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Cytotoxic Effects of Ruthenium Compounds on Human Cancer Cell Lines.

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ABSTRACT

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Chemotherapy is the most common cancer treatment. Traditionally, platinum-based drugs are used in chemotherapy. More recently, researchers have focused on ruthenium based compounds as a substitute for the platinum compounds. Ruthenium-based compounds appear to be less toxic to healthy cells than traditional platinum-based compounds. In this study, 7 ruthenium-based compounds were tested on HT-29 (colon) and MCF-7 (breast) human cancer cell lines with the specific aim of determining whether or not any of the ruthenium-based compounds exhibited cytotoxic properties. In addition, levels of vascular endothelial growth factor (VEGF) production were tested in supernate from the cancer cells treated with various ruthenium-based compounds to determine whether or not the ruthenium-based compounds had an effect their VEGF production. Our results indicate that none of the ruthenium based compounds tested had a cytotoxic effect on the cancer cell lines; however, some of the compounds did exhibit inhibition of cell growth. Results further indicate an initial decrease in VEGF production in the cell lines treated with the ruthenium compounds but that this effect was compound-cell line specific.

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CHAPTER 1

INTRODUCTION

According to the National Cancer Institute, nearly 1.4 million men and women in United States are diagnosed with cancer each year. In 2006, approximately 600,000 men and women in the United States died from cancer of all varieties. The median age of diagnosis is 67. Approximately 41% of people born today in the United States will be diagnosed with cancer at some point in their lifetime (Ries et al., 2007).

The diagnosis of cancer was not well comprehended until the late 19th century. However, there is evidence that cancer affected animals well before humans inhabited earth. The remains of a Cretaceous dinosaur and a Pleistocene cave bear were determined to have tumors in their vertebrae (Barton-Burke & Wilkes, 2006). There is documentation of cancer and tumors in humans dating as far back as the ancient Egyptians. Malignant neoplasms, uncontrolled growths of abnormal tissue, have been discovered in Egyptian mummies over 5000 years old (Barton-Burke & Wilkes). References to benign and malignant tumors have been found on papyrus scrolls along with references to different medicines and treatments used on the tumors, one such treatment being Castor oil. Hippocrates, dubbed the "Father of Medicine," and Galen, two prominent physicians of early Rome and Greece, helped revolutionize the way medicine was viewed. Disease, which was previously viewed as a mystical or supernatural occurrence, became regarded as a naturally occurring physical process. Cancer became a recognized diagnosis in the time of Hippocrates, who originally gave it the name *karkinos* or *karkinoma*, which are Greek words for "crab." It was named as such because Hippocrates was reminded of a crustacean when looking at the hard center

and spiny projections on the tumors (Chemical Heritage Foundation, 2001). Galen is credited for being one of the earliest physicians to surgically remove tumors; however, Galen took the stance that, in general, cancer was better left untreated. In the Middle Ages all diseases were considered part of four Greek bodily fluids: blood, phlegm, yellow bile, and black bile. Cancer was considered excess black bile and only curable if caught in the very early stages. During the Renaissance, doctors based medicine more on direct observation. Ambroise Pare′, the best-known surgeon of the era, recommended removal of tumors by surgery but only when the cancer could be completely removed. Although medicine advanced during this era, cancer was still believed to be the result of excess black bile and primarily incurable. In addition, different arsenic pastes were used in cancer treatment during this time period (Chemical Heritage Foundation). Although the paste had very little systematic benefit, it did have a caustic effect on tumors and it appeared that the arsenic paste did have an "antitumor" effect on the tumor itself. Because of the apparent antitumor effect, the arsenic pastes were used as a form of cancer treatment until 1865 when improvements in therapy were made (Barton-Burke & Wilkes). The 1600s brought about technology such as the microscope and the telescope, which allowed for new scientific discoveries. It was during this time that William Harvey discredited the humoral theory of disease, the concept that an imbalance of special fluids in the body resulted in diseases. Harvey, a well-respected medical leader in the scientific community and physician to King Charles I of England, did this by studying the heart and describing the continuous circulation of the blood (Weigand, 2008). Once the idea that black bile resulted in cancer was laid to rest, cancer could be studied using innovative ideas and new techniques. During this time scientist Robert Hooke described

the cell and Italian physician Gaspare Aselli discovered lymphatic vessels. Abnormalities in the lymph system were researched as the possible cause of cancer. Although anesthesia and antiseptics were not in use during this time, mastectomies were performed in patients with breast cancer and in many instances the lymph nodes of cancer patients were removed as well (Chemical Heritage Foundation). In the middle 1700s, oncology, the study of cancer, was made a specialized field of scientific study. The role that environmental factors played in the development of cancer came into question along with the safety of working around carcinogenic agents like chimney soot. The 19th century brought many dramatic developments in science and several advancements in technology. Due to autopsies performed by physicians Giovanni Margagni and Matthew Baillie, several cancers such as cancers of the breast, stomach, rectum, and pancreas were described in great detail. Advances in microscopy allowed scientists to differentiate between cancerous and normal cells and to study cellular activity. It was also during this time period that researchers in France and Italy collected the first cancer statistics. The early 1900s welcomed in advanced cell research. The discovery of chemical carcinogens such as herbicides and pesticides brought more focus to the study of cancer. It was also during this time that chemotherapy, the treatment of cancer with anti-cancer drugs, was developed (Chemical Heritage Foundation). In 1937, Congress passed the National Cancer Institute Act, which designated yearly funding for cancer research (Hektoen, 1938). In 1939, the National Cancer Institute was formed by merging of the Office of Cancer Investigations at Harvard University and the National Institutes of Health's pharmacology division. During this time the "smoking-cancer link" was initiated (Chemical Heritage Foundation). In 1955, Congress made funds available for a National

Chemotherapy program that allowed for the testing of different chemical compounds on cancer. It was during this time that the link between smoking and cancer was deemed probable. In 1964, the U.S. Surgeon General Luther L. Terry reported that lung cancer was indeed linked to smoking (Chemical Heritage Foundation). In 1971, Congress passed the National Cancer Act. This legislation declared that the incidence of cancer is rapidly increasing and is a major health concern in the United States, that cancer is the leading cause of death in the United States, and that the purpose of the Act was to "enlarge the authorities of the National Institute of Cancer and the National Institutes of Health in order to advance the national effort against cancer" (Library of Congress, 1971). In the mid-late 1900s, oncogenes such as *src* were discovered (Chemical Heritage Foundation). Oncogenes are genes that cause the transformation of normal cells into cancerous cells, especially a viral gene that transforms a host cell into a tumor cell (Oncogene, 2006).

Cancer research has come a long way in the past couple of decades; however, there is much to be learned about these quickly dividing cells that are capable of mutation and are very harmful to healthy cells in the body. Cancer research remains a fast-pace field with much knowledge to be acquired and a rewarding experience to be gained with every advancement made.

Cancer Treatment and Chemotherapy

There are three main types of treatment for cancer: surgery, radiation therapy, and chemotherapy. Chemotherapy, our main area of interest, is the treatment of cancer with anticancer drugs and is fairly effective when cancer is caught in the early stages (The Royal Marsden NHS Foundation Trust, 2007). Paul Ehrlich, the Director at the Royal

Institute of Experimental Therapy in Frankfurt, Germany, is credited as being one of the main founders of chemotherapy due to his discovery of the drug Salvarsan in 1907. Salvarsan was used for the treatment of syphilis. Ehrlich, who was already well-known for his many achievements in the field of immunology, based his work on the idea that, "the chemical constitution of drugs used must be studied in relation to their mode of action and their affinity for the cells of the organisms against which they were directed" (Nobel Lectures, 1967). Ehrlich's aim was to locate chemical substances with a high affinity for organisms of a pathogenic nature to which they would directly travel. Ehrlich referred to these chemical substances as "magical bullets" (Nobel Lectures). The first chemotherapy drug tested on patients, nitrogen mustard, was based on a poisonous gas (Cancer Research UK, 2007). The first documented use of chemotherapy as a cancer treatment was in 1942 with the use of nitrogen mustard to treat lymphoma (Goodman et al., 1984). Many advancements in chemotherapy have been made over the past decades. The most notable advancement was the discovery of platinum-based drugs to be used in anticancer activity.

In 1965, Barnett Rosenberg was working as a biophysicist at the University of Michigan. Rosenberg decided to test whether or not electrical currents had a part in cellular division. This was based upon his theory that the mitotic spindles in cell division were much like the science experiment where magnetic field lines formed when iron shavings are scattered on paper while a magnet is underneath (Alderden, Hall, & Hambley, 2006). To test his hypothesis, *Escherichia coli* (*E. coli*) was grown in an ammonium chloride buffer while a current was sent through the buffer via "inert" platinum electrodes submerged in the buffer. After a brief time period, the *E. coli* cells

failed to retain their normal shape and began to appear long and filamentous. This effect on the *E. coli* was found to be the result of inhibition of cellular division (Alderden, et al.). After a thorough study, it was determined that the electrical current did not have anything to do with the phenomenon, but rather the phenomenon was due to the formation of platinum hydrolysis formed from the platinum electrodes in the buffer. From here, a number of transition metal compounds were tested, but it was the platinum salt $(NH_4)_2[PtCl_6]$ that was found to have the same effect of inhibition of cellular division in a number of different gram-negative bacilli. After more research, it was determined that the cis form of $[PtCl_4](NH_3)_2]$, a platinum (IV) complex, was the factor responsible for inhibiting cellular division; however, in the trans form, the complex was found to be ineffective (Alderden et al.).

With these results in hand, testing began to determine whether these complexes could be used in anticancer activity. The platinum(II) complex, *cis*-$[PtCl_2](NH_3)_2]$, and the platinum(IV) complex, *cis*-$[PtCl_4](NH_3)_2]$, were tested on 180 sarcomal tumors in Swiss white mice. The results not only showed that the complexes exhibited a virulent activity and were capable of reducing large dense tumors, but that the mice survived and were healthy after testing. The most triumphant news was that after 6 months, the mice that had been cured showed no signs of cancer. These results were enough to send cisplatin, *cis*-$[PtCl_2](NH_3)_2]$, to clinical trials. Today, cisplatin is one of the most successful drugs used in chemotherapy (Alderden et al., 2006).

