Tavocept (BNP7787): A Novel Chemoprotector/ sensitizer and Radioprotector/sensitizer

---

A Thesis Presented to the Faculty of the Graduate School

University of Missouri

In partial Fulfillment
Of the Requirements for the Degree

Master of Veterinary Clinical Science

By
David Bommarito

Dr. Carolyn Henry, Thesis Supervisor

May 2011

The undersigned, appointed by the Dean of the Graduate School, have examined the Thesis entitled

Tavocept (BNP7787): A Novel Chemoprotector/sensitizer and Radioprotector/sensitizer

Presented by David Bommarito

A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance.

Professor Carolyn Henry

Professor Frederick Hausheer

Assistant Teaching Professor Kimberly Selting

ACKNOWLEDGEMENTS

The author wishes to thank the following individuals:

Drs. Carolyn Henry and Kim Selting, for their continued guidance, mentoring and support in this research and the clinical oncology residency program.

Dr. Frederick Hausheer, for giving me the opportunity to work with such an amazing drug.

Hong Liu, for her kind and gracious assistance in laboratory techniques.

Aulma Parker, for her mentorship and generous support with troubleshooting.

Dr. Craig Lewis for his statistical assistance.

TABLE OF CONTENTS

LIST OF FIGURES

Tavocept (BNP7787): A Novel Chemoprotector/ sensitizer and Radioprotector/sensitizer

David Bommarito

Dr. Carolyn Henry, Thesis Supervisor

ABSTRACT

Introduction: Tavocept is a novel chemoprotector/ sensitizer that has been used in combination with cisplatin to treat adenocarcinomas. The objectives of this study were to evaluate the interaction of radiation and Tavocept on a nasal tumor cell line and try to apply the concepts to the treatment canine nasal tumors.

Materials and Methods: Using cell culture techniques and the nasal carcinoma cell line RPMI 2650, various combinations of radiation, cisplatin chemotherapy, and Tavocept were evaluated. Cytotoxicity to the cells was established using the sulfarhodamine (SRB) dye assay.

Results: Tavocept alone did not show any cytotoxicity or increased cell growth compared to the control group. The addition of Tavocept increased the cytotoxicity of cisplatin compared to cisplatin alone. The combination of Tavocept to radiation therapy appeared no different than radiation alone. The addition of Tavocept at a low and high concentration to radiation and cisplatin increased cytotoxicity compared to radiation and cisplatin alone.

Conclusions: Tavocept by itself appears to not have any toxic effects on the cell line, but it increases the cytotoxicity of cisplatin suggesting chemosensitization. There was no apparent interaction with radiation, neither sensitization nor protection.

CHAPTER 1: INTRODUCTION

1.1 Therapeutic Index

The successful treatment of cancer relies on the ability to selectively target and damage cancer cells while sparing normal tissue. The therapeutic index embodies this concept and is defined as a measure of the relative efficacy of a therapy against a tumor when compared to normal tissue toxicity.[1] (Figure 1A) Historically, the positive therapeutic index of chemotherapy and radiation in the treatment of cancer was based on the belief that the target cells of these therapies were rapidly dividing. More current literature suggests that the actual target should be the cancer stem cell as well as cells in G_0. These cells are not rapidly dividing and have a great capacity for resistance to chemotherapy via efflux pumps (e.g., MDR, MRP, LRP, BCRP), increased ability to handle oxidative stress and avoid apoptosis, and increase DNA repair capacity.[1,2] This may explain why many solid tumors are not curable with chemotherapy or radiation therapy.

To maximize the therapeutic index, it is often necessary to combine therapies in a multimodal approach. Combination therapies such as radiation and chemotherapy allow for increased local tumor control as well as spatial cooperation to eliminate metastatic disease.[3,4] The detriment of the multimodal approach is the increased risk of normal tissue damage due to overlapping or synergistic toxicities. In order to strengthen efficacy and to minimize toxicity, both radiation and chemotherapy sensitizers and

protectors have been developed. Sensitizers and protectors promote an increased therapeutic index in both single and multimodal protocols.

