in combination with other anti-angiogenic drugs, sorafenib can be administered in smaller doses, thus reducing the opportunity for undesirable side effects.

Pazopanib is an oral anti-angiogenic agent that acts by binding and inhibiting VEGF and PDGF receptors on endothelial cells (Harris et al., 2008). In 2009, pazopanib was FDA approved for the treatment of renal cell carcinoma (Pazdur, 2012). More recently in 2012, it was approved for the treatment of soft tissue sarcomas (Pazdur, 2012). Interestingly, pazopanib is well tolerated as a topical treatment for corneal neovascularization, however, it is not yet FDA approved for this specific use (Amparo et al., 2012). In addition, oral and topical pazopanib has been shown to decrease VEGF-induced vascular leakage, particularly in mouse retinas, but did
not have an effect on retinas isolated from rabbits (Iwase et al., 2012). Literature suggests that pazopanib has a lot of potential as a treatment for both solid tumors and neovascularization in other diseases. However, the species-specific results indicate that there could be some limitations and more research in humans is necessary.

Pazopanib has been shown to decrease cellular growth, survival and migration of both tumor and endothelial cells (Podar et al., 2006). In addition, it was shown to increase cellular apoptosis and inhibit blood vessel formation in multiple myeloma (Podar et al., 2006). However, once pazopanib treatments were terminated in multiple myeloma patients, tumors grew back very rapidly (Podar et al., 2006). This finding suggests that although pazopanib has significant anti-tumor and anti-angiogenic effects, these effects are only transitory. In order for long-term effects to be seen, pazopanib must be administered chronically without any disruptions.

**Monoclonal Antibodies**

Because antibodies can be tailored to target tumor-specific antigens, monoclonal antibodies are a very promising group of potential cancer therapies. Antibodies can be engineered to target just about any molecule in the body, leading to protein-specific inhibition. One of the most widely known and used monoclonal antibodies for anti-angiogenic therapy is bevacizumab. IMC-1C11 is another example of an anti-angiogenic antibody, however, it was unsuccessful and therefore is not widely known.

VEGF is an important factor of angiogenesis and acts as a mitogen for endothelial cells. VEGF, however, is a molecule that goes by many names. It was first identified as Vascular Permeability Factor due to its ability to increase blood vessel permeability (Keck et al., 1989). Multiple names for this single molecule illustrate the numerous roles it plays in physiological processes. Although VEGF is highly expressed in normal tissues, such as lung epithelial cells
and cardiac myocytes, it is also greatly upregulated in the majority of solid tumors (Berse et al., 1992). Bevacizumab is a humanized monoclonal antibody that targets and neutralizes VEGF, thus deactivating its effects on blood vessel permeability and angiogenesis in tumors.

Early studies treating cancer with anti-VEGF antibodies provided striking evidence of tumor growth inhibition (Kim et al., 1993). In 1993, in vivo models were treated with anti-VEGF antibodies and resulted in a significant weight reduction in tumors ranging from 70 to 96 percent (Kim et al., 1993). However, these more profound effects were only seen when treatments began immediately after tumor induction (Kim et al., 1993). Therefore, these same results may not correlate with clinical trials due to the fact that most cancers are not detected until their later stages. This study speculates that angiogenesis was not blocked entirely due to the existence of other pro-angiogenic factors in addition to VEGF (Kim et al., 1993).

Later, in 2004, bevacizumab was discovered to significantly decrease tumor microvascular density in patients suffering from rectal cancer (Willett et al., 2004). This specific study provided evidence that bevacizumab can have significant effects on tumor vasculature at low doses (Willett et al., 2004). Currently, bevacizumab, more commonly known as Avastin, is FDA approved for the treatment of multiple cancers, including breast, renal cell, glioblastoma, non-small cell lung and colorectal cancer (Pazdur, 2013). It is currently undergoing clinical trials to determine if it can be used for the treatment of other cancer types or in combinational therapies.

IMC-1C11 is a chimeric monoclonal antibody that binds to the extracellular domain of VEGFR2, thereby preventing intracellular tyrosine kinase activation (Posey et al., 2003, Lu et al., 2000). Therefore, IMC-1C11 inhibits the pro-angiogenic effects of VEGFR2. When IMC-1C11 was used in phase I clinical studies, it inhibited both tumor growth and angiogenesis (Zhu and
Witte, 1999, Sullivan and Brekken, 2010). However, although IMC-1C11 did not cause any life-threatening side effects, it did have decent immunogenicity properties. For example, when used in a study for the treatment of colorectal cancer metastases, half of the patients developed an immune response to the drug by creating antibodies that targeted the chimeric IMC-1C11 antibody (Posey et al., 2003). This immunogenic response seen in patients is problematic and limits the use of IMC-1C11 in a clinical setting.

Endogenous Therapies

Much like pro-angiogenic molecules, anti-angiogenic molecules are produced endogenously as well. These endogenous inhibitors include anti-angiogenic proteins, hormone metabolites and other modulators of apoptosis (Nyberg et al., 2005). In fact, many endogenous anti-angiogenic peptides have been shown to have therapeutic effects in various cancers (Nyberg et al., 2005). Such endogenous inhibitors include endostatin and angiostatin.

In 1997, endostatin was isolated from a murine hemangioendothelioma cell line (O’Reilly et al., 1997). It is considered a matrix-derived peptide, due to the fact that it is a C-terminal fragment of collagen XVIII (Nyberg et al., 2005, O’Reilly et al., 1997). Endostatin specifically inhibits endothelial cell proliferation in a dose-dependent manner and has no significant effects on cells of non-endothelial origin (O’Reilly et al., 1997). This implies that when used as a therapeutic drug, endostatin will have limited non-specific side effects. For example, when originally tested, endostatin showed maximal anti-angiogenic effects in chick embryos without causing any toxic side effects (O’Reilly et al., 1997). In addition to its low toxicity and potent anti-angiogenic effects, endostatin was shown to inhibit the growth of several different types of primary tumors (O’Reilly et al., 1997). Moreover, endostatin has not been shown to induce drug
resistance (Boehm et al., 1997). This is an intriguing characteristic for a potential cancer therapy, since drug resistance is a re-occurring problem in most cancers.

According to past literature, endostatin has its effects through multiple mechanisms (O’Reilly et al., 1997). Firstly, endostatin has been shown to interfere with FGF-induced signaling, resulting in reduced motility of endothelial cells (Dixelius et al., 2002). For instance, when endothelial cells were treated with both endostatin and FGF-2, there was a significant reduction in actin stress fiber formation and focal adhesions (Dixelius et al., 2002). These results are speculated to be due to the phosphorylation of focal adhesion kinase and paxillin via endostatin (Dixelius et al., 2002). Reducing the motility of endothelial cells would decrease their ability to migrate towards a solid tumor, thus decreasing the tumor’s ability to recruit its own blood supply. Secondly, endostatin was found to cause endothelial cell-specific G1 cell cycle arrest through the inhibition of cyclin D1 (Dhanabal et al., 1999, Hanai et al., 2002). Cell cycle arrest could cause endothelial cells to become senescent or eventually apoptotic, both of which could lead to anti-angiogenic consequences. Thirdly, endostatin can block VEGF signaling by preventing the activation of VEGFR2, thus inhibiting the activation of its down-stream pathways (Kim et al., 2002). Finally, endostatin can down-regulate many pro-angiogenic genes including, but not limited to, cell cycle related genes, apoptosis inhibitor genes and mitogenic factor genes (Shichiri and Hirata, 2001).

More recently, endostatin has been found to be an effective cancer treatment for numerous cancer types when used in combination with cytotoxic therapy. For example, Yan and associates discovered that adenovirus-mediated combined therapy with endostatin and tumor necrosis factor-related apoptosis-inducing ligand can result in the regression of hepatocellular carcinoma (Yan et al., 2013). Combinational treatment of endostatin and chemotherapeutic drug,
docetaxel, has also been shown to be an effective therapy for cervical cancer (Qui et al., 2012). In the midst of endostatin’s success as a cancer therapy, it has unfortunately been associated with poor survival. For example, it was recently speculated that elevated levels of endostatin in blood serum is associated with poor survival of patients suffering from advanced nasopharyngeal carcinoma (Mo et al., 2013). In summary, endostatin holds great potential for the multiple cancer therapies, however, more research is necessary before its effects are fully understood. Currently, recombinant human endostatin is being used pre-clinically under the drug name Endostar (Huang et al., 2012).

In 1994, O’Reilly and associates discovered the endogenous anti-angiogenic peptide, angiostatin, circulating in the serum of Lewis lung carcinoma (LLC) tumor-bearing mice (O’Reilly et al., 1994). Angiostatin is a plasminogen fragment that specifically inhibits endothelial cell proliferation (O’Reilly et al., 1994). The nature of its production places angiostatin in the non-matrix derived category of endogenous inhibitors (Nyberg et al., 2005). Quite surprisingly, primary tumors of LLC were found to produce angiostatin, therefore inhibiting the growth of their metastases (O’Reilly et al., 1994). When these primary tumors are removed, however, metastatic growth is re-established (O’Reilly et al., 1994). These results illustrate angiostatin’s ability to prevent the metastatic spread by inhibiting the formation of vascular beds within metastases (O’Reilly et al., 1994).

Angiostatin and uncleaved plasminogen molecules bind to receptors expressed on the surface of endothelial cells (Moser et al., 1999). Plasminogen was found to bind to annexin II and angiostatin to plasma membrane-associated adenosine triphosphate (ATP) synthase (Moser et al., 1999). Moser and associates argued that the interaction of angiostatin and plasma
membrane-associated ATP synthase could reduce the production of ATP, thereby leaving endothelial cells more vulnerable to hypoxic changes and cellular damage (Moser et al., 1999).

Recently, angiostatin has been shown to decrease the expression of certain MMPs at both the gene and protein level (Radziwon-Balicka et al., 2012). MMPs are necessary during angiogenesis in order to break down the surrounding extracellular matrix. By inhibiting these MMPs, angiostatin can specifically inhibit MMP-dependent endothelial cell migration, thus preventing angiogenesis (Radziwon-Balicka et al., 2012).

**Anti-angiogenic Therapy in Epithelial Ovarian Cancer**

Epithelial ovarian cancer is the most lethal gynecological cancer affecting women today. Due to the late onset of vague symptoms and a lack of reliable screening techniques, EOC is usually diagnosed in its advanced stages. Treatment generally consists of surgical debulking, followed closely by combinational chemotherapy of paclitaxel and carboplatin. Although EOC is typically susceptible to treatment initially, approximately 70 percent of patients will have disease recurrence within a three-year period and the majority will relapse in a chemoresistant state (Ledermann et al., 2013). As a result of late detection and chemoresistance, the 5-year survival rate is easily less than 50 percent for women diagnosed with EOC (Harries and Gore, 2002). More effective treatments for advanced staged EOC is necessary in order to improve the survival rate and quality of life of women suffering with this disease. Anti-angiogenic therapies are being studied in order to determine their overall effect on progressive disease-free survival in women with chemo-sensitive and resistant disease.