Currently, the most commonly used chemotherapeutic drugs are platinum(II) compounds. The Roman numeral "II" indicates that platinum is a type II metal, meaning it can form more than one type of oxidation state (Myron, 2004). The oxidation state

refers to the degree of oxidation an atom, ion, or molecule has. For the purpose of simplicity, the oxidation state and number can be used interchangeably, and the oxidation number for simple atoms or ions is equal to the ionic charge (WordNet, 2006). In addition to cisplatin, two common platinum(II) compounds are carboplatin and oxaliplatin (National Cancer Institute, 2007). Carboplatin appears to be less toxic to healthy cells than cisplatin and has infrequent and less harsh side effects. The lessened toxicity is thought to be due in part to its molecular structure, which gives it a slower reaction time in the body. In 2002, oxaliplatin was approved in the United States to treat advanced colorectal cancer. To date, oxaliplatin is the only platinum based compound to display anticancer activity against colorectal cancer (Alderden et al.). While carboplatin and oxilaplatin are both effective and less toxic to healthy cells than cisplatin, these drugs still exhibit negative affects on healthy cells.

The main object of chemotherapy drugs is to kill cancer cells without having harmful effects on healthy cells in the body (The Royal Marsden NHS Foundation Trust, 2007). The most commonly used chemotherapy drugs are organometallic compounds. Organometallic compounds contain bonds between one or more metal atoms and one or more carbon atoms of an organyl group (IUPAC Compendium of Chemical Terminology, 1997). DNA is the primary binding site of the organometallic compounds. The backbone of the DNA is made up of four nucleotides: adenine, thymine, guanine, and cytosine. Adenine always binds to thymine, while guanine always binds to cytosine. Platinum binds to DNA by cross-linking, or forming bonds with, adjacent guanines causing a class of DNA binding proteins to adhere to the DNA and is able to interact with the nucleic acids (Bonnet & Taylor, 1989).

Three modes of association between metal complexes and DNA are generally distinguished: external binding, groove binding, and intercalation. External binding is when the compound binds to the outside of the DNA helix. Groove binding is when the compound binds in the grooves made by the double helix. Intercalation is when the compound binds between the base pairs of nucleotides, which form the double helix. The conditions of binding depend on the nature of the metal and surrounding donor groups (Miller, Taylor, & Basch, 1985).

Problems have been encountered with many of the currently used platinum based compounds, specifically cisplatin. Some of these problems include developed drug resistance by the tumor cells, which can later cause a relapse, toxic effects of the compounds on healthy tissue, inadequate tissue targeting, and impaired transport-uptake of the compound by the tumor cells (Anderson, Herman, & Rochon, 2007). Additionally, cisplatin can only treat a limited range of cancers, such as testicular, ovarian, bladder, head and neck tumors (Anderson et al., 2007). One of the primary aims of researchers in this field is to synthesize metal-based drugs with unique DNA binding activities capable of overcoming the problem of cellular resistance to cisplatin and of limited activity against common tumors, such as gastrointestinal and breast cancers (Miller et al.).

In addition to problems such as drug resistance, another major problem with platinum-based drugs is that they have several side effects including: severe nausea, vomiting, loss of appetite and taste, difficulty in eating, diarrhea, and anorexia (National Cancer Institute, 2007). Due to the traumatic side effects of platinum-based compounds, a great effort has been made to find new metals, to substitute in place of platinum (Anderson et al., 2007). Transition metals should work well because of their many

oxidative states and similar properties to platinum. The transition metals that have shown promise are titanium, vanadium, and ruthenium. Of these three, ruthenium seems to be the most promising because it demonstrates greater resistance to hydrolysis than other traditional platinum complexes and demonstrates a more selective action on tumors (Anderson et al.). It is believed that metal-based compounds that do not contain platinum, such as ruthenium based compounds, will have different biodistributions, different mechanisms of action, and be less toxic to healthy tissue due to the difference in oxidative properties (Ravera, Baracco, Cassino, Zanello, & Osella, 2004).

> The absence of direct cell toxicity, rather than being a limitation to their use might be mandatory for low side effects on healthy tissues, a depressing common bias of any pharmacological approach to cancer treatment (Capozzi et al., 1998).

Ruthenium. Ruthenium is a rare transition metal of the platinum group. Ruthenium(III) complexes are the most recent group of compounds to be studied. The metal-DNA binding of the ruthenium complexes to DNA is a function of size, shape, and hydrophobic characteristics of the complex as determined by the chemical structures of the ligands of the complex. These complexes have characteristic sulfoxide and nitrogen-donor ligands, a smaller molecule that specifically binds to a larger molecule, and have been reported to exhibit antimetastatic properties (Genentech, Inc., 2007). The two key characteristics of ruthenium(III) complexes are their exceptional antimetastatic behavior and the exhibition of antitumor activity.

The imidazolium *trans*-tetrachloro(dimethylsulfoxide)imidazoleruthenate(m) is an anionic complex known as NAMI-A, chemical formula [ImH][Ru(III)Cl_4(DMSO)(Im)]. It is the first ruthenium-based compound to complete phase I clinical trials (Bacac, 2002).

Of the ruthenium complexes tested, NAMI-A has shown the most promise as an anticancer agent because it inhibits metastasis formation and growth while being devoid of severe cytotoxic effects to healthy cells. Also, it does not appear to modify cell growth in healthy tissue (Ravera et al., 2004).

Although direct toxicity to the tumor itself is a key factor of a chemotherapeutic drug, antimetastatic properties are equally as important. Metastasis occurs as tumor cells spread from the primary tumor site to a secondary site, which is in at another part of the body (Bashyam, 2002). The dominant cause of a poor clinical outcome in cancer patients is the progression of tumors from a benign state with limited growth to an invasive state with tumor metastasis (Sava et al., 2004).

A possible mechanism for NAMI-A's activity on metastasis is not the conventional cytotoxic mechanism, but it appears that NAMI-A promotes metastatic cell changes by way of a phenotype not as invasive or malignant (Bacac, 2002). This mechanism of action was investigated using a new murine metastatic cell line (metGM), which was previously isolated, stabilized, and fully characterized. The metGM cells present two phenotypically different subpopulations, each with an opposite response to NAMI-A. The metGM cell line was used as an *in vitro* model to study the metastatic effects of NAMI-A on metastatic cells. It was found that NAMI-A is not suppressive for the host immune system, induces the metabolic activation of resting lymphocytes, and maintains induced activation of lymphocytes. This suggests that NAMI-A uses a selective and different mechanism of action than traditional platinum-based drugs in dealing with metastatic and host immune cells (Bacac, 2002). Possible explanations are the significant increase of tumor cells in the G_2/M phase where the cells are growing and

getting ready to divide, although this is not a common effect for all tumor cell lines (Bergamo et al., 1998). *In vitro*, NAMI-A shows temporary cell cycle arrest of tumor cells in the premitotic G2/M phase, while cisplatin appeared to cause a progressive interruption in the different phases of the cell cycle in a dose-dependent manner (Bergamo et al., 1998). It is hypothesized that NAMI-A shows temporary cell cycle arrest of tumor cells in the premitotic G2/M phase by tying up the DNA so that the DNA cannot replicate (Sava et al., 2003). This gives insight to new mechanisms by which other ruthenium-based compounds with similar chemical properties to NAMI-A may interact with DNA to result in antimetastatic properties.

Ruthenium-based compounds are also good antitumor agents compared to cisplatin. Testing was done *in vitro* on TLX5 lymphoma cells in order to compare ruthenium compounds NAMI-A, Na[*trans*-$RuCl_4$(TMSO)Ind] (TIND), and Na[*trans*-$RuCl_4$(TMSO)Iq] (TEQU) with cisplatin (Capozzi et al., 1998). Cell cycle modifications were observed in the lymphoma cells and the protein content was measured. The pattern of effects varied among the ruthenium compounds. TEQU and cisplatin appeared to have the similar cytotoxicity in a dose-dependent manner and DNA fragmentation, while TIND had a small increase in S and G_2M populations when the tumor cells were exposed in a time-dependent manner. NAMI-A appeared to have no effect on direct tumor cytotoxicity but was found to have strong, antimetastatic properties (Capozzi et al., 1998). In fact, NAMI-A appears to be a unique antitumor agent:

> It [NAMI-A] is as active of, or even more active than cisplatin in several experimental conditions but differs from this compound for the low or absent side-effects detectable at active dosages (Capozzi et al., 1998).

While the mechanism of action of NAMI-A is still extensively unknown, it is common knowledge that ruthenium(III) complexes are more inactive than their analogous ruthenium(II) derivatives. An "activation by reduction" mechanism has been suggested to help explain the biological activity of all chloro-amino ruthenium derivatives (Ravera, et al., 2004). Because the amount of oxygen is less and the pH is lower at tumor sites, the "activation by reduction" mechanism of ruthenium causes it to be extremely selective as the metal complexes may aggregate in the oxygen-lacking environment (Anderson et al., 2007).

Once the "activation by reduction" has taken place in a ruthenium(III) complex, or hydrolysis of at least one chloride has occurred, the complex has the capability to bind to DNA or another very important plasma protein known as transferrin (Alessio et al., 2000). Transferrin is a blood plasma protein that is responsible for iron transportation in the body, specifically for expediting transport into cells (Centers for Disease Control and Prevention, 2007). Cancer cells have a high affinity binding to transferrin (Chan & Gerhardt, 1992). Iron is required by all living systems since it is an essential component of many important enzymes. Iron is taken up into cells by way of the transferrin (Tf)-transferrin receptor (TR) complex. Once free ferric iron binds to apotransferrin, the iron-Tf complex binds to the cell surface transferrin receptor. The iron-Tf-TR complexes are then internalized in coated vesicles by way of receptor-mediated endocytosis. Once inside the endosome, the iron is released from transferrin and is taken to its site of use. The mechanism of translocation of the endosome is not yet known (Chan & Gerhardt). Cancer cells have a high number of transferrin receptors on their cell surface. Because of

this, it is thought that NAMI-A and other ruthenium complexes are taken up into the cell via this method.