1.2 Chemotherapy and Radiotherapy Protectors

Chemotherapy and radiotherapy protective agents are designed to spare the normal tissue to a greater degree than the tumor tissue and shift the normal tissue toxicity tolerance curve to the right with little or no change to the tumor cytotoxicity curve (Figure 1A). For example, the chemotherapy agent doxorubicin has potent cumulative cardiotoxic effects in humans and dogs. Doxorubicin is an anti-tumor antibiotic which has anti-tumor effect through many mechanisms of action including topoisomerase II inhibition, DNA intercalation, apoptosis induction, and damage to cellular membranes.[1] Doxorubicin also generates free radicals and the primary mechanism of cardiac myocyte damage is thought to be due to the formation of iron-dependent oxygen free radicals. The myocardium has lower levels of enzymes that can detoxify free radicals such as catalase, superoxide dismutase, and glutathione peroxidase, and is accordingly more sensitive to oxidative radical damage than other tissues.[5] Dexrazoxane is a chemoprotector that decreases the myocardial damage by acting as an iron chelator and prevents the formation of iron-dependent oxygen free radicals (e.g., peroxide, hydroxyl and superoxide). It is believed that free radical damage is not a major chemotherapeutic mechanism of doxorubicin, so dexrazoxane protects the heart without significantly lowering the cytotoxicity to the neoplastic cells.[6-9]

A second example of a chemoprotector is the drug 2-mercapto ethane sulfonate sodium, or mesna. Cyclophosphamide and ifosfamide are electrophilic alkylating agents

that undergo hepatic metabolism and activation. The major toxic metabolite that is excreted via the kidneys and accumulates in the uroepithelium and bladder is acrolein. Acrolein is a direct irritant to the uroepithelial tract and can lead to sterile hemorrhagic cystitis.[10] Mesna prevents this from occurring by covalently binding to acrolein in the urinary tract, making it more water soluble, and preventing its interaction with the urothepithelium. The anti-tumor effects of these drugs are not perturbed because acrolein does not play a role in tumor cell cytotoxicity; anti-tumor effects of the oxazaphosphoranes are believed to be mediated by phosphoramide mustard (cyclophosphamide) and isophosphoramide mustard (ifosfamide).[11]

A third example of a cytoprotector is the drug 2-[(3-aminopropyl)amino]ethanethiol dihydrogen phosphate (ester) (WR-2721, ethyol, or amifostine), which is a prodrug that is metabolized to an active free thiol form by the intracellular enzyme alkaline phosphatase (ALP). The free thiol form can help lower the nephrotoxic effects of cisplatin by inactivation of the active nephrotoxic species of cisplatin within the renal tubules. The free thiol form can also quench free radicals, thus limiting the damage caused by radiotherapy. Amifostine has protective effects that are more specifically targeted to non-cancerous tissue due to the higher pH and ALP levels in normal cells which allows for greater accumulation and activation of amifostine to its protective form.[12] The increased specificity towards normal tissues allows the drug to shift the tolerance of the normal tissue curve toward the right without changing the anti-tumor efficacy curve.

1.3 Chemotherapy and Radiotherapy Sensitizers

Ideal chemotherapy and radiotherapy sensitizing agents work by specifically interacting with tumor cells without damaging the normal tissue. This affects the therapeutic index by shifting the anti-tumor curve to the left, with little or no effect on the normal tissue tolerance curve (Figure 1A). Chemotherapy sensitizers adopt a wide variety of approaches. A few examples include proteasome inhibition, apoptosis sensitization, and disruption of tumor-mediated drug resistance. Velcade (bortezomib) is a proteasome inhibitor which prevents the degradation and reuse of proteins. Protein production requirements are high in cells that are undergoing frequent division. In addition, many pro-apoptotic proteins are targeted for proteasome destruction in cancer cells. By disrupting the destruction of pro-apoptotic proteins and recycling of amino acids for further protein production, the neoplastic cells become more vulnerable to death by chemotherapy.[13,14] Conversely, inhibiting the function of anti-apototic proteins, such as Bcl-2, can have the same effect. Bcl-2 anti-sense therapy is, therefore, another way to restore apoptotic sensitivity. Different tumor types are known to upregulate Bcl-2. The expression of Bcl-2 at high levels in cancer prevents apoptosis after exposure to therapeutics like chemotherapy and radiotherapy. Anti-sense oligonucleotides can bind to and inactivate the sense DNA strand or the mRNA product destined to be translated into the Bcl-2 protein. This allows the cell to shift to a more pro-apoptotic state and increases the response to chemotherapy.[15,16] Finally, p-glycoprotein (MDR) is a xenobiotic pump that is expressed in normal and tumor tissues and helps protect the cells by expulsing toxic drugs from the cytosol. P-glycoprotein has been shown to be strongly upregulated in cells exposed to certain xenobiotics, particularly in tumors. Verapamil and cyclosporine A are two drugs known to inhibit this pump. Combination of these

drugs with chemotherapy can help to eliminate acquired tumor resistance and restore the cancer cell chemosensitivity.[17-19]