Bevacizumab, a VEGF humanized monoclonal antibody, is commonly used in ovarian cancer clinical studies. For example, two phase II studies were completed in 2007 using bevacizumab as a monotherapy in patients with platinum resistant disease, resulting in overall
response rates of 15.9 percent and 21 percent (Burger et al., 2007, Cannistra et al., 2007).
Unfortunately these results cannot be compared to chemotherapy treatments alone as all patients received the same treatment regimen. In 2012, however, a promising phase III clinical trial was completed (Aghajanian et al., 2012). For this study, 484 patients were randomly distributed into treatment groups that either contained chemotherapy in combination with bevacizumab or chemotherapy without bevacizumab (Aghajanian et al., 2012). The patients who received bevacizumab had a median progression-free survival of 12.4 months verses 8.4 months in the untreated group (Aghajanian et al., 2012). In addition, bevacizumab was found to show little toxicity throughout these trials. The most common mild side effects reported were hypertension, proteinuria and wound healing complications (Burger et al., 2007, Cannistra et al., 2007). Gastrointestinal perforations were rare, but occurred commonly enough to be of some concern (Cannistra et al., 2007). However, it cannot be determined whether they were a result of bevacizumab or late stage disease. Overall, bevacizumab poses as a promising anti-angiogenic therapy to be used in combination with chemotherapy regimens.

Sorafenib is a small molecule tyrosine kinase inhibitor that is being assessed for its use in treating recurrent ovarian cancer. In 2011, sorafenib was utilized in a phase II clinical trial in recurrent ovarian cancer and primary peritoneal carcinomas (Matei et al., 2011). Out of the 71 patients treated, 14 showed six months of progression-free survival and two had a partial response to the drug (Matei et al., 2011). However, severe toxicities were reported including rash, hand-foot syndrome, cardiovascular and pulmonary side effects (Matei et al., 2011). Therefore, sorafenib’s antitumor effects seem to be outweighed by the excessive toxicity. Interestingly, sorafenib and bevacizumab have been looked at in combination for the treatment of ovarian cancer (Azad et al., 2008). Although the results of the phase I study were encouraging,
74 percent of patients needed to undergo a sorafenib dose reduction, suggesting that sorafenib is not ideal for long-term treatment (Azad et al., 2008). Currently, sorafenib is being used in a phase II study to assess the efficacy and safety for its use in combination with topotecan, a chemotherapy drug, for platinum-resistant ovarian cancer (Charite University, 2010).

Sunitinib malate is another small molecule tyrosine kinase inhibitor that has been used in multiple phase II clinical trials for the treatment of recurrent ovarian cancer. In 2011, a study was published stating that the median progression-free survival was 4.1 months (Biagi et al., 2011). Unfortunately these researchers did not provide any comparisons for patients who did not receive sunitinib as part of their treatment. A second clinical trial published in 2012 showed no significant differences between progression-free survival in patients treated with sunitinib versus non-sunitinib treated patients (Baumann et al., 2012). A recent study in 2013 treated patients with 37.5 mg of sunitinib daily for a 28-day treatment period (Campos et al., 2013). These researchers were looking for response rates and reported a meager rate of 8.3 percent (Campos et al., 2013). Although the toxicities reported for sunitinib are not nearly as dangerous as sorafenib, the phase II trials do not provide any convincing evidence to support the use of sunitinib in a clinical setting.

Pazopanib has been reviewed as a monotherapy for the treatment of recurrent ovarian cancer (Friedlander et al., 2010). The patients were given 800 mg of pazopanib daily until the detection of disease progression or life-threatening toxicity (Friedlander et al., 2010). It took a median of 29 days to respond to the drug and the median response duration was 113 days (Friedlander et al., 2010). The overall response rate to pazopanib was 18 percent (Friedlander et al., 2010). Pazopanib was generally well tolerated with a few reported cases of aminotransferase
elevations (Friedlander et al., 2010). Further studies looking into the efficacy and safety of pazopanib are currently in progress.

In summary, anti-angiogenic therapies have potential as future treatments for reoccurring ovarian cancer. Currently these drugs do not provide a significant impact on the overall survival, but many studies are looking into the combination of these drugs with chemotherapeutic drugs, both in a max-tolerated dose (MTD) regimen as well as a low-dose, metronomic (MET) regimen.

**Tumor Vasculature and Vessel Normalization**

In physiologically normal tissues, there is a fine balance between pro-angiogenic and anti-angiogenic molecules. This creates an equilibrium between the formation of new blood vessels and the metabolic needs of cells. Solid tumors, however, lack this equilibrium, leading to rapid and dysregulated vessel formation, which can lead to areas of tumor that receive insufficient blood flow (Helmlinger et al., 1997). This chaotic vessel organization typically results in large areas of hypoxia and acidosis.

Heterogeneity is a term that best describes tumor vasculature (Jain, 2003). In comparison to normal endothelial cells, the expression of adhesion molecules can be variable and unpredictable in tumor endothelium. This makes the vasculature a difficult, but not impossible, target for cancer therapy. Furthermore, tumor blood vessels are thought to be a “mosaic” due to the fact that both endothelial cells and tumor cells are exposed to the lumen (Chang et al., 2000). The mosaic quality of vessels has implications in tumor metastasis, as cancer cells are exposed to blood flow. In fact, while studying mosaic vessels in tumors, Chang and associates observed that approximately $10^6$ cancer cells are shed from each gram of tumor on a daily basis (Chang et al., 2000).
Blood vessel diameter also varies throughout the tumor. Padera and associates believe that highly proliferative tumor cells surrounding the vessels affect lumen diameter (Padera et al., 2004). The pressure of increasing tumor size compresses not only blood vessels, but also lymphatic vessels (Padera et al., 2004). The collapse in lymphatic vessels impairs fluid absorption, in turn causing an increase in interstitial fluid pressure. Additionally, increased interstitial fluid pressure has also been linked to the collapse of vasculature (Boucher and Jain, 1992). Evidently, the collapse in tumor vasculature may factor in to the large amount of tissue hypoxia observed in solid tumors.

Leakiness is a common characteristic observed in tumor blood vessels. VEGF is believed to be a major reason for blood vessel leakage in solid tumors and has also been implicated in the accumulation of abdominal ascites (Senger et al., 1983). In fact, when studying vascular permeability, VEGF is 50,000 times more potent than histamine, an endogenously produced inflammatory molecule (Senger et al., 1990). In addition, abnormal endothelial cell arrangement in the mosaic blood vessels also results in leakage. Endothelial cells in tumor vessels are disorganized, loosely connected and overlapped, which causes openings and allows blood to leak into interstitial spaces (Hashizume et al., 2000). Finally, pericytes are abnormally arranged or absent from tumor vasculature.

Overall, the abnormal, leaky and chaotic tumor vasculature results in very poor perfusion throughout the tumor. This has detrimental effects on cancer therapy because chemotherapy drugs cannot penetrate into the solid tumor. In addition, the increase in hypoxic tissues negatively impacts the effects of radiation and may even select for aggressive cancer cells (Bottaro and Liotta, 2003). Anti-angiogenic therapy, however, has been shown to have normalizing effects on tumor vasculature, which improves the efficacy of conventional therapy.
Anti-angiogenic therapy aims to balance the expression of pro- and anti-angiogenic molecules in order to return the tumor microenvironment to a more physiologically normal state. This results in vasculature that is less leaky, less torturous and more efficient, thereby relieving hypoxia and increasing the efficacy of other cancer therapies (Jain, 2005). The use of anti-angiogenics alone has been shown to have minor effects on patient survival, however, the effects are enhanced when used in combination with other cytotoxic drugs. For example, administration of bevacizumab in combination with fluorouracil in patients with metastatic colorectal cancer resulted in an overall survival improvement that was not only statistically significant, but also clinically meaningful (Hurwitz et al., 2004).

**Metronomic Chemotherapy**

Chemotherapeutic drugs rule the world of cancer therapy. Patients undergoing treatment for various malignancies will generally receive cytotoxic drugs at an extremely high dosing concentration, followed by two to three weeks of drug-free intervals. This dosing regimen is known as max-tolerated dose (MTD) and is specifically designed in order to kill rapidly dividing cells by inducing DNA damage or microtubule destabilization. Prolonged drug-free intervals are intended to allow normal tissue in the body to recover, however, evidence has shown that this time also allows the damaged tumor vasculature to recover as well. In addition, MTD results in many undesirable side effects, such as damage to hair follicles and gut epithelia. Many patients who receive MTD chemotherapy will develop a drug resistant form of the disease shortly after remission. On the other hand, current literature argues that administering chemotherapy drugs more frequently at a substantially lower concentration may have many more benefits than traditional MTD.
Dose-dense metronomic chemotherapy has many advantages. For instance, the considerably lower concentration of drug results in decreased side effects and morbidity due to lower toxicity (Klement et al., 2000). In addition, lower doses administered more frequently were found to be safe and efficacious (Man et al., 2002). In 2007, Kamat and associates studied the effects of metronomic chemotherapy versus MTD in ovarian cancer. They discovered that although the cumulative metronomic dose was as little as one-fifth of the total concentration of MTD, metronomic chemotherapy alone still had an impact on disease progression and when combined with a multiple kinase inhibitor, it significantly improved survival when compared to MTD alone (Kamat et al., 2007).

Traditional MTD chemotherapy targets highly proliferative cells, such as the rapidly dividing cancer cells. However, due to the slow proliferative nature of endothelial cells, tumor vasculature is only mildly affected and is able to recover during the long drug-free intervals (Hanahan et al., 2000). Browder and associates hypothesized that the recovery of endothelial cells during the MTD drug-free breaks could promote the regrowth of tumors and even encourage the development of drug-resistant cells (Browder et al., 2000). When cytotoxic drugs are administered in a metronomic regimen, however, they are able to target the cells that divide more slowly. In addition, when chemotherapy drugs are administered on a more frequent basis, they can hinder the recovery of tumor vasculature by decreasing the length of drug-free interludes (Hanahan et al., 2000). Metronomic chemotherapy’s ability to target tumor endothelial cells and inhibit tumor vasculature is what lead scientists to classify it as anti-angiogenic chemotherapy.

Many experiments have been completed, both in vitro and in vivo, in order to test the anti-angiogenic effects of certain chemotherapy drugs, such as paclitaxel, topotecan,
camptothecin and vinblastine (Klement et al, 2000, Belotti et al., 1996, Clements et al., 1999). Paclitaxel, a microtubule inhibitor, has been shown to inhibit endothelial cell proliferation, motility, invasiveness and cord formation in vitro (Belotti et al., 1996). Camptothecin and topotecan were both shown to inhibit endothelial cell growth in vitro (Clements et al., 1999). Another microtubule-affecting chemotherapeutic drug, vinblastine, has been described as a significantly potent inhibitor of angiogenesis at very small concentrations (Vacca et al., 1999). Vacca and associates used picomolar concentrations of vinblastine and found that it inhibited endothelial cell proliferation, chemotaxis and morphogenesis in vitro (Vacca et al., 1999). In addition, these small concentrations inhibited angiogenesis in an in vivo chick embryo model (Vacca et al., 1999).

Acquired drug resistance is one of the biggest downfalls to plague cancer therapy. Due to the genetically unstable nature of cancer cells, they are able to acquire genetic mutations that allow them to become immune to the effects of cytotoxic drugs. Therefore, cancer therapy must begin to target the genetically stable cells that are necessary for solid tumors and malignancies to flourish (Kerbel, 1991). Endothelial cells have very stable genetic material and rarely undergo mutations, thereby making them perfect targets for therapy. By utilizing anti-angiogenic chemotherapy, physicians can improve the efficacy of treatment against drug-resistant forms of malignancies (Browder et al., 2000). Currently, metronomic chemotherapy is being used in many phase I and II clinical studies in order to treat various types of cancer (Yoshimoto et al., 2012, Kummar et al., 2012).