Role of VEGF. Tumor growth and metastasis survival are strongly dependent on nourishment provided by the bloodstream. Because cancer cells multiply very rapidly, they require a high blood flow to provide nutrients for this cell division. Thus, the ability to produce an endless blood supply is a valuable asset for tumors. Tumors ensure this continual blood supply by forming new capillaries from preexisting blood vessels, a process known as angiogenesis (Quantikine Human VEGF Immunoassay, 2006). Angiogenesis is regulated by vascular endothelial growth factor (VEGF).

VEGF is a heparin-binding glycoprotein. It is homodimeric and is approximately 34 – 42 kDa in size. There are several isoforms of VEGF, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PIGF. VEGF-A, the most common variant simply referred to as VEGF, was the interest of our study as it is the signaling protein for angiogenesis. VEGF works by stimulating cellular responses by binding to tyrosine kinase receptors on the cell surface, causing them to dimerize and become activated through transphosphorylation (Quantikine Human VEGF Immunoassay, 2006). VEGF is produced in nearly all cancer cell lines (Rad et al., 2007).

There are two different methods of blood vessel formation in embryos: vasculogenesis and angiogenesis. In angiogenesis, new blood vessels are formed from preexisting blood vessels (Ferrera & Alitalo, 1999). During embryonic development a primary vascular plexus is formed. Later the endothelial channels are remodeled and a hierarchy of large and small blood vessels is formed. New capillaries form by sprouting or splitting from their original vessels. This process is known as intussusception. Wang,

Tu, Wang, and Zhou, (2006) reiterate the fact that angiogenesis is necessary for tumor growth and promotes the progression and metastasis of tumors. It is known that VEGF is produced for the stimulation of tumor vascularization. VEGF has been observed in adenocarcinomas (Wang, Tu, et al., 2006). Angiogenesis is necessary in adult women for the female reproductive cycle. It also is necessary for repair, remodeling, and regeneration of tissues. The two classifications of known endothelial growth factors are VEGF and angiopoietin. Of the two, VEGF is most important for the development and differentiation of the vascular system. If even one VEGF allele is lost, the result is embryonic lethality. If one of the heparin binding isoforms of VEGF is selectively inactivated still leaving one functional isoform ($VEGF_{120}$), the cardiovascular system will not be able to form properly and the result will be myocardial ischemia, perinatal, or early postnatal death (Ferrera & Alitalo). VEGF production is significantly increased in most cancer cell lines thus ensuring a continuous blood supply to the cancer cells. This results in an increased microvascular density (Ferrera & Alitalo). *In vitro*, VEGF has potent mitogenic activities specific for endothelial cells and *in vivo*, VEGF can induce angiogenesis as well as increase vascular permeability.

VEGF expression can be regulated in multiple ways. Well-known regulators of VEGF expression are peroxisome proliferator-activated receptors (PPARs). VEGF expression is also regulated by growth factors, cytokines, oncogenes, and tumor-suppressor genes (Wang, Wang, et al., 2006).

VEGF is expressed physiologically in different tissues. Tissue oxygen tension is the balance between oxygen supply and demand. When more oxygen is needed, VEGF production is induced. Therefore, tissue oxygen tension is a regulator of VEGF

production. Low oxygen tension occurs in solid tumors when the oxygen supply and oxygen consumption become imbalanced (Nakayama et al., 2006). When exposed to hypoxia, VEGF expression is induced by increased transcription and stabilization of mRNA (Pellizzaro, Coradini, & Daidone, 2002). The principle inducer of VEGF synthesis is hypoxia-inducible factor (HIF-1), which binds to elements that are responsive in the promoter region of specific genes in order to increase the transcription of the gene (Nakayama et al.).

HIF-1 plays a major role in angiogenesis and tumor growth. HIF-1 is a transcription factor that is heterodimeric and consists of HIF-1α and HIF-1β subunits (Pellizzaro et al., 2002). *In vivo*, the inhibition of HIF-1α leads to the reduced tumor size while the expression of HIF-1α causes a growth in tumor size. VEGF protein expression is mediated by phosphoinositide 3-kinase (PI3K) at the transcriptional level through the expression of HIF-1α in human ovarian cancer cells (Skinner, Zheng, Fang, Agani, & Jiang, 2004). PI3K inhibition by LY294002 decreased the transcriptional activation of VEGF while the forced expression of AKT, a cancer gene regulatory subunit, completely reversed the inhibitory effect. This is another example of how VEGF is regulated by HIF-1α and demonstrates another method by which blocking pathways can inhibit VEGF expression (Skinner et al., 2004).

The short-chain fatty acid sodium butyrate (NaB) is present in the human colon. It is able to modulate many cellular processes including cell cycle arrest, differentiation, and apoptosis in colorectal cancer cells. Pellizzaro et al. (2002) examined NaB to see whether it could regulate the levels of angiogenesis-related factors, specifically VEGF

and HIF-1α, in HT29 cells, a colon cancer cell line. NaB showed a dose-dependent decrease in VEGF and a dose-dependent increase in HIF-1α mRNA (Pellizzaro et al.).

Using two different shRNA expression vectors, expression of VEGF was inhibited in a line of colorectal adenocarcinoma cells, HT-29, by reducing the transcription levels of VEGF mRNA, which reduced VEGF protein levels (Li et al., 2007). First, this demonstrates that HT-29 cells do in fact produce VEGF. Second, if the ruthenium compounds being tested in Forsman's lab at East Tennessee State University were able to target RNA and interfere with the VEGF gene, VEGF production would stop or be reduced, thereby cutting off the blood supply to the cancer cells.

VEGF receptors are expressed in human colorectal carcinoma (CRC) cell lines as shown by analysis of real-time quantitative PCR (Wang, Tu, et al., 2006). Different cell lines exhibit different VEGF receptors (Wang, Tu, et al.). It is possible that the ruthenium compounds being tested, such as NAMI-A, bind to certain receptors, which could inhibit VEGF expression in cancer cells, thereby inhibiting cancer cell growth (Wang, Tu, et al.).

While there are multiple isoforms of VEGF, VEGF-A is a positive regulator for angiogenesis. Generally, VEGF-A binds two high affinity receptor tyrosine kinases (RTKs), VEGFR-1 (Flt-1) and VEGFR-2 (Flt-1/KDR). VEGFR-2 is the major intermediary of VEGF-A's mitogenic and angiogenic signals (Nguyen et al., 2006). Microarray analysis recently showed that VEGFR is expressed in human colon cancer cells, HCT8/S11 (Nguyen et al.). This suggests that colon cancer cells may be targeted by several angiogenic factors.

VEGF functions as a mitogenic and permeability factor as well as an antiapoptotic survival factor by initiating intracellular signaling. Thus, VEGF and VEGFR signaling pathways are being targeted in the treatment of solid tumors. In animal models, a VEGFR tyrosine kinase inhibitor has been shown to hinder angiogenesis and tumor progression. Out of several models studied by Yokoi et al. (2005) many specimens expressed several growth factors including VEGF, VEGF receptor (VEGFR), and phosphorylated VEGFR (pVEGFR).

VEGF causes angiogenesis in human colon cancer (Ellis et al., 1998). There has been recent evidence to suggest that regulatory factors of VEGF expression may in part depend on signal transduction pathways mediated by c-*src* (Ellis et al.). In most colon tumors and cell lines the tyrosine kinase activity of Src is activated. In subclones of HT29 cells, Src expression and activity were decreased as a direct result of a transfected antisense expression vector (Ellis et al.). Ellis' group looked at whether or not VEGF expression is decreased in the HT29 cell line as well as whether or not the reduced size and growth rate of antisense vector-transfected cell lines *in vivo* may partially by the result of reduced vascularization of tumors. Results indicate that VEGF mRNA expression in this cell line was decreased in proportion to the decrease in Src kinase activity (Ellis et al.).

Zhang, Wei, Xu, Wang, and Wu, (2007) looked at growth factors-cytokines and their receptors that are known to play a role in upregulation in human tumors, specifically colorectal carcinoma. VEGF seems to be a key cytokine upregulated in CRC. They also noted that VEGF has a role in proliferation and migration induction of epithelial cells. In tumors, VEGF enhances vascular permeability that allows the cancer cells to enter the

bloodstream and possibly filtrate to places far from the original site. It is a possibility that VEGF is a "trigger" and receptor tyrosine kinases (RTK's) may play a role in the regulation of VEGF expression. Many researchers are attempting to look at specific or broad receptors that can be targeted to shut off VEGF expression (Zhang et al.).

VEGF is commonly known to promote pathogenesis of all cancer types looked at. In breast cancer cells, it has been suggested that VEGF is able to act as an internal autocrine (intracrine) survival factor when bound to VEGFR1 (Lee et al., 2007). This is the first evidence of this unique survival system. Breast cancer cells express both VEGF and VEGF receptors VEGFR1, VEGFR2, and NRP1; however, there are differing opinions about the expression of these receptors in breast cancer. While some reports say VEGFR1 and VEGFR2 are strongly expressed in breast cancer tissue, others disagree and say there is a low expression of these receptors (Lee et al.). New reports have stated that VEGF acts as an autocrine growth and survival factor, although more research is being done to validate this claim (Lee et al.).

Colon carcinoma and other solid tumors depend upon neovascularization for initiation, progression, and metastasis. HT-29 is a model cell line to study. Of several angiogenetic inducers described in the HT-29 cell line as well as other colon carcinomas, VEGF is the most important (Pellizzaro et al., 2002). VEGF plays a primary role in the development of tumors and is associated with progression and metastasis of colon cancer. Many VEGF isoforms have been produced through alternative exon splicing of the gene. Of these, VEGF165 is the main form connected to the progression of colon cancer (Pellizzaro et al.).

Because most types of cancer cells produce VEGF, new methods are being examined for blocking VEGF production by tumor cells (Rad et al., 2007). Scientists have come up with several methods to block VEGF production in cancer cells. Some of these treatments include tyrosine kinase receptor inhibitors, soluble receptors, and mAbs against VEGF. To date, all of the above-mentioned methods have been used in clinical trials. Although they seem to be somewhat effective in counteracting some of the pathogenic effects of VEGF, negateve side effects such as thrombosis, bleeding, hypertension, and proteinuria have occured. Rad et al. looked at immunizing mice using a new method known as an "mVEGF kinoid." The kinoid consists of a keyhole limpet hemocyanin (KLH)-mVEGF heterocomplex. The result of the immunization with the kinoid was that neutralizing autoantibodies to mVEGF were induced, resulting in the inhibition of lung metastases. More tests will be run to determine whether this method will be a suitable way to inhibit VEGF production in cancer cells (Rad et al.).