Radiation sensitizers include chemotherapy, hypoxic cell sensitizers, and hypoxic cytotoxins. Drugs like 5-fluorouracil (5-FU) and cisplatin have been used to sensitize cancer cells prior to radiation therapy. 5-FU selectively sensitizes neoplasia because it targets cells with dysfunctional cycle regulation, that overexpress thymidylate synthetase, and that have large amounts of mRNA expression. After chemotherapy exposure, cancer cells preferentially progress into and through the S phase, attempting DNA replication in the face of an antimetabolite instead of arresting at the G1/S checkpoint. Radiation therapy will cause sublethal and potentially lethal damage which will not be repaired due to the inability to synthesize new thymidine bases; 5-FU can synergize with radiation therapy because it can directly form a covalent complex with thymidylate synthetase, thereby further interfering with thymidine production.[20] Another theory suggests that 5-FU is metabolized and activated more quickly and remains inside tumor cells longer after being exposed to radiation.[21,22] Cisplatin, by formation of intrastrand DNA purine-purine cross links, is proposed to inhibit the repair of sublethal and potentially lethal ssDNA and dsDNA damage created by radiation therapy creating enhanced damage to the locally irradiated tissue. [23] Hypoxia is a major cause of radiation resistance and many solid tumors have large areas of hypoxia.[24] Low linear energy transfer radiation such as x-rays used in traditional radiation therapy causes a majority of its damage to the DNA through free radical formation with oxygen molecules.[24] The nitroimidazole drug misonidazole acts as an oxygen donor in hypoxic cells. The drug remains oxidized in normoxic tissues, but becomes reduced and immobile in hypoxic tissues. When the molecule is irradiated,

it will serve as a source of free radical formation and restore some of the free radical damage lost due to the hypoxic environment. This is specific to tumor cells because very little normal tissue is hypoxic.[25] Hypoxic cytotoxins such as tirapazimine and mitomycin are not traditional sensitizers, but they spatially cooperate with radiation to attack both normoxic and hypoxic cancer cells. Tirapazimine and mitomycin in their oxidized forms are less toxic, and selectively accumulate in hypoxic tissues via reductive mechanisms. This reduction causes them to bind to the hypoxic tissues and activate to a more toxic species. The hypoxic cytotoxins in combination with local radiation therapy may achieve better tumor control compared to either treatment modality alone.[26,27] These are just a few of the ways cancer cells can be selectively sensitized to increase the therapeutic index.

1.4 Canine Nasal Tumors

Canine nasal tumors are the fifth most commonly treated cancer with external beam radiation therapy, despite a low overall occurrence of about 1% of all tumors in dogs.[28,29] Radiation therapy alone results in median survival times (MST) of approximately 12-14 months.[30-34] The majority of canine nasal tumor patients are euthanized or die due to local recurrence and not metastatic disease.[35] Due to lack of effective local control, other additional treatment options in combination with radiation are needed. Cisplatin has been used as a radiosensitizer in dogs. Low dose cisplatin in combination with radiation to treat nasal tumors was well tolerated, but no statistically significant increase in survival time was noted.[36] Cisplatin has also been safely used as a radiosensitizer in the form of open-cell polylactic acid (OPLA)-platinum, but no definitive survival advantage compared to historical controls was demonstrated in these

two studies.[37,38] Higher dosages of cisplatin may make for a more effective sensitizer and allow for an increase in tumor control, however, damage to the normal local tissues and systemic toxicities may also be increased. In order to mitigate the toxicity from higher doses of chemotherapy without decreasing efficacy, a chemo/radioprotective drug could be used. Additionally, the use of a chemo/radiosensitizing drug may increase tumor cytotoxicity without affecting normal tissue damage. The combination of these multimodal approaches with sensitizing and protecting agents would be expected to help increase the therapeutic index and gain better local tumor control.

CHAPTER 2: Tavocept Pharmacology and Mechanisms of Action

2.1 Tavocept Pharmacology

Tavocept (disodium 2'-dithio-bis-ethane sulfonate) is a water soluble disulfide drug that is delivered intravenously to reduce normal tissue damage from chemotherapeutics like cisplatin, as well as to increase cytotoxicity to cancer cells.[39] The safety and efficacy of this drug depends greatly on its metabolism in the plasma and cellular environment, as well as its organs of distribution. The plasma and extracellular environment are normally oxidized due to the high oxygen concentration and lack of reductive enzymes. This oxidative environment causes thiol-containing molecules to predominantly exist as (oxidized) disulfide forms. Additionally, Tavocept is far more water soluble than mesna, and therefore the solvation free energy is more stable in favor of Tavocept over mesna in an aqueous environment. Common disulfides in the plasma include cystine, homocystine, and glutathione disulfide, with cystine being the most

abundant at a concentration of 200-300μM. Because of the high partial pressure of oxygen present, only 5% of thiol containing molecules are reduced in the plasma. Within the cellular environment, the presence of reductive enzymes and the lower oxygen concentration create a reduced environment wherein approximately 99% of the thiol molecules exist as free thiols. Cysteine, homocysteine, and glutathione are examples of intracellular free thiols and of these, glutathione is the most abundant at a concentration of 1 mM.[40-43]