**Thrombospondin-1: Structure and Mechanisms**

In 1990, an adhesive glycoprotein by the name of thrombospondin-1 (TSP-1) was found to act as an endogenous inhibitor of angiogenesis (Good et al., 1990). Not only did TSP-1 inhibit
neovascularization in vivo, but it was also found to inhibit endothelial cell migration in vitro (Good et al., 1990). In fact, TSP-1 regulates various cellular processes ranging from proliferation and migration to apoptosis (Chen et al., 2000). Thrombospondin-1 is a large protein that includes N-terminal and C-terminal domains, procollagen homology domains and three different repeated sequence motifs (Chen et al., 2000). For a schematic diagram of TSP-1’s structure, please see Figure 1. TSP-1 triggers several signal transduction pathways by the formation of cell surface receptor clusters and multi-protein complexes (Chen et al., 2000). As a whole, TSP-1 plays many different roles in the systemic environment, however, the anti-angiogenic properties of TSP-1 have been linked to the type-1 repeated sequence motif, a peptide commonly known as 3TSR (Iruela-Arispe et al., 1999, Tolsma et al., 1993).

TSP-1 initiates its anti-angiogenic effects through various mechanisms, yet, interactions with CD36 seem to play a vital role in its activities. For example, Jimenez and associates discovered that TSP-1 could effectively inhibit neovascularization in mice that expressed the CD36 receptor (Jimenez et al., 2000). However, the exact opposite was observed in CD36 null mice (Jimenez et al., 2000). CD36 activation is believed to induce cellular apoptosis of activated endothelial cells. Once bound to CD36, TSP-1 initiates the CD36-Fyn-caspase apoptosis cascade resulting in cell death (Jimenez et al., 2000). In vitro studies have also supported this relationship. Dawson and associates presented that TSP-1 inhibited endothelial cell migration and tube formation via CD36 (Dawson et al., 1997). They also demonstrated that TSP-1 effects could be masked by competition with a CD36 antibody. Finally, they showed that transfection of CD36 into cells that do not normally express CD36 rendered them sensitive to the effects of TSP-1 (Dawson et al., 1997).
Figure 1: Thrombospondin-1 structure with active peptide sequences.

Thrombospondin-1 is made up of an N-terminal domain, a procollagen homology domain, three different repeat regions and a C-terminal domain. The three type-1 repeat regions make up the 3TSR peptide. Active peptide sequences include a TGF-β activation site, a protein and glycosaminoglycan binding site, CD36 binding site, and an area that inhibits endothelial cell migration towards pro-angiogenic signals. Adapted from (Lawler, 2002 and Resovi et al., 2014)
Angiogenesis inhibition is supported by other activities of TSP-1 as well. For example, TSP-1 can modify the extracellular matrix by binding to fibronectin, collagen and other extracellular proteins, such as MMP-2 (Bein and Simons, 2000). Thrombospondin-1 inhibits MMP-2 catalytic activity by preventing the conversion of MMP-2 zymogens to their activated form (Bein and Simons, 2000). This assists the anti-angiogenic activity of TSP-1 by decreasing the amount of ECM remodeling.

Thrombospondin-1 negatively impacts VEGF signaling through various mechanisms. Thrombospondin-1 indirectly affects VEGF bioavailability by preventing its release from the extracellular matrix. It does this by binding to assorted MMPs, such as MMP-9 (Rodriguez-Manzaneque et al., 2001). Without properly functioning MMPs, VEGF stores remain trapped in the ECM and unable to activate the pro-angiogenic pathways. According to Greenaway and associates, TSP-1 will also bind directly to VEGF and internalize it via the low-density lipoprotein receptor-related protein located on endothelial cells (Greenaway et al., 2007). Internalization results in the degradation of VEGF molecules, thereby directly diminishing VEGF concentrations in the ECM microenvironment. Moreover, TSP-1 decreased VEGFR2 phosphorylation in a dose-dependent manner in vitro and showed the same inhibitory effects in vivo (Zhang et al., 2009).

In addition to VEGF, TSP-1 has also been found to bind to FGF-2 and other hepatic growth factors in the ECM (Margosio et al., 2003). This suggests that TSP-1 acts as a scavenger of pro-angiogenic molecules, thereby altering the location and function of such growth factors.

TSP-1 contains an amino acid sequence that allows it to bind to latent TGF-β and transform it into its biologically active form (Schultz-Cherry et al., 1994). TGF-β is involved in many different cellular pathways including, but not limited to, proliferation, differentiation and
apoptosis. However, the role of TGF-β in tumor progression is a common topic for debate. For example, Miao and associates argue that the tumor inhibitory effects of TSP-1 are mediated by the activation of TGF-β in B16F10 melanoma (Miao et al., 2001). On the other hand, Tsushima and associates believe that high levels of TGF-β are associated with the progression of colon cancer (Tsushima et al., 1996). Due to the promiscuous nature of TGF-β, it is difficult to draw a single conclusion about its role in angiogenesis inhibition by TSP-1. In general, the actions of TGF-β seem to be cell specific and, therefore, more research is necessary in order to fully understand this molecule.

In 2013, CD47 was identified as another TSP-1 target (Kaur et al., 2013). CD47 is expressed on endothelial cells and is believed to be an upstream regulator of the tumor promoter gene, c-Myc (Kaur et al., 2013). Thrombospondin-1 has a specific CD47 binding site that results in the suppression of c-Myc and other stem cell promoter genes, limiting both cell growth and self-renewal (Kaur et al., 2013). In vitro studies illustrated TSP-1’s potent effect of c-Myc expression. For instance, picomolar concentrations of TSP-1 successfully down regulated c-Myc in cells expressing CD47, but not in cells lacking CD47 (Kaur et al., 2013). Thrombospondin-1 also decreased the release of nitric oxide, a potent vasodilator, through CD47 mechanisms (Isenberg et al., 2006). These relationships offer more mechanisms of TSP-1’s anti-angiogenic effects.

**Thrombospondin-1 in Cancer**

The therapeutic use of TSP-1 has been a topic of research for many years. Thrombospondin-1 and its mimetic molecules have inhibited the growth of many experimental tumors of melanoma, pancreatic, lung and ovarian origins (Miao et al., 2001, Zhang et al., 2005, Greenaway et al., 2009). In fact, some TSP-1 mimetic peptides have already made the jump into
phase I clinical studies. In 2005, TSP-1 mimetic, ABT-510, was administered subcutaneously in patients suffering from advanced stage malignancies (Hoekstra et al., 2005). Although this study was not intended to look at drug efficacy, there were many patients that benefitted from ABT-510 administration (Hoekstra et al., 2005). In addition, this clinical trial allowed for a favorable toxicity profile to be compiled. The major side effects included skin irritation at the site of continuous injections and fatigue (Hoekstra et al., 2005). Therefore, ABT-510 and other TSP-1 mimetic peptides hold a lot of potential for the future therapies due to their low toxicities.

3TSR has had profound effects on solid tumors in vivo. For example, when used in an orthotopic mouse model of pancreatic cancer, 3TSR had significant therapeutic effects at 3mg/kg (Zhang et al., 2005). Pancreatic tumor volume was significantly smaller in mice treated with 3TSR in comparison to controls. In addition, the area of necrotic tissue increased from 25.9 percent in controls to 51.0 percent in 3TSR treated mice (Zhang et al., 2005). When tissues were analyzed with CD31, an endothelial cell marker, and TUNEL, a marker for apoptosis, 3TSR was found to specifically increase apoptosis in tumor endothelial cells, supporting its anti-angiogenic role (Zhang et al., 2005). However, 3TSR did not impact proliferation or apoptosis directly in pancreatic cancer cells (Zhang et al., 2005). In another study, 1mg/kg 3TSR was administered to a xenograft mouse model of colon cancer in combination with Lexatumumab, a tumor necrosis factor-related apoptosis-inducing ligand receptor agonist (Ren et al., 2009). Combination treatment showed the most significant inhibition of tumor growth compared to treatments given alone (Ren et al., 2009). This evidence suggests that 3TSR may be even more effective at inhibiting tumor growth when used in combination with other apoptosis-inducing molecules. In addition, 3TSR administered at 1mg/kg resulted in significant apoptosis in tumor endothelial cells, showcasing its potency (Ren et al., 2009).
RATIONALE

The poor prognosis of women diagnosed with late stage EOC has called for more efficient treatments. Currently, anti-angiogenic treatments in combination with traditional chemotherapy have had some success in prolonging progression free survival. More specifically, 3TSR has many unique effects on EOC both in vitro and in vivo that could rationalize its used as a novel therapy for EOC.

Previous in vitro work has shown that 3TSR acts in a dose-dependent manner. When spontaneously transformed mouse ovarian cancer cells were treated with increasing concentrations of 3TSR, there was a significant decrease in VEGF expression (Petrik, unpublished). In addition, there was a significant up-regulation of pro-apoptotic molecules, such as FasL and cleaved caspase-3, as well as decreased pro-survival molecules such as Bcl-2 (Petrik, unpublished). TUNEL staining confirmed 3TSR induced apoptosis (Petrik, unpublished). Finally, a cellular proliferation marker known as Ki67, illustrated a significant decrease in proliferation after treatment with 3TSR (Petrik, unpublished). The effects of 3TSR on ovarian cancer cells in vitro lead to its use in an orthotopic syngeneic mouse model of EOC.

When used in vivo, 3TSR had profound effects on the progression of EOC. 3TSR treatments significantly inhibited primary tumor growth in comparison to an untreated control (Petrik, unpublished). In addition, 3TSR lessened disease progression by decreasing the number of visible secondary lesions throughout the abdominal cavity as well as inhibiting the accumulation of abdominal ascites (Petrik, unpublished). Closer examination of primary tumors showed that 3TSR induced tumor cell apoptosis, thereby supporting the results found in vitro (Petrik, unpublished). In addition, 3TSR decreased blood vessel density throughout the tumor by inducing endothelial cell apoptosis (Petrik, unpublished). Blood vessel normalization and
increased pericyte recruitment post 3TSR treatments resulted in both enhanced tumor perfusion and increased chemotherapy drug uptake, thereby justifying its use with combinational chemotherapy (Petrik, unpublished).

This project was designed to study the effects of 3TSR and low-dose metronomic chemotherapy on advanced stage EOC. The results of this study will allow researchers to have a better understanding of the effects of 3TSR and combinational chemotherapy in a pre-clinical model of EOC, thereby providing a rationale for its use in a clinical setting. The studies in this thesis test the hypothesis that due to blood vessel normalization and increased tissue perfusion, 3TSR will increase tissue uptake of chemotherapy drugs and enhance metronomic chemotherapy delivery to induce regression of advanced stage disease. In order to test this hypothesis, the following objectives were addressed:

1. Determine the effects of 3TSR and combinational chemotherapy on disease progression in an orthotopic syngeneic mouse model of advanced stage EOC.
2. Evaluate the effects of 3TSR and combinational chemotherapy on survival in an orthotopic syngeneic mouse model of advanced stage EOC.
3. Characterize the mechanisms by which 3TSR and combinational chemotherapy induce tumor regression in an orthotopic syngeneic mouse model of advanced stage EOC.
MATERIALS AND METHODS

Animal Model

C57Bl6 mice (Charles River Laboratories) were housed in the Central Animal Facility at the University of Guelph under standard conditions and maintained under the guidelines of the Canadian Council on Animal Care. At the time of tumor induction, mice were anesthetized with isoflurane and a small incision was made at the midline on the dorsal side, allowing access to the left ovaries. 1.0 x 10^6 ID8 cells suspended in 5 µl sterile phosphate buffered saline (PBS; Life Technologies Inc., Burlington, ON) were injected under the bursa of the left ovary in each mouse with the use of a Hamilton syringe (Fisher Scientific, Whitby, ON) and 30-gauge needle. ID8 cells are spontaneously transformed mouse ovarian cancer cells that were a kind gift from Drs. Katherine Roby and Paul Terranova (University of Kansas). Cells were grown in T-75 flasks (Fisher Scientific, Whitby, ON) with Dulbecco’s Modified Eagles Medium (DMEM; Life Technologies Inc., Burlington, ON) supplemented with 10 percent fetal bovine serum, 2 percent L-glutamine, and 1 percent antibiotic-antimycotic (Life Technologies Inc., Burlington, ON).