One of the major problems in the application of several newly targeted anticancer drugs, including inhibitors of angiogenesis, is the ability to determine a biological-therapeutic dose that is effective. The reason for this is that the optimum therapeutic activity of many new drugs is expressed below the maximum tolerated dose, if the dose can be defined at all. Therefore, surrogate markers are necessary to establish optimal dosing. In tumor-bearing mice, a rapid increases of mouse VEGF was observed within 24 hours after injections of an anti-VEGF receptor, VEGFR-2, which is a monoclonal antibody. Similarly, an increase of human plasma VEGF was seen in tumor-bearing mice. Plasma VEGF levels increase in a dose-dependent manner in mice when injected with VEGFR-2 (Bocci et al., 2004).

Currently, ruthenium complexes are being developed and tested in hopes of reducing tumor growth and metastasis as well as ultimately interrupting or blocking VEGF production. Once an effective therapeutic dose is determined from many newly developed chemotherapy drugs, steps can be taken to determine whether or not an effective way of stopping the development and spread of cancer will be found. There is hope that one day we will gain control over cancer and be able to save many lives.

In the present study, we examined the cytotoxicity of seven ruthenium compounds on two human cancer cell lines, HT-29 and MCF-7. Cytotoxicity can be defined as the external cell killing by any compound causing cell death (Altweb, 2007). The cytotoxicity was determined in human cell lines HT-29 and MCF-7 by using MTT assays. The HT-29 and MCF-7 cell lines are of particular interest as these cell lines have been found to be resistant to treatment with cisplatin. Therefore, it is our hope that the ruthenium compounds tested in this study will have a cytotoxic or inhibitory effect on the cell growth and possibly go on for further testing as potential chemotherapeutic agents.

CHAPTER 2

MATERIALS AND METHODS

Cancer Cell Lines

The cell lines used in these studies were Human Colon Adenocarcinoma (HT-29) and Human Breast Carcinoma (MCF-7)(cat. #'s HTB-38 and HTB-22, ATCC, Manassas, Virginia). The HT-29 cells were originally harvested from a white female and the MCF-7 cells were originally harvested from a white male. Both of these cell lines are adherent cell lines, which means they adhere to the surface of the container in which they are grown.

Cell line LM929 was generously donated by Stephen Keith Chapes (Kansas State University, Manhattan, KS). LM929 is a macrophage cell line and was used in this study as a negative control as it does not produce VEGF.

Cell Preparation and Treatment

HT-29 cell lines were maintained by culture in McCoy's 5A medium supplemented with 10 % FBS and ampicillen-streptomycin at 100mg/ml and 25 mg/ml, respectively. Passage of the cells was accomplished using the following method. To remove cellular debris from the culture the 25 cm^2 flasks (Fisher Sci, Pittsburg, Pennsylvania) were rinsed with 3ml of sterile PBS. The cells were rinsed again with 4ml of sterile PBS as this line of cells had a lot of cell debris and needed to be rinsed twice. The PBS was then suctioned off. 3ml Trypsin (1X) (cat. # 25-050-Cl, Mediatech, Inc., Herndon, Virginia) was added to the flask. Trypsin is used to remove adherent cells from the flask. To assist in cellular release the flask was placed in an incubator for 10 minutes at 37°C and 5% CO_2. Cells were rinsed from the flask with 4 ml McCoy's 5A complete

media. The resulting suspension (cells, 3 ml Trypsin, and 4 ml media) was transferred to a 15 mL conical tube (Fisher Sci, Pittsburgh, Pennsylvania). An additional 5 mL of complete media was added to the conical tube for a total volume of 12 mL. The additional media was added to inhibit the Trypsin because the media contains Trypsin inhibitor and prolonged exposure to Trypsin is harmful to the cells. The cell suspension was centrifuged for 10 minutes at 8°C and 1000 RPM. Once centrifugation was complete, the supernate was suctioned off and disposed of and the cell pellet was resuspended in 6 mL complete media and returned to the culture flask and placed in the incubator.

The MCF-7 cell line was maintained in the same manner as the HT-29 cell line with the exception of the media used. This cell line was cultured in DMEM medium supplemented with 10% FBS and carbenicillin/streptomycin at 100mg/ml and 25 mg/ml, respectively.

The LM929 cell line was maintained by culture in DMEM medium supplemented with 10% FBS and carbenicillin/streptomycin at 100mg/ml and 25 mg/ml, respectively. Passage of the cells was accomplished using the following method. The media was suctioned off and 3 mL Trypsin (1X) was added to the flask. After 30 seconds the Trypsin was suctioned off, leaving the cells mildly adhered to the flask. Cells were rinsed from the flask with 6 mL DMEM complete media. The cells were pipetted up and down in the complete media and placed in the incubator.

Prior to starting an MTT assay, cells were moved to a Corning 75 cm^2 flask (Fisher Sci, Pittsburg, Pennsylvania) to mass produce cells. Cell lines were maintained in the same manner as above; however, once cells were resuspended in 6 mL complete

media, the cell suspension was divided evenly between two 75 cm^2 flasks and filled with complete media to bring the total volume of each flask to 25 mL.

Synthesis of Ruthenium Compounds

The organometallic compounds used in these studies were synthesized by Radhey Srivastava (University of Louisiana, Lafayette) and sent to the laboratory of Allan Forsman (East Tennessee State University, Johnson City) for use in cytotoxicity assays. The compounds assayed are listed in Table 1.

Table 1

List of Ruthenium Compounds

Compound Name	Molecular Formula	Molecular Weight (g/mol)
NG2	$RuCl_3$(TMSO)(1,10-phenanthroline)	Unknown
QL19	K[$RuCl_3$(bpy)(SCN)]	359.64
RS216	[$RuCl_3$(bpy)(NCOPh)]	Unknown
RS242	K[$RuCl_2$(TMSO)(bpy)(methyl p-tolylsulfide)]	609.66
RS244	K[$RuCl_3$($TMSO)_3$]	559.03
RS247	K[$RuCl_3$($TMSO)_3$]	559.03
RS250	[$RuCl_3$($TMSO)_2$(NO)]	445.76

Note: Structures in blue have not been established yet.

A brief description of compound synthesis is as follows:

Compound NG2 was made via the reaction of a molar ratio of 1:1 of $H(TMSO)]\{RuCl_4(TMSO)_2$ and phenanthroline in 95% ethanol at room temperature. This reaction yielded a precipitate, which was named NG2.

Compound QL19 was made by dissolving mer-$[RuCl_3(DMSO)(bpy)]$ and KSCN separately in acetone and water, respectively. The two solutions were mixed together and heated to 80°C for 4 hours. The solvent was placed on a rotary evaporator then dried in a vacuum. The resulting product was recrystallized in hot acetone.

Compound RS216 was made by dissolving mer-$[RuCl_3(DMSO)(bpy)]$ and benzohydroxamic acid separately in dichloromethane. The clear benzohydroxamic acid solution was slowly added to the mer-$[RuCl_3(DMSO)(bpy)]$. The solution was heated at 80°C for 19 hours. The solvent was placed on a rotary evaporator then dried in a vacuum. The solid product was recrystallized in dichloromethane and hexane.

Compound RS242 was made by dissolving a molar ratio of 1:1 of $RuCl_3(TMSO)(bpy)$ and $AgNO_3$ separately in acetonitrile. The $AgNO_3$ solution was slowly added to the $RuCl_3(TMSO)(bpy)$ solution over a 1-hour time period. AgCl precipitated, was filtered out, and rejected. Methyl-p-tolyl sulfide was added to the solution and stirred at room temperature over a period of several hours. This yielded a yellow precipitate that was filtered out, washed with cold acetonitrile, and dried in a vacuum. The reaction was carried out in a N_2 atmosphere in a 1:1:1 molar ratio of the reactants. Based on infrared (IR) spectroscopy analysis, the formula for this compound was determined to be $K[RuCl_2(TMSO)(bpy)$(methy-p-tolyl sulfide].

Compound RS244 was made by adding solid potassium salt of oxalic acid to a hot solution of cis-[$RuCl_2(TMSO)_4$] in methanol. The mixture was refluxed for 5 hours. KCl precipitated, was filtered out, and rejected. The mixture was placed on a rotary evaporator. This yielded a sticky yellow solid that was stirred overnight in a mixture of acetone and diethylether. The yellow solid was filtered and dried in a vacuum.

Compound RS247 was made by adding solid potassium salt of malonic acid to a hot solution of cis-[$RuCl_2(TMSO)_4$] in methanol. The mixture was refluxed for 2.5 hours. KCl precipitated, was filtered out, and rejected. The volume was then reduced to approximately 3 mL on a rotary evaporator and placed in a hood for slow evaporation. This procedure yielded a yellow crystalline produce. The X-ray analysis of the compound revealed the structure to be K[$RuCl_3(TMSO)_3$].

Compound RS 250 was made by dissolving a molar ratio of 1:1 of $RuCl_3(TMSO)_3$ and benzohydroxamic acid separately in acetonitrile. The mixture was refluxed for 4 hours. The volume was then reduced to approximately 5 mL and placed in a freezer overnight. This procedure yielded a brown solid precipitate, which was filtered, washed with cold acetonitrile and diethlether, and dried in a vacuum. The assumed formula for the compound is [$RuCl_3(TMSO)_2(NO)$].

Preparation of Ruthenium Compounds

The ruthenium compounds were received in powder form. Compounds were weighed on an electronic scale. Calculations were made to reconstitute the compounds at a concentration of 10 mg/ml and the appropriate amount of solvent was added to each compound. The compounds were placed on a vortex mixer to thoroughly mix and dissolve the compounds. Within minutes, compounds QL19, RS216, RS242, RS247, and

RS250 settled out of solution: however, the remaining liquid for each was no longer clear, indicating that some compound had gone into solution. The tubes containing compound were then placed in a water bath for 10 minutes at 37°C and then vortexed. The compounds settled out once again. This procedure indicated to us that these solutions were supersaturated. For definition purposes, the maximum amount of compound that would go into solution will be referred to as "saturated" and the maximum saturation concentration cannot be higher than 10 mg/ml. Compound NG2 had previously been diluted at 10 mg/ml and was also determined to be supersaturated. Compound RS244 was the only compound to go into solution when diluted at 10mg/ml.