Disulfide drugs such as Tavocept mostly remain as disulfides when they enter an oxidized environment such as the plasma. The normal plasma maintains a 95%:5% oxidized to reduced ratio, and the administration of additional disulfide drug can only shift the balance by a small degree. The theoretical maximum increase in the oxidized:reduced species ratio when injecting disulfide drug into the blood would be 5% with a final balance of 100%:0%.[44] The relatively small change in oxidized:reduced disulfide:thiol balance allows large doses (18.4 g/m^2) of Tavocept to be given safely, resulting in very high plasma concentrations (e.g., ca. 10 to 20 mM) of the drug and therefore more drug is available to enter the cells. At this dosage, approximately 10 mM of drug in the plasma is achieved, and this concentration far exceeds that of the most abundant physiologically generated disulfides.[45-47] Free thiol drugs like mesna are more likely to disrupt and lower the amount of oxidized species relative to the reduced species of disulfide:thiols in the plasma because mesna will undergo rapid oxidation in an oxidative environment and thereby deplete the concentrations of oxidized cystine, homocystine and glutathione disulfide. The initial low concentration of reduced molecules in the plasma makes significantly shifting the normal homeostatic balance

much easier. The equilibrium relationship of disulfides to free thiols in plasma, and in the cells appears to obey a Law of Mass Action. This relationship seems to be physiologically conserved in mammals. The administration of mesna creates toxicity at much lower plasma concentrations as compared to a disulfide drug because it will cause a larger disruption in the opposite direction of the disulfide to free thiol proportions. Administration of mesna will transiently have a greater effect in perturbing and increasing the free thiol concentration, thereby reducing the proportion of disulfides in plasma. For example, a single 6.0 g/kg and a 4.0 g/kg dose of Tavocept can be given intravenously to a rat and a dog, respectively, with no toxicity. Doses of 1.8 g/kg of Mesna resulted in 50% lethality in the rat, and 0.4 g/kg of mesna was lethal in the dog.[48,49] The lack of plasma or extracellular reduction of Tavocept also prevents the drug from binding to chemotherapy agents in the extracellular compartments. This is in contrast to a reduced drug like Mesna, which is more prone to bind chemotherapeutics in the plasma. The disulfide form (Tavocept) is far less reactive compared to the free thiol form (mesna).[44,48]

After intravenous injections, only small amounts of Tavocept undergo reduction to free thiols in the plasma. This occurs via a non-enzymatic thiol transfer (SN2 displacement) and results in the production of mesna and mesna disulfide heteroconjugates. Much more reduction occurs once Tavocept enters the cytoplasm of the cell. The balance of the intracellular environment is 1%:99% oxidized to reduced, and the entry of an oxidized disulfide like Tavocept will transiently disrupt this ratio. The shifting to a more oxidized environment is responsible for several of the drug's effects.[44]

2.2 Tavocept Mechanism of Action- Chemosensitization

Thioredoxin (TRX) and glutaredoxin (GRX) are intracellular signaling molecules that initiate pathways that can act as key drug resistance and survival signals in normal and neoplastic tissue. GRX is coupled to TRX metabolically, and TRX overexpression leads to increased tumor resistance to oxidative stress and chemotherapy induced apoptosis, and increased RNA to DNA conversion, increased VEGF expression, and increased nuclear expression of cell division signaling. Overexpression of these pathways is observed in almost all adenocarcinomas and it may also be increased in other tumors.[50-55] In the oxidized form, TRX/GRX pathways are inactive and no downstream signaling occurs. Thioredoxin reductase and glutathione reductase use energy from NADPH to generate reduced and active TRX and GRX respectively. GRX is reduced directly by glutathione, and glutathione is reduced by glutathione reductase.[50,54] When reduced, these molecules can serve to reduce other downstream signaling molecules important in the initiation of RNA to DNA precursor conversion and gene expression, anti-apoptosis, anti-oxidative stress, increased VEGF production, and increased glutathione and precursor production. These factors can all promote drug resistance and proliferation in the face of chemotherapy induced stress.[50,54] Additionally, when a disulfide drug such as Tavocept enters the cell, the balance is shifted from a reduced to a more oxidized environment which is an augmentation of the oxidative stress. Tavocept and its metabolites promote covalent oxidation of TRX/GRX keeping them in an inactive non-signalling state, thereby restoring sensitivity to oxidative stress and apoptosis promoted by subsequent chemotherapy treatment. In addition, Tavocept is intracellularly