After surgery, mice were closely monitored for the first 24 hours and routinely monitored daily thereafter. Tumors were allowed to progress for 60 days without therapeutic intervention in order to replicate late stage disease before commencing treatments. Animals were weighed and underwent health assessments on a weekly basis.

Daily 3TSR injections began 60 days post tumor induction. 3TSR was a kind gift from Dr. Jack Lawler (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA). Mice either received daily intraperitoneal (IP) injections of 3TSR (1.0 mg/kg/day in 200µl PBS), or sterile PBS (200µl) as a vehicle control. Fourteen days later, chemotherapy regimens commenced. Metronomic (MET) chemotherapy treatment groups received 2.0 mg/kg of the
platinum-based alkylating agent carboplatin (Sigma-Aldrich Canada Ltd., Oakville, ON) and 1.0 mg/kg of the microtubule inhibitor paclitaxel (Biolyse Pharma Corporation, St. Catharines, ON), four times per week. Max-tolerated dose (MTD) chemotherapy groups received 10.0 mg/kg of carboplatin and 10.0 mg/kg of paclitaxel, two times per week. At 90 days post tumor induction, following 30 days of treatment, 30 mice were euthanized (n=5/group) and tissues were collected for time-point comparisons. The remaining mice were allocated to a survival study and were removed once they became moribund in order to determine changes in survival following anti-angiogenic or chemotherapy treatments. For a schematic diagram of the dosing schedule please refer to Figure 2.

**Tissue Collection and Preparation**

Mice were humanely euthanized using cervical dislocation. At the time of death, tissues and fluid samples were collected from each mouse, including ascites, serum, primary tumors and visible secondary lesions. Initially, primary tumors were measured and weighed and then cut in half. Half of the tumors were fixed in 10 percent buffered formalin phosphate (Fisher Scientific, Whitby, ON) for histological analysis, the other half were flash frozen in liquid nitrogen for protein collection. Visible secondary lesions were counted and then either formalin fixed or flash frozen. Secondary lesions were graded on a scale where no visible lesions received a grade of zero, one to two were designated one plus, three to ten were assigned two plus and more than ten received a three plus. Blood was collected in vacutainer serum separator tubes for serum collection (Becton Dickinson and Company, Franklin Lakes, NJ). Ascites, serum and flash frozen samples were stored at -80°C. Formalin fixed samples remained in formalin overnight and were then stored in 70 percent ethanol (ETOH; Greenfield Ethanol Inc., Brampton, ON) until time of embedding. They were then paraffin embedded and cut on a rotary microtome.
Figure 2: Schematic diagram of in vivo dosing schedule

Tumor induction surgery was performed on day 0 and tumors were given 60 days to develop into stage III EOC. At day 60, daily 3TSR injections began, followed by administration of chemotherapy drugs commencing at day 74. Thirty mice were removed at the 90 day time point and the remaining mice were removed at onset of morbidity until the experiment was terminated on day 150.
Five micrometer sections were mounted on Superfrost Plus glass slides (Fisher Scientific, Whitby, ON) for immunohistochemical analysis.

In order to collect protein from flash frozen tumors, tissues were immersed in a RIPA lysis buffer (Appendix II) and homogenized over ice. Homogenized tissues sat on ice for approximately 30 minutes and were then spun down at 21,000 x g for 10 minutes at 6°C in the micro centrifuge. Aqueous phases were aliquoted over ice into nuclease-free microcentrifuge tubes (Fisher Scientific, Whitby, ON) and stored in the -80°C freezer and the pellet was discarded. DC Bio-Rad Protein Quantification Kit (Bio-Rad, Mississauga, ON) was used to quantify protein concentration.

**Immunohistochemistry**

On the first day, paraffin embedded tissues were deparaffinized in three xylene (Fisher Scientific, Whitby, ON) washes at five minutes each. Tissues were then rehydrated using decreasing concentrations of ETOH, beginning with two 100 percent ETOH baths, followed by two 90 percent ETOH baths and ending with one 70 percent ETOH bath, each bath lasting two minutes. Once rehydrated, tissues were washed in PBS (Appendix II) for three minutes, followed by immersion in 1.0 percent hydrogen peroxide (Appendix II) for ten minutes in order to block endogenous peroxidase activity. Before antigen retrieval, tissues were washed in PBS two times for three minutes. Tissues were transferred to the antigen retrieval buffer (Appendix II) and incubated at 90°C for twelve minutes then allowed to cool in the buffer at room temperature for twenty minutes. Tissues were again washed two times in PBS for three minutes and outlined with an ImmEdge hydrophobic barrier pen (Vector Laboratories, Burlington, ON). Non-specific binding was inhibited by 5 percent bovine serum albumin (BSA) blocking solution (Appendix II) for ten minutes. After blocking, slides were incubated with appropriate dilutions
of primary antibodies overnight in humidity chambers at 4°C. Detailed descriptions of immunohistochemical antibodies can be found in Table 2.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody Identification</th>
<th>Species</th>
<th>Primary Conc.</th>
<th>Secondary Conc.</th>
<th>Antigen Retrieval</th>
<th>DAB Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td>ab28364</td>
<td>Rabbit</td>
<td>1/25</td>
<td>1/100</td>
<td>Citrate Buffer</td>
<td>30 sec</td>
</tr>
<tr>
<td>CD36</td>
<td>NB400-144</td>
<td>Rabbit</td>
<td>1/500</td>
<td>1/100</td>
<td>Citrate Buffer</td>
<td>30 sec</td>
</tr>
<tr>
<td>Cleaved Caspase-3</td>
<td>9661</td>
<td>Rabbit</td>
<td>1/200</td>
<td>1/100</td>
<td>Citrate Buffer + Tween20</td>
<td>1 min + 30 sec</td>
</tr>
<tr>
<td>Flk-1/VEGFR2</td>
<td>sc6251</td>
<td>Mouse</td>
<td>1/600</td>
<td>1/100</td>
<td>Tris-EDTA Buffer</td>
<td>30 sec</td>
</tr>
<tr>
<td>Ki67</td>
<td>ab15580</td>
<td>Rabbit</td>
<td>1/200</td>
<td>1/200</td>
<td>Citrate Buffer + Tween20</td>
<td>30 sec</td>
</tr>
<tr>
<td>TSP-1</td>
<td>sc81755</td>
<td>Mouse</td>
<td>1/50</td>
<td>1/100</td>
<td>Citrate Buffer</td>
<td>30 sec</td>
</tr>
<tr>
<td>VEGF</td>
<td>ab46154</td>
<td>Rabbit</td>
<td>1/400</td>
<td>1/100</td>
<td>Citrate Buffer + Tween20</td>
<td>30 sec</td>
</tr>
<tr>
<td>Von Willebrand Factor</td>
<td>A 0082</td>
<td>Rabbit</td>
<td>1/400</td>
<td>1/100</td>
<td>Citrate Buffer</td>
<td>1 min</td>
</tr>
</tbody>
</table>

The next day, slides were washed twice in PBS for three minutes each. Afterwards appropriate dilutions of biotinylated secondary antibodies (Sigma-Aldrich Canada Ltd., Oakville, ON) were added and incubated for 2 hours in humidity chambers at room temperature. After two hours, tissues were washed two times in PBS for three minutes each and incubated with ExtrAvidin-Peroxidase (Sigma-Aldrich Canada Ltd., Oakville, ON). ExtrAvidin was used at a 1/50 dilution and incubated for one hour in humidity chambers at room temperature. Slides were then washed in PBS and antibody localization was visualized with the 3,3’-diaminobenzidine
DAB incubation time varied with each individual antibody and exact duration can be viewed in Table 2. Following DAB, slides were washed in PBS twice for three minutes and then stained with Carazzi’s Hematoxylin (Appendix II) for 20 seconds. Tap water was used to wash off excess Hematoxylin staining and increasing ETOH baths dehydrated tissues. Slides were dipped six times in 50 percent ETOH, then immersed in 70 percent ETOH twice for 30 seconds each, followed by 90 percent ETOH for one minute and 100 percent ETOH twice for three minutes each. Finally, tissues were immersed in xylene two times for three minutes each and mounted using Cytoseal-XYL mounting medium (Thermo Fisher Scientific, Nepean, ON).

Slides were imaged at 200x magnification. Tissues stained with CD31 and von Willebrand Factor were analyzed by hand using ImageJ software. Blood vessels were outlined and percentage of blood vessel area was calculated. Images of Ki67 and cleaved caspase-3 staining were analyzed using a web application called ImmunoRatio in order to calculate percent immunopositive. Finally, the remaining antibodies were analyzed using ImageScope to quantify percent positive staining.

**Western Blot Analysis**

Protein samples collected from primary tumors were mixed with equal volumes of reducing buffer (Appendix II) and boiled in water for five minutes. Once denatured, the samples were loaded into either pre-made Bio-Rad 4-15 percent gradient gels (Bio-Rad, Mississauga, ON) or hand-made 8 percent and 12 percent separating PAGE gels (Appendix II) and separated using gel electrophoresis. Precision plus protein dual color standard (Bio-Rad, Mississauga, ON) was used for protein identification. Proteins were resolved in the gel with application of 125V for approximately one hour or until the bromophenol blue dye (Sigma-Aldrich Canada Ltd., Ontario, ON).
Oakville, ON) reached the bottom of the gel. Once separated, the proteins were then transferred onto an Amersham Hybond ECL nitrocellulose blotting membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) for two hours at 100V. Afterwards, the membrane was washed in Tris buffered saline with tween (TBST; Appendix II) to remove any traces of methanol (Fisher Scientific, Whitby, ON), followed by blocking with 5 percent skim milk in TBST (Appendix II) or 5 percent BSA in TBST (Appendix II). Membranes were then incubated overnight with the appropriate dilutions of primary antibodies at 4°C. For complete details of antibodies used in western blot analysis, refer to Table 3.