Cytotoxicity Assay

For both the HT-29 and MCF-7 cell lines, 96 well microplates were seeded at 2 x 10^4 cells per well with a final well volume of 106 µl. 102 µl complete medium was placed in the first three wells and 53 µl complete medium was placed in the remaining wells across two rows for each compound tested. For accuracy, a multitip pipette was used throughout the experiment. 4.24 µl of a saturated compound was added to each of the first three wells for each compound tested, giving each of the first three wells a volume of 106.24 µl. The compounds were serial diluted across 2 rows of the 96-well plate by removing 53 µl from each of the first three wells and pipetting it into the next triplicate of wells. This procedure was repeated across two rows in triplicate. The remaining 53 µl from the last three wells of the dilution was discarded. Cells were added to each well at a density of 2 x 10^4 cells/53 µl complete medium. Controls used in the assay consisted of serial dilutions of HCl to establish a percent kill curve, medium only control, cell in medium control, acetone control, acetonitrile control, dimethyl sulfoxide

(DMSO) control, and methylene chloride control. Acetone, acetonitrile, DMSO, and methylene chloride were necessary controls as these four were used as solvents to dissolve some of the ruthenium compounds and it needed to be shown that the solvents did not cause cell death or growth inhibition in either cancer cell line. Assays were incubated for 72 hrs. at 37℃ and 5% CO_2. Following the incubation period, assays were developed using a CellQuanti-MTT cell viability assay kit (Bioassay Systems, Hayward, CA). The assays were developed by adding 20 µl of MTT reagent to each well. The 96-well microplates were then incubated for an additional 4 hours. 100 µl of solubilization buffer was placed in each well and the plate put on a plate shaker for 1 hour to dissolve the formazan crystals. The 96-well microplates were read on a Cary 50 plate reader (Varian, Palo Alto, California) at 590nm using Cary WinUV software. Several assays were read at 620nm as cited by the literature as an acceptable value; however, upon comparison between the suggested value of 590nm and the alternate value of 620nm, a major discrepancy was observed and tests read at 620nm were discarded and results removed from the data. After the plates were read, a standard curve was established via linear regression (Mathematica 7.1, Wolfram Research) using the HCl values. Next, the percent kill (% kill) of compounds and controls tested was determined using the % kill formula. The % kill formula is as follows: (Exp - Ctl)/(Max - Ctl) x 100 =, where Max is acid control and Ctl is the media control. Because no % kill was observed in our assays, percent inhibition was determined using the formula (1-A/B) x 100%. In this equation, mean absorbance values of the treated and control wells are represented by A and B, respectively.

The MTT assay is a colorimetric assay that is based on the conversion of tetrazolium salt MTT, which is a pale yellow substrate, to formazan, which is a purple dye (Figure 1).

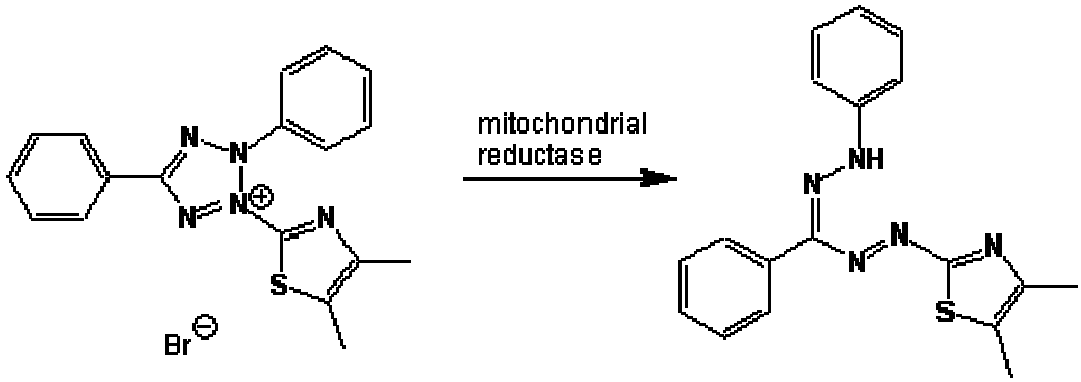

Figure 1. Conversion of MTT to formazan. (Wikipedia, 2006)

The assay involves a cellular reduction reaction involving two pyridine nucleotide factors, NADH and NADPH. The reduction occurs only when mitochondrial reductase enzymes are active, making the conversion specifically related to the number of living cells. Because MTT is only catalyzed by living cells, it is a good way to measure cell growth (CellQuanti-MTT Cell Viability Assay Kits, 2004). In the final incubation period living cells produce a crystalline formazan product. When a solubilization solution is added in the final step of the assay to dissolve the formazan product, the result is a purple solution. The absorbance is quantified spectrophotometrically by measuring the wavelength at 590 nm. Comparing the amount of purple formazan produced by cells treated with the ruthenium-based compounds to the amount of formazan produced by control cells that underwent no treatment, allows the calculation of the effectiveness of the ruthenium-based compounds in causing cell death by using a dose-response curve generated from the control cells (CellQuanti-MTT Cell Viability Assay Kits). The

ruthenium compounds were further tested to see whether or not there was an effect on VEGF production.

Vascular Endothelial Growth Factor Assay

The VEGF assays were performed using a Quantikine Human VEGF Immunoassay (catalog number DVE00, R&D Systems, Minneapolis, Minnesota). The assay is used to quantitatively determine the concentration of human VEGF in cell supernates, serum, and plasma.

In preparation for the first VEGF assay, 96-well microplates were set up in the same manner as an MTT assay and incubated for 72 hours. However, the 96-well microplates containing HT-29 cells were seeded at different cell concentrations. Separate microplates were seeded 5×10^3, 1×10^4, 5×10^4, 8×10^4, and 2×10^5 cells per well in order to test which cell concentration yielded the most VEGF. Plates were incubated at 37℃ and 5% CO_2. Supernate was collected for 4 consecutive days from wells treated with compounds NG2, QL19, RS216, RS242, RS244, RS247, and RS250. The supernates were placed into individual 1 ml capped tubes, labeled, and stored at -80℃ until time of use. Plates containing MCF-7 cells were seeded at concentration of 8×10^4 cells per well, incubated at 37℃ and 5% CO_2, and supernates were collected for 4 consecutive days. Supernates for the second VEGF assay were collected in the same manner; however, based upon review of data from the first VEGF assay, the HT-29 cell line was seeded at 5×10^4 cells per well and the MCF-7 cell line was seeded at 8×10^4 cells per well. Collected supernates were placed into individual 1 ml capped tubes, labeled, and stored at -80℃ until time of use. Supernate from HT-29 cells and MCF-7 cells that underwent no treatment was used as a positive control and supernate from

LM929 cells was used as a negative control. Compound RS242 was not used in the second VEGF assay as the supply had run out.

Prior to the start of the VEGF assay, all reagents and working standards were prepared via instructions provided in the kit (see Appendix 1). The 1 mL tubes containing cell supernates treated with the different ruthenium-based compounds were removed from storage and thawed in a water bath. The 96-well plate was removed from its foil pouch and placed in the hood. 50 µL Assay Diluent RD1W was placed in each well. 200 µL of the Standard, control, or previously collected cell supernate was added to each well. The 96-well plate was covered with the adhesive strip provided in the assay kit and incubated for 2 hours at room temperature to allow any VEGF present in the standard, control, or cell supernate to bind to the immobile VEGF-specific monoclonal antibody, which came precoated on the 96-well plate. Each well was aspirated and washed with 400 µL Wash Buffer a total of three times to wash away any substances that were not bound to the immobile antibody. Complete removal of liquid from all wells during each wash was necessary for good test results. After the final wash, any remaining Wash Buffer was removed by inverting the plate and blotting it against a clean paper towel. Next, 200 µL of VEGF Conjugate was placed in each well. The VEGF Conjugate is an enzyme-linked polyclonal antibody that is specific for VEGF. The VEGF Conjugate will bind to the VEGF that is bound from the standard, control, or cell supernate in the above step. All wells were covered with a new adhesive strip and the plate was incubated for 2 hours at room temperature to allow the VEGF Conjugate time to bind to the VEGF present on the plate. Each well was aspirated and washed using the above procedure. 200 µL of Substrate Solution was then placed in each well. The

reaction of the Substrate Solution and the antibody-enzyme reagent yields a colored product. Once the Substrate Solution was added, the plate was protected from light by foil and incubated for 20 minutes at room temperature to allow time for the color to develop. Lastly, 50 µL of Stop Solution was placed in each well to stop the color development. The optical density of each well was taken at 450 nm on a Cary 50 microplate reader (Varian, Palo Alto, California) as the color develops in proportion to the amount of VEGF bound from the substrate, control, or cell supernate. Readings were also taken at 540 nm and a wavelength correction was made by subtracting the readings at 450 nm from the readings at 540 nm. The wavelength subtraction is necessary to correct for optical imperfections in the plate.

CHAPTER 3

RESULTS

MTT Assays

Data were pooled from five MTT assays from which the 6 ruthenium compounds were tested on the HT-29 cell line (Table 2). The lower the optical density (O.D.) reading, the higher the level of cell growth inhibition. A one-way ANOVA was then performed for each compound to test whether or not the means of each ruthenium compound at the highest concentration showed a significant difference from the mean of cells that underwent no treatment.

Table 2

HT-29 Mean Absorbance Values

Compound	Solution	$O.D._{590\ nm}$*
Cell Only	NA	1.6756
NG2	Saturated**	0.2901
QL19	Saturated**	1.4559
RS216	Saturated**	1.2694
RS244	Saturated**	0.3131
RS247	Saturated**	1.6008
RS250	Saturated**	0.9112

* Mean absorbance values of 5 MTT assays

** Saturation as defined in Materials and Methods

Data were also pooled from five MTT Assays for each compound tested on the MCF-7 cell line (Table 3) and a one-way ANOVA was performed to see whether or not

the means from cells treated with the individual compounds varied significantly from the mean of cells that underwent no treatment. The results of the one-way ANOVA for both cell lines are shown in Table 4. The data from all cells tested with the compound RS242 were discarded as the solvent used with the compound, DMSO, caused cell death. This made it impossible to determine if any cell death was related to the compound itself.