The next day, membranes were washed in TBST and incubated with appropriate secondary antibodies for one hour at room temperature. Afterwards, membranes were washed once more in TBST before addition of Western Lightning plus ECL enhanced luminol reagent (Perkin Elmer, Waltham, MA) or Clarity Western ECL substance (Bio-Rad, Mississauga, ON). Radiographic film (Electro-Medical Equipment Company, Ltd., Richmond Hill ON) and Alpha Imager system or the ChemiDoc MP imager (Bio-Rad, Mississauga, ON) and Image Lab software were used to image and quantify membranes.
Table 3: Western blot analysis antibody details

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody Identification</th>
<th>Protein Loaded (ug)</th>
<th>Species</th>
<th>Primary Conc.</th>
<th>Secondary Conc.</th>
<th>Blocking Solution</th>
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<tr>
<td>Akt</td>
<td>9272</td>
<td>20</td>
<td>Rabbit</td>
<td>1/4000 in 5% skim milk</td>
<td>1/2000 in 5% skim milk</td>
<td>5% skim milk</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>NB100-92142</td>
<td>20</td>
<td>Rabbit</td>
<td>1/500 in 5% skim milk</td>
<td>1/2000 in 5% skim milk</td>
<td>5% skim milk</td>
</tr>
<tr>
<td>Beta Actin</td>
<td>4967</td>
<td>20</td>
<td>Rabbit</td>
<td>1/5000 in 5% skim milk</td>
<td>1/2000 in 5% skim milk</td>
<td>5% skim milk</td>
</tr>
<tr>
<td>CD36</td>
<td>NB400-144</td>
<td>20</td>
<td>Rabbit</td>
<td>1/2000 in 5% skim milk</td>
<td>1/5000 in 5% skim milk</td>
<td>5% skim milk</td>
</tr>
<tr>
<td>GAPDH</td>
<td>2118</td>
<td>20 or 40</td>
<td>Rabbit</td>
<td>1/1000 in 5% BSA</td>
<td>1/2000 in 5% BSA</td>
<td>5% BSA</td>
</tr>
<tr>
<td>Phosphorylated Akt</td>
<td>9271</td>
<td>20</td>
<td>Rabbit</td>
<td>1/1000 in 5% BSA</td>
<td>1/2000 in 5% BSA</td>
<td>5% skim milk</td>
</tr>
<tr>
<td>TSP-1</td>
<td>sc81755</td>
<td>40</td>
<td>Mouse</td>
<td>1/200 in 5% skim milk</td>
<td>1/2000 in 5% skim milk</td>
<td>5% skim milk</td>
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<tr>
<td>VEGF</td>
<td>sc153</td>
<td>20</td>
<td>Rabbit</td>
<td>1/500 in 5% skim milk</td>
<td>1/2000 in 5% skim milk</td>
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<td>VEGFR2</td>
<td>2479</td>
<td>20</td>
<td>Rabbit</td>
<td>1/2000 in 5% BSA</td>
<td>1/4000 in 5% skim milk</td>
<td>5% skim milk</td>
</tr>
</tbody>
</table>

Statistics

All data was analyzed using a one-way analysis of variance (ANOVA) and comparisons between each treatment group were made using the Tukey’s multiple comparison test. To calculate statistical significance in survival data, a log-rank test was performed.
RESULTS

Effects of 3TSR and chemotherapy treatments on EOC progression

At 90 days post tumor induction, mice were sacrificed and ovaries were removed, measure and weighed. All treatment groups had primary ovarian tumors that weighed significantly (p<0.05) less than tumors removed from PBS treated control mice (Figure 3). Tumor area was roughly calculated by multiplying the length of the tumor by its width. These measurements suggested that tumor volume decreased significantly (p<0.05) in all treated mice when compared to untreated controls. In addition, 3TSR and metronomic chemotherapy significantly decreased tumor volume in comparison to mice treated with MTD alone (Figure 3).

Visible secondary lesions throughout the abdominal cavity were counted in order to assess metastatic disease progression. PBS treated mice had significantly (p<0.05) more secondary lesions present throughout the abdominal cavity compared to all treatment groups. In addition, mice treated with 3TSR in combination with metronomic chemotherapy had significantly (p<0.05) fewer visible lesions than all other treatment combinations except for 3TSR in combination with MTD (Figure 4).

Effects of 3TSR and chemotherapy on EOC survival

All mice were removed at onset of morbidity in order to test the effects of 3TSR and chemotherapy on survival (Figure 5). Mice treated with PBS had statistically shorter lives than mice in all other treatments groups. Mice treated with metronomic chemotherapy or MTD chemotherapy alone were statistically different from other treatment groups, but similar to each other. When 3TSR was administered alone or in combination with chemotherapy, there was a
At 90 days post surgery, mice were sacrificed and ovaries were removed, weighed and measured.

A) Representative images of primary tumors both in situ and excised for each treatment group.

B) Tumors in PBS mice weighed significantly \((p<0.05)\) more than all treatment groups. C) Approximate tumor volume was measured and PBS mice had significantly larger tumor volumes than all treatment groups. 3TSR and metronomic chemotherapy significantly decreased tumor volume when compared to MTD alone \((p<0.05)\). Different letters indicate statistical significance. \(n=5\) per treatment group. Error bars represent standard error.
Figure 4: Evaluation of EOC disease progression by secondary lesion counts at 90 days post surgery

Secondary lesions were graded on a scale where no visible lesions received a grade of zero, one to two were designated one plus, three to ten were assigned two plus and more than ten received a three plus. PBS mice had significantly (p<0.05) more visible lesions present throughout the abdominal cavity than other treatment groups. 3TSR and metronomic chemotherapy resulted in significantly (p<0.05) fewer secondary lesions than all treatment combinations other than 3TSR and MTD. Different letters represent statistical significance. n=5 per treatment group. Error bars represent standard error.
Figure 5: Kaplan-Meier survival curve of orthotopic syngeneic mouse model of EOC treated with 3TSR and combinational chemotherapy

Mice were removed at onset of morbidity until the experiment was terminated on day 150. PBS mice lived statistically shorter lives than other treatment groups. MET and MTD groups were similar to each other and statistically different from other groups. 3TSR resulted in significant survival advantages. 3TSR alone or in combination with MTD were similar to each other. Treatments of 3TSR in combination with MET chemotherapy significantly improved survival compared to all other treatment groups. The pink dotted line at day 60 represents the initiation of daily 3TSR treatments and black dotted line at day 74 represents beginning of chemotherapy treatments. A log rank test was used to assess statistical significance.
significant survival advantage in comparison to PBS treatments or chemotherapy treatments alone. The use of 3TSR alone and in combination with MTD chemotherapy was similar to each other. Finally, administering 3TSR in combination with metronomic chemotherapy significantly improved survival when compared to all other treatment groups.

**Blood vessel density in EOC tumors**

In order to study the effects on blood vessel density, two endothelial cell markers were analyzed. CD31 and von Willebrand factor stains vascular tumors with uniformity and were, therefore, chosen to analyze blood vessels in ovarian tumor tissue (Miettinen et al., 1994). According to CD31 staining, all treatments except for MTD chemotherapy alone resulted in a significant (p<0.05) decrease in blood vessel density in comparison to controls (Figure 6). In tissues that were stained with von Willebrand factor, all treatment groups resulted in a significant (p<0.05) decrease in blood vessel density compared to controls (Figure 7). In addition, blood vessel density in tumors treated with 3TSR in combination with metronomic chemotherapy was significantly (p<0.05) lower than all other treatment combinations (Figure 6 & 7).

**Expression of angiogenic factors in EOC tumors**

A combination of immunohistochemistry and western blot analysis were utilized in order to evaluate the expression of angiogenic factors. VEGF and its receptor, VEGFR2, as well as TSP-1 and its proposed receptor, CD36, were all analyzed using these methods. VEGF is a potent pro-angiogenic factor and was found to be significantly (p<0.05) up regulated in the PBS treated control tumors (Figure 8). Furthermore, immunohistochemical analysis illustrated that VEGF expression was significantly (p<0.05) down regulated in tumors that were treated with 3TSR in combination with metronomic chemotherapy (Figure 8). Western blot analysis showed
Figure 6: Immunohistochemical analysis of blood vessel density with CD31 endothelial cell marker

Immunohistochemistry for CD31 expression in tumors from mice treated with 3TSR and chemotherapy at 90 days post tumor induction.  A) Representative images at 200x magnification, including tissues with omitted primary and secondary antibodies as controls.  B) Quantification of CD31 staining.  Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error, different letters represent statistical significance.
Figure 7: Immunohistochemical analysis of blood vessel density staining for von Willebrand Factor

Immunohistochemistry for von Willebrand factor expression in tumors from mice treated with 3TSR and chemotherapy at 90 days post tumor induction. A) Representative images at 200x magnification, including tissues with omitted primary and secondary antibodies as controls. B) Quantification of von Willebrand factor staining. Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error, different letters represent statistical significance.
Figure 8: Immunohistochemical analysis of VEGF expression in EOC primary tumors

Immunohistochemistry for VEGF protein expression in tumors from mice treated with 3TSR and chemotherapy at 90 days post tumor induction. A) Representative images at 200x magnification, including tissues with omitted primary and secondary antibodies as controls. B) Quantification of VEGF staining. Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error, different letters represent statistical significance.
no significant trends (Figure 9). Single bands for VEGF appeared at approximately 20 kDa and GAPDH was used as a loading control.

In histologically stained tissues, VEGFR2 was up regulated in tumors treated with 3TSR both alone or in combination with metronomic chemotherapy. Tumors that were treated with PBS had the lowest expression of VEGFR2 followed closely by tumors treated with 3TSR in combination with MTD chemotherapy (Figure 10). Western blot analysis showed no significant trends (Figure 11). Two bands appeared at approximately 200 kDa and beta-actin was utilized as a loading control.

Expression of TSP-1 was analyzed using an antibody that binds to a segment of the endogenously produced molecule, thereby specifically analyzing the amount of TSP-1 produced by the mouse and not injected with the 3TSR drug. Immunohistochemical analysis showed that TSP-1 expression was significantly up regulated in mice that were treated with metronomic chemotherapy both alone or in combination with 3TSR (Figure 12). The lowest amount of TSP-1 expression was seen in tumors treated with PBS and 3TSR in combination with MTD. Western blot analysis showed no significant trends (Figure 13). Thrombospondin-1 appeared to be cleaved during the denaturing process and was present as two bands at approximately 50 kDa and 25 kDa. GAPDH was utilized as the loading control.

When evaluating CD36 expression in both immunohistochemistry and western blot analysis, no significant differences were seen between treatment groups (Figure 14). According to western blot analysis, there was a slight up-regulation in CD36 expression in tissues treated with 3TSR in combination with metronomic chemotherapy, however, this increase was not statistically significant (Figure 15). CD36 appeared as a single band at approximately 50 kDa.
Figure 9: Western blot analysis of VEGF expression in EOC primary tumors

A) Immunoblot of VEGF expression in primary tumors isolated from each treatment group. Denaturing conditions revealed 20 kDa (approx.) bands with the use of GAPDH as a loading control. B) Densitometry measurements of three blots with different tumor samples are presented in the bar graph with no significant differences (p<0.05). Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error.
Figure 10: Immunohistochemical analysis of VEGFR2 expression in EOC primary tumors

Immunohistochemistry for VEGFR2 expression in tumors from mice treated with 3TSR and chemotherapy at 90 days post tumor induction. A) Representative images at 200x magnification, including tissues with omitted primary and secondary antibodies as controls. B) Quantification of VEGFR2 staining. Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error, different letters represent statistical significance.
Figure 11: Western blot analysis of VEGFR2 expression in EOC primary tumors

A) Immunoblot of VEGFR2 expression in primary tumors isolated from each treatment group. Denaturing conditions revealed 200 kDa (approx.) bands with the use of beta-actin as a loading control. B) Densitometry measurements of three blots with different tumor samples are presented in the bar graph with no significant differences (p<0.05). Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error.
Figure 12: Immunohistochemical analysis of thrombospondin-1 expression in EOC primary tumors

Immunohistochemistry for TSP-1 protein expression in tumors from mice treated with 3TSR and chemotherapy at 90 days post tumor induction. A) Representative images at 200x magnification, including tissues with omitted primary and secondary antibodies as controls. B) Quantification of TSP-1 staining. Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error, different letters represent statistical significance.
**Figure 13: Western blot analysis of thrombospondin-1 expression in EOC primary tumors**

A) Immunoblot of TSP-1 expression in primary tumors isolated from each treatment group. Denaturing conditions revealed two bands at 50 kDa and 25 kDa (approx.) with the use of GAPDH as a loading control.  

B) Densitometry measurements of three blots with different tumor samples are presented in the bar graph with no significant differences (p<0.05). Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error.
**Figure 14: Immunohistochemical analysis of CD36 expression in EOC primary tumors**

Immunohistochemistry for CD36 expression in tumors from mice treated with 3TSR and chemotherapy at 90 days post tumor induction.  
A) Representative images at 200x magnification, including tissues with omitted primary and secondary antibodies as controls.  
B) Quantification of CD36 staining. Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error.
Figure 15: Western blot analysis of CD36 expression in EOC primary tumors

A) Immunoblot of CD36 expression in primary tumors isolated from each treatment group. Denaturing conditions revealed 50 kDa (approx.) bands with the use of GAPDH as a loading control. B) Densitometry measurements of three blots with different tumor samples are presented in the bar graph with no significant differences (p<0.05). Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error.
and GAPDH was used for the loading control. Overall there was little variability in CD36 over the six treatment groups.

**Survival factors do not vary between treatment groups**

To determine the expression of survival factors, immunohistochemistry and western blot analysis was utilized. In mice, Ki67 is expressed solely in proliferating cells, therefore, immunohistochemical staining for this marker allowed visualization and quantification of proliferating tissue within primary tumors (Winking et al., 2004). Ki67 expression did not vary greatly between treatment groups and no significant differences were seen (Figure 16).

Western blot analysis of Akt, activated phosphorylated Akt and Bcl-2 showed slight up regulation in 3TSR and metronomic chemotherapy treatment groups, however, there were no significant differences in expression of these survival molecules (Figure 17). Akt and phosphorylated Akt appeared as a single band at approximately 50 kDa, while, Bcl-2 appeared as a single band at approximately 25 kDa. Beta-actin was utilized as a loading control and emerged as a single band at approximately 37 kDa.

**Cell death up regulated in primary tumors after 3TSR treatments alone and in combination with metronomic chemotherapy**

Caspase-3 is a crucial mediator of cellular apoptosis, thereby providing a convenient marker for visualizing and quantifying cell death in tissues (Porter and Janicke, 1999). Immunohistochemical staining of cleaved caspase-3 was utilized to examine cell death in primary tumors of varying treatment groups. Cleaved caspase-3 expression was significantly (p<0.05) up regulated in tumors treated with 3TSR alone and in combination with metronomic chemotherapy (Figure 18). Cleaved caspase-3 was slightly up regulated in tissues treated with
Figure 16: Immunohistochemical analysis of proliferation in EOC primary tumors

Immunohistochemistry for Ki67 protein expression in tumors from mice treated with 3TSR and chemotherapy at 90 days post tumor induction. A) Representative images at 200x magnification, including tissues with omitted primary and secondary antibodies as controls. B) Quantification of Ki67 staining. Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error.
Figure 17: Western blot analysis of survival proteins in EOC primary tumors

A) Immunoblot of Akt expression in primary tumors. Denaturing conditions revealed 50 kDa (approx.) band with the use of beta-actin as a loading control. Densitometry measurements of three blots with different tumor samples are presented in the bar graph with no significant differences (p<0.05).  

B) Immunoblot of phosphorylated Akt expression in primary tumors. Denaturing conditions revealed 50 kDa (approx.) band with the use of beta-actin as a loading control. Densitometry measurements of three blots with different tumor samples are presented in the bar graph with no significant differences (p<0.05).  

C) Immunoblot of Bcl-2 expression in primary tumors. Denaturing conditions revealed 25 kDa (approx.) band with the use of beta-actin as a loading control. Densitometry measurements of three blots with different tumor samples are presented in the bar graph with no significant differences (p<0.05). Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error.
Figure 18: Immunohistochemical analysis of cell death in EOC primary tumors

Immunohistochemistry for cleaved caspase-3 protein expression in tumors from mice treated with 3TSR and chemotherapy at 90 days post tumor induction. A) Representative images at 200x magnification, including tissues with omitted primary and secondary antibodies as controls. B) Quantification of cleaved caspase-3 staining. Data was analyzed using a one-way ANOVA with Tukey’s test, n=3, error bars represent standard error, different letters represent statistical significance.
only metronomic chemotherapy and was least expressed in tumors treated with PBS and MTD alone or in combination with 3TSR.
DISCUSSION

This study has demonstrated an effective and novel treatment for advanced stage EOC. The use of 3TSR in combination with metronomic chemotherapy not only impacts disease progression, but also results in a statistically significant survival advantage.

The application of TSP-1 has been studied in many solid tumors such as pancreatic cancer, squamous cell carcinomas, prostate cancer and ovarian cancer (Zhang et al., 2005, Streit et al., 1999, Jin et al., 2000, Greenaway et al., 2009). Dose response curves performed on B16F10 tumor-bearing mice showed that the 3TSR mimetic peptide was more effective at inhibiting tumor growth than TSP-1 as a whole when administered at concentrations of 1.0 mg/kg/day or higher (Miao et al., 2001). Ideal 3TSR activity was seen at 2.5 mg/kg/day with an 81 percent reduction in tumor volume, illustrating the potency of this molecule (Miao et al., 2001). When administered alone in an orthotopic human pancreatic cancer model at 3.0 mg/kg/day, 3TSR showed a significant 69 percent reduction in tumor volume as well as an increase in necrotic tumor tissue (Zhang et al., 2005). However, when 3TSR was used in combination with an apoptosis-inducing ligand, doses as small as 1.0 mg/kg/day could have significant effects on tumor growth (Ren et al., 2009). For example, when used in combination with Lexatumumab, a tumor necrosis factor-related apoptosis-inducing ligand, 3TSR at 1.0 mg/kg/day inhibited tumor growth by 93 percent (Ren et al., 2009). Because this study examined the effects of 3TSR in combination with chemotoxic drugs, 3TSR was administered at a concentration of 1.0 mg/kg/day.

Doses for metronomic and MTD chemotherapy were chosen after careful consideration of both the literature and the challenges faced within this particular study. In the literature, carboplatin was administered intraperitoneally at concentrations ranging from 7.0 mg/kg to 50.0
mg/kg (Alagkiozidis et al., 2011, Alami et al., 2008, Chang et al., 2013, Jandial et al., 2009). Intraperitoneal injections of paclitaxel ranged from 12.0 mg/kg to 20.0 mg/kg (Alagkiozidis et al., 2011, Chang et al., 2013, Hu et al., 2002, Kubota et al., 1997). Because this study administered both carboplatin and paclitaxel simultaneously, each drug concentration was chosen below the individual MTD in order to decrease the chances of overlapping toxicities. Therefore, MTD concentrations of 10.0 mg/kg were used for both carboplatin and paclitaxel.

Metronomic concentrations, on the other hand, were chosen to be substantially smaller than the MTD concentrations. Paclitaxel was administered at 1.0 mg/kg and carboplatin was administered at 2.0 mg/kg. In a seven-day period, MTD chemotherapy was administered two times and metronomic chemotherapy was administered four times. Although the cumulative dosage of MTD chemotherapy outweighed metronomic chemotherapy, this was not considered to be a negative influence on the results. In fact, previous literature argued that a cumulative metronomic dose as little as one-fifth of the MTD cumulative dose still had a significant impact on therapeutic response (Kamat et al., 2007). Therefore, when studying metronomic chemotherapy one can consider the popular saying, “less is more”.

Paclitaxel is a naturally occurring anti-tumor agent that was discovered after its isolation from the Pacific yew tree (Wani et al., 1971). Paclitaxel acts by promoting the assembly and stabilization of microtubules in cells, thereby inhibiting the dynamic nature of microtubule activity (Huizing et al., 1995). It is a highly hydrophobic molecule and is, therefore, usually suspended in a mixture of Cremophor EL and dehydrated ethanol (Sparreboom et al., 1996). This vehicle, however, impacts the pharmacokinetics of paclitaxel and is even believed to be the cause of paclitaxel’s nonlinear pharmacokinetic behavior (Sparreboom et al., 1996). In fact, the Cremophor EL vehicle limits the MTD of paclitaxel that can be administered intravenously due
to its acute lethal toxicities (Eiseman et al., 1994). Analysis of paclitaxel administered intravenously in mice at two concentrations of 2.0 mg/kg and 10.0 mg/kg not only showed a 30-fold increase in peak plasma concentration, but also revealed a bi-exponential decay (Sparreboom et al., 1996). In addition, the terminal half-life of paclitaxel suspended in Cremophor EL was found to be approximately 17 hours (Sparreboom et al., 1996).

Carboplatin is a platinum-based anti-tumor analogue derived from its parent compound cisplatin. Platinum drugs covalently bind to DNA, forming DNA adducts, thereby disrupting DNA function and repair and ultimately resulting in cellular apoptosis (Kelland, 2007). When administered intravenously in humans, carboplatin has a terminal half-life of approximately 5.8 days (van der Vijgh, 1991). In addition, when administered intraperitoneally in humans, carboplatin is cleared at approximately 15 ml/min from the peritoneal cavity (van der Vijgh, 1991). However, terminal half-life for carboplatin is shorter in mice than humans (van Hennik et al., 1987). In addition, following intravenous administration, carboplatin renal clearance occurs at approximately 9.3 ml/min/kg in mice (Siddik et al., 1987).

Intraperitoneal (IP) injections of chemotherapeutics allow the drug to be administered directly into the peritoneal cavity. This method offers a biochemical advantage to patients suffering with advanced stage ovarian cancer where the disease is disseminated throughout the peritoneal cavity. Pharmacokinetic calculations suggested that IP administration allows the drug to sustain significantly higher concentrations in the peritoneal cavity than in the blood circulation (Dedrick et al., 1978). In addition, localized administration of the drug offers some protection from adverse side effects at distant sites of the body, such as the bone marrow (Armstrong et al., 2006). In fact, IP administration of chemotherapy drugs has been shown to significantly improve survival in women with advanced stage disease (Armstrong et al., 2006). This data was so
compelling that the National Cancer Institute suggested that IP administration should be used in all women with optimally debulked stage III disease (Bowles et al., 2014). Therefore the use of IP injections in this study is extremely relevant to clinical applications. Nevertheless, intravenous (IV) administration of combinational chemotherapy remains the standard procedure for EOC treatments today (Ledermann et al., 2013).

Thrombospondin-1 has been analyzed for its use in both IV and IP injections in mice. For example, in 1998, TSP-1 was injected intraperitoneally and, thereafter, the presence of TSP-1 in blood circulation impacted the effects on tumor burden (Volpert et al., 1998). Volpert and associates documented that 6.2 µg of TSP-1 per milliliter of plasma in circulation inhibited metastatic growth (Volpert et al., 1998). In addition, they noted that 0.82 µg/ml slowed metastatic growth and finally 0.13 µg/ml had no effects (Volpert et al., 1998). Following this study, Milao and associates discovered that high concentrations of TSP-1 are less effective at inhibiting tumor growth when injected IP, but more active when administered intravenously at the same concentration (Milao et al., 2001). It was suggested that the discrepancies in effects were not necessarily a result of the direct interaction of the drug with the tumor, but the drug’s ability to access the blood stream after IP injections (Milao et al., 2001). However, TSP-1 did, in fact, decrease tumor volume after both IP and IV administration (Milao et al., 2001). It is important to highlight that both of these studies were completed with tumors that were injected subcutaneously and located outside of the peritoneal cavity, therefore, may not be applicable to peritoneal diseases, such as ovarian cancer. Currently, research is being performed by Dr. Jack Lawler to determine the bioavailability of specifically 3TSR in the blood circulation. In addition, little is known about the use of 3TSR by oral administration. More research into this
area of administration could prove to be clinically relevant, as oral administration would be a convenient route for daily doses.

The orthotopic syngeneic mouse model of EOC utilized in this study closely resembles the phenotype of human EOC, thereby providing an effective pre-clinical model (Greenaway et al., 2008). The primary tumors isolated from this model are classified as serous adenocarcinomas, the most common type of EOC in humans (Lee et al., 2003). After surgical induction of EOC, mice were given 60 days to develop large primary tumors, visible secondary lesions and abdominal ascites before treatments commenced. Women are usually diagnosed in stage III or stage IV, therefore, this wait period allowing mice to reach advanced stage disease is essential in order to make this study more clinically relevant.