Table 3

MCF-7 Mean Absorbance Values

Compound	Solution	$O.D._{590\ nm}$*
Cell Only	NA	1.7238
NG2	Saturated**	0.4654
QL19	Saturated**	1.585
RS216	Saturated**	1.3534
RS244	Saturated**	0.8768
RS247	Saturated**	1.5268
RS250	Saturated**	1.5039

* Mean absorbance values of 5 MTT assays

** Saturation as defined in Materials and Methods

Table 4

Results of the one-way ANOVA for the HT-29 and MCF-7 cell lines

Cancer Cell Line	Compound	Significance
HT-29	NG2	$F = 26.45$, $P < 0.0001$
HT-29	RS250	$F = 13.37$, $P < 0.001$
MCF-7	NG2	$F = 56.62$, $P < 0.001$

Difference between the means of cells treated with ruthenium compounds NG2 and RS250 and the mean of cells that underwent no treatment

A one-way ANOVA is a statistical test to determine whether or not the means of two or more populations are significantly different (Keller, 2005). A significant ANOVA, meaning the p-value is below the level of significance, usually .05, allows for the null hypothesis to be rejected. The p-value is a quantitative measurement of the evidence that does not support the null hypothesis.

While none of the 7 ruthenium-based compounds tested was responsible for causing cell death in either cell line, 2 of the 7 ruthenium-based compounds tested on the HT-29 cell line caused inhibition of cell growth at the highest concentration of the compounds tested *in vitro*. Compounds NG2 and RS250 were responsible for causing cell growth inhibition in the HT-29 cell line (Table 5).

Table 5

Percent Inhibition in HT-29 cell line

Compound	Solution	Percent Inhibition
NG2	Saturated*	82.7 %
RS250	Saturated*	45.62 %

*Saturation as defined in Materials and Methods.

In the MCF-7 cell line compound NG2 showed a 73.01% inhibition of cell growth at the highest concentration tested *in vitro*, which is a saturated level. Saturation is defined in Materials and Methods.

VEGF Assays

Results of the first VEGF assay indicated that in the HT-29 cell line, cells seeded at 5 x 10^4 cells per well produced the most VEGF. As indicated in the literature, the first VEGF assay confirmed results that MCF-7 cells seeded at 8 x 10^4 cells per well produced a high amount of VEGF. Supernate from each cell line that underwent no treatment was used as a positive control, while the noncancerous cell line, LM929, was used as a negative control as it is not known to make VEGF. Results of the first VEGF assay for the HT-29 cell line are shown in Table 6. Results of the first VEGF assay for the MCF-7 cell line are shown in Table 7. The lower the optical density (O.D.) reading, the higher the level of cell growth inhibition.

Table 6

Results of first VEGF/HT-29 assay

Cell Line	Cell Concentration	$O.D._{.450nm}$*
HT-29	5×10^3	0.457
HT-29	1×10^4	1.059
HT-29	5×10^4	3.742
HT-29	8×10^4	3.077
HT-29	2×10^5	2.218

* Mean absorbance of two assays

Table 7

Results of first VEGF/MCF-7 assay

Cell Line	Cell Concentration	$O.D._{.450nm}$
MCF-7	8×10^4	3.247
MCF-7	8×10^4	3.490

* Mean absorbance of two assays

Once the highest level of VEGF produced in both cell lines was confirmed, a second VEGF assay was run in the same manner as the first; however, all HT-29 cells were seeded at 5×10^4 cells per well and all MCF-7 cells were seeded at 8×10^4 cells per well. The results of the second VEGF assay for the HT-29 and MCF-7 cell lines are shown in Figure 2 and Figure 3, respectively.

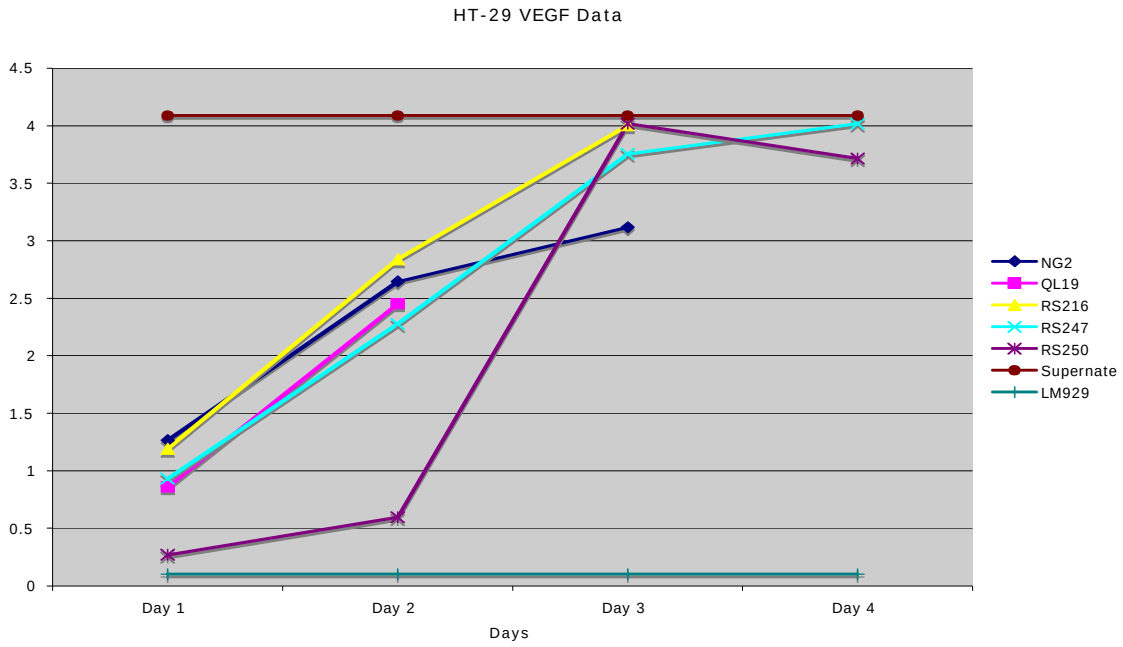

Figure 2. Results of second VEGF/HT-29 assay.

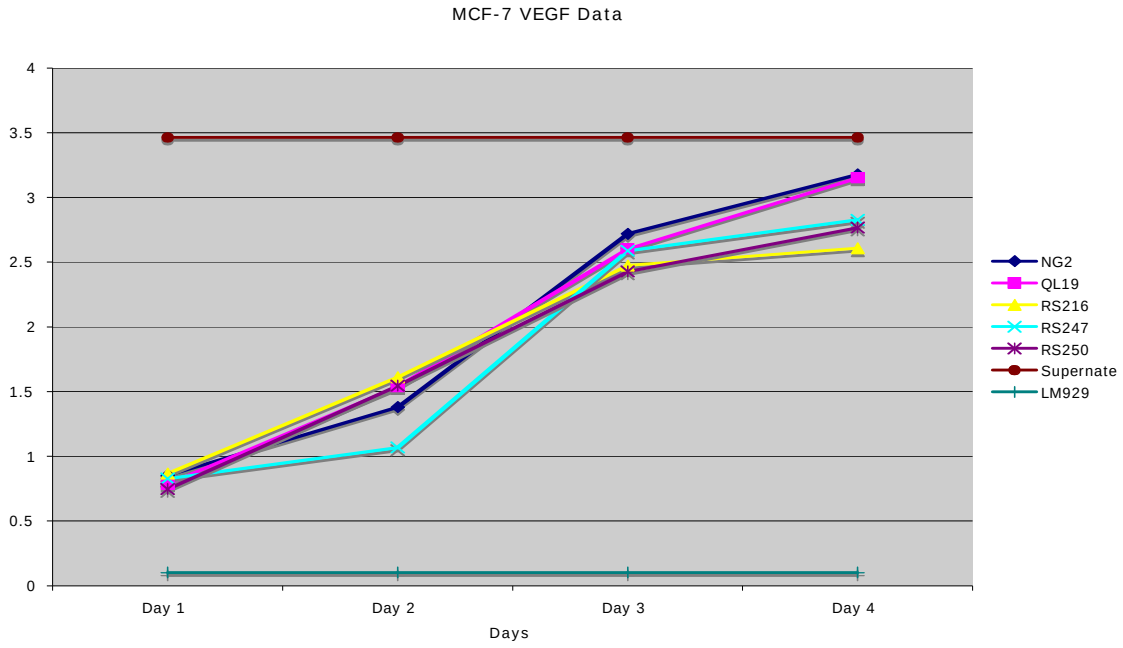

Figure 3. Results of second VEGF/MCF-7 assay.

Data from the second VEGF assay were used in a two-way ANOVA with replication to determine if there was a statistical difference between cells treated with

individual ruthenium compounds within the separate cancer cell lines. An individual two-way ANOVA with replication was run using the data from Day 1, 2, 3, and 4, to determine whether or not there was a significant decrease in VEGF production after treatment of cells with the individual ruthenium compounds. Each cancer cell line was tested separately.

A two-way ANOVA is used when there is one measurement variable present and two nominal variables, or factors, used in an experiment. In this experiment, we are testing the hypothesis that the ruthenium compounds have an effect on the human cancer cell lines. The amount of VEGF was measured in cells treated with the various ruthenium compounds as well as cells that underwent no treatment. The two nominal variables are the various ruthenium compounds and the two different human cancer cell lines. The two-way ANOVA with replication was used because there were multiple observations made in the assay (Coolidge, 2000).

When determining levels of significance, the two-way ANOVA with replication compares several important things. First, the two-way ANOVA with replication looks for a significant difference in sets of data or columns. A column (set of data) is defined as each ruthenium compound separately tested on one human cancer cell line. The two-way ANOVA with replication compares columns and determines whether there is a significant p-value in one cancer cell line treated with different ruthenium compounds. The two-way ANOVA with replication also looks for a significant difference between samples. A sample is defined as the sets of data for each human cancer cell line used. For this study we have two samples, the MCF-7 and HT-29 human cancer cell lines. The two-way ANOVA with replication also determines whether the ruthenium compounds

and individual cell lines had an effect on one another. This is known as the interaction variable.