At 90 days post tumor induction, tumor size was evaluated. All treatments significantly inhibited primary tumor growth in comparison to PBS treated controls (Figure 3). For example, primary tumors treated with MTD chemotherapy alone decreased in size by about 60 percent. Treatments with 3TSR in combination with MTD produced an approximate decrease of 72 percent in tumor size. This was followed by a 75 percent and 76 percent decrease when treated with metronomic chemotherapy alone and 3TSR alone respectively. Finally, when treated with 3TSR in combination with metronomic chemotherapy, primary tumors shrunk by approximately 85 percent.

In addition to primary tumor size, disease progression was also assessed at the 90 day time point (Figure 4). By counting the number of visible secondary lesions throughout the abdominal cavity, each mouse was graded on a three plus scale. Mice having no secondary lesions were assigned a zero, one to two lesions were designated as a one plus, three to ten lesions were given a two plus and more than ten lesions earned a three plus. All mice in the PBS
control group were assigned a three plus and no other mice in any other treatment groups presented with more than ten visible lesions. This suggests that the untreated controls presented with the most advanced stage EOC at 90 days. A median rating of two plus was seen in mice treated with 3TSR alone and metronomic chemotherapy alone. A median of one plus was seen in both MTD alone and in combination with 3TSR. Finally, a median of zero was seen in mice that were treated with a combination of 3TSR and metronomic chemotherapy, suggesting the most regressed state of EOC at 90 days. These results suggested that all treatment groups impacted the progression of EOC and even resulted in the regression of advanced stage disease. The time-point data collected at 90 days also provided some insight into the survival data.

Treatment groups with fewer visible secondary lesions at the 90 day time point generally survived longer than mice with more lesions. For instance, mice treated with 3TSR in combination with metronomic chemotherapy had the fewest visible lesions at the 90 day time point, suggesting that the disease had regressed. In addition, this treatment group survived significantly longer than all other treatment groups (Figure 5). On the other hand, PBS treated control mice had the highest concentration of secondary lesions throughout the abdominal cavity and thereby became moribund significantly earlier than all other treatment groups. All treatment groups had a significant impact on disease progression as seen by the size of primary tumors and severity of secondary disease and these effects lead to varying survival advantages.

Previous data has shown that 3TSR significantly effects blood vessel density in orthotopic models (Zhang et al., 2005, Petrik, unpublished). For example, in an orthotopic model of pancreatic cancer, 3TSR was found to significantly decrease microvessel density from 8 percent to 3.7 percent (Zhang et al., 2005). In addition, previous work in the Petrik lab illustrated that administration of 3TSR alone not only decreased blood vessel density, but also
resulted in the normalization and possible maturation of the vasculature in an orthotopic model of ovarian cancer (Petrik, unpublished). The data collected in this study supports the anti-angiogenic effects of 3TSR. All treatments that included 3TSR, either alone or in combination, resulted in a significant reduction in blood vessel density (Figure 6 & 7). In addition, metronomic chemotherapy seemed to enhance these anti-angiogenic effects. It is believed that the administration of chemotherapy drugs at more frequent intervals targets tumor endothelial cells, thereby directly resulting in anti-angiogenic effects (Hanahan et al., 2000). Therefore, treatments that include both 3TSR and metronomic chemotherapy resulted in significantly lower blood vessel densities (Figure 6 & 7).

Previous work in the Petrik laboratory showcased 3TSR’s ability to reduce VEGF expression in a dose-dependent manner in vitro (Petrik, unpublished). Moreover, TSP-1 is documented in the literature to inhibit VEGF expression by various mechanisms (Greenaway et al., 2007, Rodriguez-Manzaneque et al., 2001). The immunohistochemical results of this study support previous findings as 3TSR administration either alone or in combination decreased VEGF expression in ovarian tumors in comparison to the untreated control (Figure 8). Surprisingly, this inhibition was enhanced by the addition of metronomic chemotherapy, suggesting that the anti-angiogenic effects of 3TSR are magnified when used in combination with anti-angiogenic chemotherapy. On the other hand, western blot analysis did not support previous findings and showed no significant differences in VEGF expression between treatment groups (Figure 9). This could be due to the antigen specificity in each experimental procedure. For example, in western blot analysis, it is possible to analyze a single band at a specific molecular weight, but in immunohistochemical analysis there may be non-specific binding.
Therefore, more work will need to be done in the future in order to determine which experimental procedure is more accurate.

Immunohistochemical analysis of primary tumors unveiled a novel trend in VEGFR2 expression. Treatments of 3TSR alone or in combination with metronomic chemotherapy caused a significant increase in VEGFR2 expression (Figure 10). This up-regulation in VEGFR2 could possibly be a response to compensate for the decrease in ligand availability. According to the literature, surface levels of receptors are regulated by the availability of ligands (Condic and Letourneau, 1997). Therefore, as 3TSR and metronomic chemotherapy decrease the prevalence of VEGF, tumor cells may respond by increasing the concentration of VEGF receptors. However, these results were not supported by other experiments in this study. According to western blot analysis, there were no significant differences in VEGFR2 expression between treatment groups (Figure 11). Moreover, previous in vitro work showed that even with increasing concentrations of 3TSR, VEGFR2 expression did not significantly change (Petrik, unpublished). The discrepancy between the immunohistochemistry and western blot data may be a result of the limitations of the experiments utilized. For example, immunohistochemistry looks at a single section in an otherwise heterogeneous tumor, whereas western blot analysis analyzes the tumor as a whole.

Thrombospondin-1 is believed to play a crucial role in the anti-angiogenic effects of metronomic chemotherapy (Bocci et al., 2003). When endothelial cells are exposed to metronomic chemotherapy in vitro, they induce expression of TSP-1 both at the gene and protein levels (Bocci et al., 2003). In addition, metronomic chemotherapy administration in vivo resulted in an up-regulation of TSP-1 in blood circulation (Bocci et al, 2003). Therefore, TSP-1 is thought to be a mediator of anti-angiogenic chemotherapy. The immunohistochemical data
presented in this study support previous findings, as endogenous production of TSP-1 was upregulated in mice that were given metronomic chemotherapy either alone or in combination with 3TSR (Figure 12). However, western blot analysis did not reveal the same trend and found no significant differences (Figure 13). The discrepancies between immunohistochemical data and western blot data may be due to the experimental limitations described previously and more work needs to be done in the future to determine the more accurate results.

Interactions between TSP-1 and its receptor CD36 are essential for regulating angiogenesis in ovarian tissue (Osz et al., 2014). Therefore, it was important to analyze the expression of CD36 between treatment groups. No significant variations in CD36 expression were reported in either immunohistochemistry or western blot analysis (Figure 14 & 15). This data could be seen in a positive light, because little variation in TSP-1 receptors may suggest that the overall tumor growth suppression and decreased blood vessel density was a treatment-specific result and not due to the overexpression or absence of CD36. However, the data was somewhat surprising, as the TSP-1 receptor does not vary between tissues even though ligand availability does. One explanation may be due to the activity of ligand binding and receptor recycling in the cell at the time of tissue fixation.

Currently, there is conflicting data when it comes to the effects of 3TSR on cellular proliferation. Zhang and associates treated pancreatic cancer cells in vitro with 3TSR and found that there was no significant effect on cell cycle progression (Zhang et al., 2005). On the other hand, Petrik and associates studied the expression of Ki67 in ovarian cancer cells and found that 3TSR significantly decreased the number of cells undergoing proliferation (Petrik, unpublished). However, the results outlined in this study support the findings of Zhang and associates.
Immunohistochemical staining of Ki67, a known marker of cellular proliferation in mice, showed no significant differences in proliferation between treatment groups (Figure 16).

One of the mechanisms that allow TSP-1 to induce endothelial cell death and inhibit angiogenesis is the alteration of survival protein gene expression (Nör et al., 2000). Specifically, TSP-1 has been documented to down-regulate the expression of Bcl-2, a well-known survival protein (Nör et al., 2000). Previous work in the Petrik laboratory has also confirmed that 3TSR decreased Bcl-2 expression in a dose-dependent manner when used in ovarian cancer cells in vitro (Petrik, unpublished). Both Bcl-2 and Akt survival proteins were analyzed by western blot analysis in this study, however, no significant differences were seen in Akt, activated Akt or Bcl-2 expression (Figure 17).

In addition to cellular proliferation, this study analyzed apoptosis. Previous TUNEL data confirmed that 3TSR induced apoptosis in both endothelial cells and ovarian cancer cells (Zhang et al., 2005, Petrik, unpublished). In addition, it is believed that 3TSR can enhance both internal and external apoptosis pathways (Nör et al., 2000, Petrik, unpublished). Thrombospondin-1, as a whole, has been shown to activate caspase-3, thus activating the internal apoptosis caspase cascade (Nör et al., 2000). Moreover, 3TSR is documented to increase the amount of cleaved caspase-3 expressed in ovarian cancer cells in a dose-dependent manner in vitro (Petrik, unpublished). In addition, 3TSR significantly increased the expression of Fas ligand, thereby increasing the chances of activating the external apoptosis pathway (Petrik, unpublished). This study has illustrated that 3TSR administration alone and in combination with metronomic chemotherapy significantly up regulated the expression of cleaved caspase-3, suggesting that there was a significant up-regulation in cellular apoptosis (Figure 18).
increase in apoptosis may explain not only the decrease in blood vessel density, but also the
decrease in overall primary tumor size.

**Study Limitations**

One of the most prominent limitations in this study was the availability of tumor
tissue. When the 90 day tumors were collected, half of the tumors were formalin fixed and
paraffin embedded for immunohistochemical analysis and half were flash frozen in liquid
nitrogen for protein collection. The tumors themselves were fairly small, thereby, limiting the
amount of tissue for replicates. In addition, tissue preparation limited the experimental methods
that could be performed. If more tissue were available, it would have been flash frozen for
cryosectioning. Cryosectioning is known to better preserve the antigenicity of tissues in
comparison to paraffin embedding, which leads to superior antigen detection (Fischer et al.,
2008). Cryosectioning would have been a more efficient method for staining and analyzing
blood vessel density.

Previous work in the Petrik laboratory has documented that 3TSR decreased the
accumulation of phosphorylated VEGFR2 (Petrik, unpublished). Unfortunately, this study does
not include data regarding the phosphorylated state of VEGFR2. Multiple VEGFR2 antibodies
were utilized, however, none could be optimized for the ovarian cancer tissue during the duration
of the study.

**Future Directions**

This study focused on the analysis of the 90 day primary tumor tissue, however, blood
serum was also collected at the 90 day time point. Analysis of the serum for circulating
angiogenic molecules would add an interesting dimension to this study and may provide some
insight into disease progression between treatment groups.
One exciting branch of research is looking at the use of adeno-associated viruses as a method of sustained drug release (Zhang et al., 2007). In fact, the use of 3TSR in a recombinant adeno-associated virus (rAAV) in a model of pancreatic cancer has already shown promise for this method of administration, resulting in both localized and systemic anti-angiogenic effects (Zhang et al., 2007). It would be interesting to see the effects of rAAV 3TSR gene therapy in combination with metronomic chemotherapy, as the chronic release of 3TSR would eliminate the need for daily injections.

In addition, 3TSR has been incorporated into a fusion protein, which increases the stability of the drug in vivo. Utilizing the 3TSR fusion protein may prove to be superior over 3TSR alone. However, more research needs to be completed in order to determine the effects of this fusion protein on ovarian cancer cells both in vitro and in vivo.

Finally, pre-clinical data has provided strong evidence for the use of 3TSR in a clinical setting. In the future, we hope to see 3TSR utilized in humans with advanced stage disease.
SUMMARY AND CONCLUSIONS

This study analyzed the efficacy of 3TSR and combinational metronomic chemotherapy on advanced stage EOC. Previous work in the Perik laboratory illustrated 3TSR’s ability to normalize tumor vasculature, increase tissue perfusion and increase chemotherapy uptake in ovarian tumors (Petrik, unpublished). Intraperitoneal 3TSR was administered with combinational chemotherapy in an orthotopic syngeneic mouse model of EOC and primary tumors were collected at the 90 day time point. The data showed that all treatment groups significantly decreased primary tumor size in comparison to the untreated control (Figure 3). In addition, analysis of secondary disease showed evidence of disease regression in mice treated with 3TSR in combination with metronomic chemotherapy (Figure 4). Disease regression at the 90 day time point directly correlated to survival advantages. For example, untreated mice had the largest tumors at the 90 day time point and became moribund significantly earlier than the other treatment groups (Figure 5). Moreover, mice treated with 3TSR and metronomic chemotherapy had the smallest tumors and fewest secondary lesions at 90 days and survived statistically longer than other treatment groups (Figure 5). Tumors collected at 90 days were analyzed for blood vessel density and mice treated with 3TSR in combination with metronomic chemotherapy appeared to have significantly lower microvascular densities when compared to the majority of other treatments (Figure 6 & 7). Treatments did not significantly impact cellular proliferation within tumor tissue, nor did they affect the expression of the TSP-1 receptor CD36 (Figure 14 & 16). However, 3TSR alone or in combination with metronomic chemotherapy significantly down regulated the expression of VEGF, a potent pro-angiogenic marker, and significantly up regulated the expression of cleaved caspase-3, suggesting increased apoptosis (Figure 8 & 18). In conclusion, the results from this study suggest that 3TSR in combination
with metronomic chemotherapy has a significant effect on tumor burden and may be a possible clinical treatment for EOC in the future.
LITERATURE CITED


Boll F. Das, Princip des Wachstum. Berlin (1876).


Iruela-Arispe ML, Lombardo M, Krutzsch HC, Lawler J, Roberts DD. Inhibition of angiogenesis by thrombospondin-1 is mediated by 2 independent regions within the type 1 repeats. Circulation. 1999 Sep 28;100(13):1423-31.


Sparreboom A, van Tellingen O, Nooijen WJ, Beijnen JH. Nonlinear pharmacokinetics of paclitaxel in mice results from the pharmaceutical vehicle Cremophor EL. Cancer Res. 1996 May 1;56(9):2112-5.


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Cytoseal-XYL mounting media
Thermo Fisher Scientific, Nepean, ON

DC Protein Quantification kit
Bio-Rad, Mississauga, ON

Dithiothreitol (DTT; western blot)
Life Technologies Inc., Burlington, ON

Dulbecco’s Modified Eagle Medium (DMEM)
Life Technologies Inc., Burlington, ON

Ethanol
Greenfield Ethanol Inc., Brampton, ON

Ethylene diaminetetraacetic acid (EDTA)
Fisher Scientific, Whitby, ON

ExtrAvidin - Peroxidase
Sigma-Aldrich Canada Ltd., Oakville, ON

Fetal bovine serum (FBS)
Life Technologies Inc., Burlington, ON

Glycerol
Fisher Scientific, Whitby, ON

Glycine
Fisher Scientific, Whitby, ON

Hamilton syringe
Fisher Scientific, Whitby, ON

Hematoxylin
Fisher Scientific, Whitby, ON

ImmEdge Hydrophobic Barrier Pen
Vector Laboratories, Burlington, ON

L-glutamine
Life Technologies Inc., Burlington, ON

Leupeptin
Sigma-Aldrich Canada Ltd., Oakville, ON

Methanol
Fisher Scientific, Whitby, ON

Nuclease-free microcentrifuge tubes
Fisher Scientific, Whitby, ON

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Paclitaxel
Biolyse Pharma Corporation, St. Catharines, ON

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Sigma-Aldrich Canada Ltd., Oakville, ON

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APPENDIX II – RECIPES FOR SOLUTIONS

20x Phosphate Buffered Saline (PBS; Stock; Immunohistochemistry)

- Sodium chloride ........................................................................................................... 60.0g
- Potassium chloride ....................................................................................................... 4.0g
- Sodium potassium dibasic anhydrous ....................................................................... 23.0g
- Potassium phosphate monobasic ............................................................................... 4.0g
- Reverse osmosis water (RO H₂O) .............................................................................. up to 1L

1x PBS (Immunohistochemistry)

- 20x PBS .................................................................................................................... 200mL
- RO H₂O ..................................................................................................................... 3.8L

Antibody Diluting Fluid (1% BSA)

- BSA powder ............................................................................................................... 1.0g
- Sodium azide ............................................................................................................ 0.02g
- 1x PBS ....................................................................................................................... up to 100mL

Immunohistochemistry Blocking Solution (5% BSA)

- BSA powder ............................................................................................................... 10g
- Sodium azide ............................................................................................................ 0.2g
- 1x PBS ....................................................................................................................... up to 200mL

1.0% Hydrogen Peroxide

- 30% Hydrogen peroxide solution ............................................................................. 6.6mL
- 1x PBS ....................................................................................................................... up to 200mL

Citrate Buffer

Stock A – Citrate Buffer
Citric Acid ......................................................................................................................2.1g
RO H₂O .........................................................................................................................up to 100mL

Stock B – Citrate Buffer
Sodium citrate dihydrate ...............................................................................................14.7g
RO H₂O .........................................................................................................................up to 500mL

Working Citrate Buffer
Stock A .........................................................................................................................18mL
Stock B .........................................................................................................................82mL
RO H₂O .........................................................................................................................up to 1L
pH to 6.0

Working Citrate with Tween-20 Buffer
Stock A .........................................................................................................................18mL
Stock B .........................................................................................................................82mL
RO H₂O .........................................................................................................................up to 1L
pH to 6.0 then continue
Tween-20 ......................................................................................................................0.5mL

Tris-Ethylenediaminetetraacetic acid with Tween-20 Buffer
Tris base ..........................................................................................................................1.21g
Ethylenediaminetetraacetic acid (EDTA) ......................................................................0.37g
RO H₂O .........................................................................................................................up to 1L
pH to 9.0 then continue
Tween-20 ......................................................................................................................0.5mL

Carazzi’s Hematoxylin
Hematoxylin ...........................................................................................................0.25g
Glycerol ................................................................................................................50mL
Aluminum potassium sulfate ..............................................................................12.5g
Potassium iodate .................................................................................................0.05g
RO H₂O ...........................................................................................................200mL

**RIPA Lysis Buffer**

10mM Tris HCl .................................................................................................0.788g
RO H₂O ...........................................................................................................up to 495mL

pH to 7.6 then continue

5mM EDTA ....................................................................................................0.7306g
50mM Sodium chloride ................................................................................1.461g
30mM Tetrasodium pyrophosphate ...............................................................3.988g
1% Triton X-100 .........................................................................................5mL

Protease Inhibitors (add fresh before each use) ............................................Per mL RIPA

Aprotinin (2.5 mg/mL) ................................................................................2µL
Phenylmethanesulfony fluoride (PMSF; 0.871 g/mL) ..................................20µL
Sodium Orthovanadate (0.1 mM) ...............................................................2µL
Sodium fluoride (50.0 mM) ........................................................................50µL
Pepstatin A (1 mg/mL) .................................................................................1µL
Leupeptin (2 mg/mL) ...................................................................................1µL

**3x Reducing Buffer**

10% SDS ......................................................................................................2mL
Glycerol ........................................................................................................1mL
1M Tris HCl ..........................................................................................................................0.5mL

Tris HCl ..............................................................................................................................1.576g

RO H₂O ................................................................................................................................up to 10mL

pH to 6.8

Bromeophenol Blue .............................................................................................................10mg

RO H₂O ................................................................................................................................up to 9mL

Dithiothreitol (DTT) ...........................................................................................................1:7 ratio

12% Separating PAGE Gel

RO H₂O ..................................................................................................................................3.3mL

30% Acrylamide ...................................................................................................................4mL

1.5M Tris buffer ....................................................................................................................2.5mL

Tris base ..............................................................................................................................18.16g

RO H₂O ................................................................................................................................up to 100mL

pH to 8.8

10% SDS ............................................................................................................................0.1mL

10% Ammonium persulfate (APS) ......................................................................................0.1mL

APS ......................................................................................................................................0.1g

RO H₂O .................................................................................................................................1mL

Tetramethylethylenediamine (TEMED) ...........................................................................8µL

Pour into gel mold, leaving space for stacking gel. Fill mold to top with RO H₂O until gel resolved

8% Separating PAGE Gel

RO H₂O ..................................................................................................................................4.6mL

103
30% Acrylamide ........................................................................................................2.7mL

1.5M Tris buffer ........................................................................................................2.5mL

Tris base ...................................................................................................................18.16g

RO H₂O ...........................................................................................................up to 100mL

pH to 8.8

10% SDS ............................................................................................................0.1mL

10% Ammonium persulfate (APS) ............................................................................0.1mL

APS .....................................................................................................................0.1g

RO H₂O ...........................................................................................................1mL

Tetramethylethylenediamine (TEMED) ................................................................12µL

Pour into gel mold, leaving space for stacking gel. Fill mold to top with RO H₂O
until gel resolved

5% Stacking PAGE Gel

RO H₂O ............................................................................................................2.1mL

30% Acrylamide ....................................................................................................0.5mL

1.0M Tris buffer ...................................................................................................0.38mL

Tris base .............................................................................................................12.12g

RO H₂O ...........................................................................................................up to 100mL

pH to 6.8

10% SDS .........................................................................................................30µL

10% APS .........................................................................................................30µL

APS ..................................................................................................................0.1g

RO H₂O .......................................................................................................1mL
TEMED ....................................................................................................................................... 6µL

Pour into mold on top of separating gel. Place comb into mold and allow to resolve.

**5x Running Buffer (Tris-Glycine Buffer)**

- Tris base .................................................................................................................................... 15.1g
- Glycine ......................................................................................................................................... 72.1g
- 10% SDS ..................................................................................................................................... 10mL
- RO H₂O ...................................................................................................................................... up to 1L

**1x Running Buffer (Tris-Glycine Buffer)**

- 5x Running buffer ......................................................................................................................... 200mL
- RO H₂O .................................................................................................................................... 800mL

**10x Transfer Buffer**

- Tris base .................................................................................................................................... 30.2g
- Glycine ........................................................................................................................................ 144g
- RO H₂O ...................................................................................................................................... up to 800mL

**1x Transfer Buffer**

- Methanol ................................................................................................................................... 200mL
- RO H₂O ...................................................................................................................................... up to 900mL
- 10x Transfer buffer ......................................................................................................................... 100mL

**10x Tris buffered saline (TBS)**

- Tris base .................................................................................................................................... 24.2g
- Sodium chloride ............................................................................................................................ 80.0g
- RO H₂O ...................................................................................................................................... up to 1L
pH to 7.6

1x Tris buffered saline with Tween-20 (TBST)

- 10x TBS .................................................................................................................. 100mL
- RO H₂O ............................................................................................................... up to 1L
- Tween-20 ............................................................................................................. 1.0mL

5% Skim milk in TBST

- Skim milk powder ............................................................................................... 5.0g
- TBST .................................................................................................................. up to 100mL

5% BSA in TBST

- BSA .................................................................................................................... 5.0g
- TBST ................................................................................................................ up to 100mL