The results of the two-way ANOVA with replication are shown in Table 8. Data on Day 4 could not be evaluated as the readings from the VEGF assay were regarded as inaccurate because the VEGF readings were too high to be accurately measured against the VEGF standard curve.

Table 8

Results of two-way ANOVA with replication

	Treatment w/ Ruthenium Compounds	Difference Between Cell Lines	Interaction Variable
Significance on Day 1	F =188.08, P < 0.0001	X	X
Significance on Day 2	F =189.06, P < 0.0001	F = 196.45, P < 0.0001	F = 46.43, P < 0.0001
Significance on Day 3	F = 16.54, P < 0.0001	F = 123.28, P < 0.0001	F = 12.37, P < 0.0001
Significance on Day 4	NA	NA	NA

Although the two-way ANOVA with replication allows for determination of significance to be made, it does not give indication as to which factor is responsible. Upon finding that the interaction variable was significant, a multiple comparisons test was used on the individual cell line data to further determine which ruthenium compound(s) showed significant VEGF reduction.

Multiple comparison tests, also referred to as *a posteriori tests* or *post hoc tests*, are commonly used after a significant ANOVA in which the null hypothesis has been rejected (Coolidge, 2000). Multiple comparison tests aid in finding a pattern of significant differences among the means and maintain the Type 1 Error rate at an acceptable level ($p = .05$ or less) while looking at the Least Significant Difference (LSD) and the omega squared (ω^2). The Least Significant Difference is a test that does a pair-wise comparison of the means of cells treated with the various ruthenium compounds (Coolidge). The Least Significant Difference is determined by this comparison. Based on the sample size a value is determined. The determined value is a benchmark value of significance. For a mean to be considered significant, the difference must be greater than the benchmark value. A Tukey's multiple comparison test was also done to determine the ω. A Tukey's multiple comparison test is a method similar to the LSD test for finding a benchmark value of significance. The Tukey's test is considered more conservative than a LSD test for lowering the Type 1 Error rate and is used to reaffirm the significance. If the ω α is greater than the benchmark value, then the mean is considered significant. In the Tukey's multiple comparison test, the ω^2 value represents the magnitude of the effect of the independent variable upon the dependent variable. Table 9 shows the values by which the magnitude of effect is determined.

Table 9

Values for ω^2 that determine magnitude of effect

$\omega^2 > .15$	Large Effect
$\omega^2 > .06$	Medium Effect
$\omega^2 > .01$	Small Effect

If the absolute value of the difference is greater than the LSD α and the Omega α, then the two tests are in agreement that the treatment means are significant.

On Day 1, the results of the multiple comparison tests in the HT-29 cell line revealed a significant difference between the mean of cells treated with compounds NG2, QL19, RS216, RS247, and RS250 compared with the mean of the cells that underwent no treatment. The most significant difference was found between cells that underwent no treatment and cells that were treated with RS250. Cells treated with compounds QL19, RS247, RS216, and NG2, showed a decreasing significance, respectively (Table 10).

Table 10

HT-29 Multiple Comparison Test Results - Day 1

Treatment	Difference
NG2	-2.73625
QL19	-3.1423
RS216	-2.81095
RS247	-3.0685
RS250	-3.73155

The difference refers to the optical density of the cells treated with individual ruthenium compounds as compared with the optical density of the cell supernate for cells that underwent no treatment. LSD α = 0.041343368, where p = 0.05; Omega α = 0.067263679, where p = 0.05.

On Day 1, the results of the multiple comparison tests in the MCF-7 cell line revealed a significant difference between the mean of cells treated with compounds NG2, QL19, RS216, RS247, and RS250 compared with the mean of cells that underwent no treatment. The most significant difference between cells treated with compounds and cells that underwent no treatment was found in cells treated with QL19, followed by RS250, NG2, RS247, and RS216, respectively (Table 11).

Table 11

MCF-7 Multiple Comparison Test Results - Day 1

Treatment	Difference
NG2	-2.6109
QL19	-2.990375
RS216	-2.50415
RS247	-2.54415
RS250	-2.6248

The difference refers to the optical density of the cells treated with individual ruthenium compounds as compared with the optical density of the cell supernate for cells that underwent no treatment. LSD α = 0.600629911, where p = 0.05; Omega α = 0.977196089, where p = 0.05.

On Day 2, the results of the multiple comparison tests in the HT-29 cell line revealed a significant difference between the mean of cells treated with compounds NG2, QL19, RS216, RS247, and RS250 compared with the mean of cells that underwent no treatment. The most significant difference between cells treated with compounds and cells that underwent no treatment was found in cells treated with RS250, followed by RS247, QL19, NG2, and RS216, respectively (Table 12).

Table 12

HT-29 Multiple Comparison Test Results - Day 2

Treatment	Difference
NG2	-1.357
QL19	-1.5505
RS216	-1.1635
RS247	-1.7246
RS250	-3.40605

The difference refers to the optical density of the cells treated with individual ruthenium compounds as compared with the optical density of the cell supernate for cells that underwent no treatment. LSD α = 0.331967951, where p = 0.05; Omega α = 0.540095952, where p = 0.05.

On Day 2, the results of the multiple comparison tests in the MCF-7 cell line revealed a significant difference between the mean of cells treated with compounds NG2, QL19, RS216, RS247, and RS250 compared with the mean of cells that underwent no treatment. The most significant difference between cells treated with compounds and cells that underwent no treatment was found in cells treated with RS247, followed by NG2, QL19, RS250, and RS216, respectively (Table 13).

Table 13

MCF-7 Multiple Comparison Test Results - Day 2

Treatment	Difference
NG2	-2.0838
QL19	-1.83385
RS216	-1.76235
RS247	-2.30425
RS250	-1.82615

The difference refers to the optical density of the cells treated with individual ruthenium compounds as compared with the optical density of the cell supernate for cells that underwent no treatment. LSD α = 0.287341626, where p = 0.05; Omega α = 0.467491058, where p = 0.05.

On Day 3, the results of the multiple comparison tests in the HT-29 cell line revealed a significant difference between the mean of cells treated with compounds NG2, QL19, RS216, RS247, and RS250 compared with the mean of cells that underwent no treatment. The most significant difference between cells treated with compounds and cells that underwent no treatment was found in cells treated with NG2, followed by RS247, RS216, and RS250, respectively (Table 14). Data obtained from cells treated with compound QL19 on Day 3 were determined to be inaccurate as the readings were too high to be considered accurate when compared against the VEGF standard curve, and thus was removed.

Table 14

HT-29 Multiple Comparison Test Results - Day 3

Treatment	Difference
NG2	-0.8857
RS216	-0.00095
RS247	-0.2467
RS250	-0.01565

The difference refers to the optical density of the cells treated with individual ruthenium compounds as compared with the optical density of the cell supernate for cells that underwent no treatment. LSD α = 0.499455614, where p = 0.05; Omega α = 0.778994231, where p = 0.05.

On Day 3, the results of the multiple comparison tests in the MCF-7 cell line revealed a significant difference between the mean of cells treated with compounds NG2, QL19, RS216, RS247, and RS250 compared with the mean of cells that underwent no treatment. The most significant difference between cells treated with compounds and cells that underwent no treatment was found in cells treated with RS250, followed by RS216, RS247, QL19, and NG2, respectively (Table 15).

Table 15

MCF-7 Multiple Comparison Test Results - Day 3

Treatment	Difference
NG2	-0.74375
QL19	-0.77145
RS216	-0.89865
RS247	-0.78445
RS250	-0.94475

The difference refers to the optical density of the cells treated with individual ruthenium compounds as compared with the optical density of the cell supernate for cells that underwent no treatment. LSD α = 0.464371289, where p = 0.05; Omega α = 0.755509839, where p = 0.05.

On Day 4, the results of the multiple comparison tests in the HT-29 cell line revealed a significant difference between the mean of cells treated with compounds NG2, QL19, RS216, RS247, and RS250 compared with the mean of cells that underwent no treatment. The most significant difference between cells treated with compounds and cells that underwent no treatment was found in cells treated with RS247 followed by cells treated with RS250 (Table 16). Data obtained from cells treated with compounds NG2, QL19, and RS216 on Day 4 were determined to be inaccurate because the readings were too high to be considered accurate when compared against the VEGF standard curve, thus the results were removed.

Table 16

HT-29 Multiple Comparison Test Results - Day 4

Treatment	Difference
RS247	-0.2467
RS250	-0.01565

The difference refers to the optical density of the cells treated with individual ruthenium compounds as compared with the optical density of the cell supernate for cells that underwent no treatment. LSD α = 0.344898349, where p = 0.05; Omega α = 0.452899764, where p = 0.05.

On Day 4, the results of the multiple comparison tests in the MCF-7 cell line revealed a significant difference between the mean of cells treated with compounds NG2, QL19, RS216, RS247, and RS250 compared with the mean of cells that underwent no treatment. The most significant difference between cells treated with compounds and cells that underwent no treatment was found in cells treated with RS216, followed by RS250, RS247, NG2, and QL19, respectively (Table 17).

Table 17

MCF-7 Multiple Comparison Test Results - Day 4

Treatment	Difference
NG2	-0.28565
QL19	-0.2219
RS216	-0.7655
RS247	-0.545
RS250	-0.6054

The difference refers to the optical density of the cells treated with individual ruthenium compounds as compared with the optical density of the cell supernate for cells that underwent no treatment. LSD α = 0.292483749, where p = 0.05; Omega α = 0.475857047, where p = 0.05.

CHAPTER 4

DISCUSSION

The most commonly used chemotherapeutic drugs are platinum(II) compounds (National Cancer Institute, 2007). Cisplatin is the most commonly used platinum based compound in chemotherapy. Due to the problems that have been encountered with many of the currently used platinum based compounds such as developed drug resistance by the tumor cells, toxic effects of the compounds on healthy tissue, inadequate tissue targeting, and impaired transport-uptake of the compounds by the tumor cells, a great effort has been made to find new metals to substitute in place of platinum (Anderson et al., 2007).

Ruthenium is a rare transition metal of the platinum group. The two key characteristics of ruthenium(III) complexes are their exceptional antimetastatic behavior and the exhibition of antitumor activity. Of the ruthenium complexes tested, NAMI-A has shown the most promise as an anticancer agent because it inhibits metastasis formation and growth while being devoid of severe cytotoxic effects to healthy cells. Also, it does not appear to modify cell growth in healthy tissue (Ravera et al., 2004). Our findings are consistent with this.

Out of the 7 ruthenium compounds tested on the HT-29 and MCF-7 cell lines, our data indicates that compounds NG2 and RS250 exhibit antitumor activity as they inhibited cell growth in the HT-29 colon cancer cell line. Compound NG2 also appears to exhibit antitumor activity as it inhibited cell growth in the MCF-7 breast cancer cell line. These are promising results as breast cancer and colon cancer are not treatable with cisplatin. More testing will need to be done to determine whether or not compounds NG2 and RS250 are devoid of toxic effects to healthy cells. Future tests can also be run to

determine the mechanism by which our ruthenium compounds bind to DNA causing an interruption in the cell cycle, thus inhibiting cell growth in the cancer cells. We know that DNA is the primary binding site of the compounds. However, the mode of association between the ruthenium compounds and DNA is unknown and needs to be distinguished between external binding, groove binding, and intercalation (Miller et al., 1985).

The mechanism by which ruthenium compounds NG2 and RS250 inhibit cell growth is yet to be determined, but it is known that ruthenium(III) complexes are more inactive than their analogous ruthenium(II) derivatives. An "activation by reduction" mechanism has been suggested in order to explain the biological activity of all chloro-amino ruthenium derivatives (Ravera et al., 2004). Because the amount of oxygen is less and the pH is lower at tumor sites, the "activation by reduction" mechanism of ruthenium causes it to be extremely selective as the metal complexes may aggregate in the oxygen-lacking environment (Anderson et al., 2007). Once the "activation by reduction" has taken place in a ruthenium(III) complex, or hydrolysis of at least one chloride has occurred, the complex has the capability to bind to DNA or transferrin (Alessio et al., 2000). Because of the large number of transferring receptors located on the cell surface of cancer cells, it is thought that ruthenium complexes are taken up into the cell via this method.

Although direct toxicity to the tumor itself is a key factor of a chemotherapeutic drug, antimetastatic properties are equally as important. The major cause of a poor clinical outcome in cancer patients is the progression of tumors from a benign state with limited growth to an invasive state with tumor metastasis (Sava et al., 2004). Ruthenium

compounds NG2 and RS250 need to be tested to determine if they work by causing transient cell cycle arrest of tumor cells in the premitotic G2/M phase, as does NAMI-A (Bergamo et al., 1998). It is hypothesized that NAMI-A shows transient cell cycle arrest of tumor cells in the premitotic G2/M phase by tying up the DNA so that the DNA cannot replicate (Sava et al., 2003). Compounds NG2 and RS250 might use this mechanism of action as they are similar in properties to NAMI-A.

Tumor growth and metastasis survival are strongly dependent on nourishment provided by the bloodstream. Because cancer cells multiply very rapidly, they require a high blood flow to provide nutrients for this cell division. Tumors ensure this continual blood supply by forming new capillaries from preexisting blood vessels, a process known as angiogenesis (Quantikine Human VEGF Immunoassay, 2006). Angiogenesis is regulated by vascular endothelial growth factor (VEGF). VEGF production is significantly increased in most cancer cell lines thus ensuring a continuous blood supply to the cancer cells (Ferrara et al., 1999).

Five of the 7 ruthenium compounds were tested to see if VEGF production had decreased in the cells treated with the various compounds. If the VEGF level had decreased, it would be an indication that the ruthenium-based compounds had interfered with VEGF production at some level. The ruthenium compounds were further examined to determine whether the level of VEGF production was decreased. This would have little effect on cells grown in the lab because the cells are well fed by the growth medium and would have no need to make VEGF; however, this would be very important to cancer cells growing *in vivo*. Cancer cells grown *in vivo* rely on VEGF to produce blood vessels from preexisting blood vessels in order that the necessary nutrients be obtained by the

cancer cells, resulting in optimal cell growth (Ferrara et al., 1999). Thus, the ruthenium compounds that can decrease the amount of VEGF produced by the cancer cell lines *in vitro* are of interest because they may be able to cause cell growth inhibition *in vivo* by effectively reducing VEGF production to levels too low to nutritionally sustain human cancer cells.

We were able to initially reject our null hypothesis that the ruthenium compounds would not interfere with the cell's ability to produce VEGF. In the HT-29 cell line, cells treated with compound RS250 exhibited the highest inhibition of VEGF production on Day 1 and Day 2. In the MCF-7 cell line, cells treated with QL19 appeared to produce the least amount of VEGF on Day 1, followed closely by cells treated with compound RS250. On Day 2, cells treated with RS250 appeared to produce less VEGF than cells treated with compound QL19; however, while both compounds appeared to inhibit VEGF production in the cell line above, it was not in a truly significant manner from cells treated with the other ruthenium compounds. By Day 4, VEGF production did not appear to be inhibited in either human cancer cell line. This would indicate that while the ruthenium compounds were able to inhibit VEGF production initially, the amount of VEGF produced during that time was still great enough to sustain the cancer cells. More research needs to be done to determine why the cancer cells treated with compounds RS250 and QL19 were initially inhibited. One possibility is that the cancer cells that were seeded on Day 1 took up all of the ruthenium compound and VEGF production was inhibited. However, once the cells divided, the new cells were able to make enough VEGF to allow the cells to flourish. Another possibility is that the cells initially seeded took up most of the ruthenium compound. Once those cancer cells divided, the daughter

cells took up the remaining compound. In either scenario, once the cancer cells had divided multiple times, enough VEGF was produced to sustain the cell line.

Although the mechanism of action is not known as to how the ruthenium compounds acted upon the cancer cell lines to cause inhibition of VEGF production, there are several factors known to regulate VEGF production. Hypoxia inducible factor (HIF-1) plays a major role in angiogenesis and tumor growth. HIF-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits (Pellizzaro et al., 2002). *In vivo*, the inhibition of HIF-1α leads to the reduced tumor size while the expression of HIF-1α causes a growth in tumor size (Pellizzaro et al.).

Using two different shRNA expression vectors, expression of VEGF was inhibited in a line of colorectal adenocarcinoma cells, HT-29, by reducing the transcription levels of VEGF mRNA, which reduced VEGF protein levels (Li et al., 2007). Our ruthenium compounds could target RNA and interfere with a gene used in VEGF production.

While there are multiple isoforms of VEGF, VEGF-A is a positive regulator for angiogenesis. It primarily binds two high affinity receptor tyrosine kinases (RTKs), VEGFR-1 (Flt-1), and VEGFR-2 (Flt-1/KDR). The ruthenium compounds may be acting as RTK inhibitors by blocking the receptor sites, thus inhibiting the binding of VEGF to the receptor site. More research will need to be done to determine the method by which our ruthenium compounds are inhibiting VEGF production in both cancer cell lines.

The overall hypothesis for the first experiment was that ruthenium compounds would have a cytotoxic effect on human carcinomas. More specifically, we hypothesized that the ruthenium compounds would have a cytotoxic effect on human breast carcinoma

and human colon adenocarcinoma, while our null hypothesis was that the ruthenium compounds would have no effect on the two cancer cell lines we worked with. While none of the compounds tested had a complete cytotoxic effect on either of the cell lines, compounds NG2 and RS250 caused inhibition of cell growth in the HT-29 cell line and compound NG2 caused inhibition of cell growth in the MCF-7 cell line.

The overall hypothesis for the second experiment was that the various ruthenium compounds would interfere with the cell's ability to produce VEGF. More specifically, we hypothesized that the ruthenium compounds would interfere with cell's ability to produce VEGF in the human breast carcinoma and human colon adenocarcinoma cell lines, while our null hypothesis was that the ruthenium compounds would not interfere with the cell's ability to produce VEGF in either human cancer cell line. In the HT-29 cell line, compound RS250 did interfere with the cell's ability to produce VEGF. In the MCF-7 cell line, compounds QL19 and RS250 appeared to inhibit VEGF production as well. Therefore, initially our null hypothesis can be rejected on Days 1-3 in both cell lines, but not rejected on Day 4 as inhibition of VEGF production was not observed.

Future Research

Two of the ruthenium-based compounds, NG2 and RS250, appear to have an inhibitory effect on cell growth in the HT-29 cell line, while one ruthenium-based compound, NG2, appears to have an inhibitory effect on cell growth in the MCF-7 cell line. The antitumor properties of these ruthenium compounds need to be further tested to determine how they might be employed in the future. Compounds NG2 and RS250 will need to be characterized to determine whether or not they are devoid of toxic effects on healthy tissue. Compound NG2 exhibited inhibition of VEGF production on Day 1 in the

HT-29 cell line, while compounds QL19 and RS250 exhibited inhibition of VEGF production on Day 1 in the MCF-7 cell line. Compound RS250 was the only compound tested to cause cell growth inhibition and inhibition of VEGF production in the HT-29 cell line. A possible explanation for the inhibition of cell growth in HT-29 cells treated with compound RS250 is that the VEGF production was inhibited enough to cause cell growth inhibition but not enough to cause cell death. Further testing needs to be done to determine the mechanism of action for compound RS250. It is our hope that one day these compounds might be effectively used in cancer treatment.

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APPENDIX

Reagent Preparation in VEGF Assay

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution – Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

VEGF Standard – Reconstitute the VEGF Standard with 1.0 mL of Calibrator Diluent RD5K (*for cell culture supernate samples*) or Calibrator Diluent RD6@ (*for serum/plasma samples*). This reconstitution produces a stock solution of 2000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

For Cell Culture Supernate Samples: Use polypropylene tubes. Pipette 500 µL of Calibrator Diluent RD5K into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL dilution serves as the high standard. Calibrator Diluent RD5K serves as the zero standard (0 pg/mL).

For Serum/Plasma Samples: Use polypropylene tubes. Pipette 500 µL of Calibrator Diluent RD6U into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (2000 pg/mL). Calibrator Diluent RD6U serves as the zero standard (0 pg/mL).

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