metabolized into mesna and mesna heteroconjugates, and mesna can covalently bind to TRX/GRX. This binding can prevent the use of these TRX/GRX molecules in further signaling, and requires that further energy be expended in the form of a NADPH molecule to remove the mesna and regenerate TRX/GRX to their active forms.[56] There are two major end results from the cytoplasmic interactions of Tavocept. By inactivating/delaying the reduction of TRX/GRX, Tavocept helps to restore and normalize apoptotic sensitivity and reduce proliferation of cancer cells that overexpress these pathways. Furthermore, Tavocept is depleting cellular energy in the form of NADPH when TRX/GRX reduction uses NADPH to regenerate the active and reduced forms.[56]

Only about 5% or less of the administered Tavocept enters the cancer cells, yet, potentiation with chemotherapy is still observed.[57] When treating rats bearing subcutaneous WARD colon carcinoma, the use of Tavocept and cisplatin together increased the response dramatically compared to cisplatin alone, while concurrently preventing and mitigating sublethal or lethal toxicities. Tavocept given alone at 1000 mg/kg resulted in no anti-tumor responses or toxicities. Cisplatin given at dosages of 6 and 9 mg/kg resulted in a 25% complete response (CR) rate and a 75% partial response (PR) rate with no toxicity in the 6 mg/kg group and a 40% acute lethal toxicity rate in the 9 mg/kg group. The combination of 1000 mg/kg Tavocept with cisplatin at either of the two concentrations resulted in a 75% complete response rate and a 25% partial response rate with no acute lethal toxicity observed.[58] In another rat model, advanced (3 g tumor size) subcutaneously implanted colon adenocarcinoma was treated with oxaliplatin and Tavocept either alone or in combination. This model again demonstrated anti-tumor

enhancement when the drugs were used together. As expected, Tavocept alone did not have anti-tumor activity or toxicity while oxaliplatin at a dose of 15 mg/kg resulted in a 25% CR and 75% PR with no deaths. When given at a higher dose of 25 mg/kg, the oxaliplatin resulted in a 100% PR, but with a 100% animal lethality. When Tavocept at 2000 mg/kg was added to either dose of oxaliplatin, no lethality was observed and 100% CR was seen with the 15 mg/kg group, and 75% CR and 25% PR was seen with the 25 mg/kg group.[59] The beneficial effects are not limited to the platinum chemotherapeutics. When combined with 46.5 mg/kg of paclitaxel, Tavocept at 1000 mg/kg significantly delayed tumor growth in a cervical adenocarcinoma rat xenograft model when compared to no drug or to paclitaxel alone.[60] In human clinical trials, a meta-analysis of a Phase II United States study and a Phase III Japanese study in patients with advanced (inoperable) non-small cell lung cancer revealed improved response rates and significant increases in overall survival in the adenocarcinoma subgroups with the addition of Tavocept. The data demonstrated that the addition either 40 g IV (USA) or 18.4 mg/m^2 (Japan) of Tavocept to the combination chemotherapy protocol of cisplatin and paclitaxel significantly extended the overall and one year survival time compared to chemotherapy alone in patients with advanced adenocarcinoma, the most common subtype of lung cancer in the world. It was also noted that this outcome was most significant in adenocarcinomas, which have been shown to upregulate TRX/GRX as a survival/resistance mechanism.[39]

2.3 Tavocept Mechanism of Action- Nephroprotection

Cisplatin has been used in human and veterinary cancer therapy for years. It attacks cancer cells by binding preferentially to two adjacent intrastrand purine residues,

guanine-guanine in particular, and acting as a physical roadblock to DNA replication machinery, interfering with DNA repair as well as mRNA transcription.[1] The major toxicities of cisplatin include nephrotoxicity, myelosuppression, immediate emesis, ototoxicity, and peripheral neurotoxicity.[1] When cisplatin is administered intravenously, most of the drug will stay in its native form due to the high chloride content (ca. 110 mM) of the plasma. When the drug enters the intracellular space of either cancer or normal tissue, the chloride concentration significantly drops (e.g., to zero) and the drug will undergo aquation or hydroxyl displacement on one of the chloride leaving groups, creating very reactive aquo or hydroxyl derivatives of cisplatin.[61] When aquated cisplatin is present in the renal tubules, the cells attempt to detoxify the drug. Aquated cisplatin becomes attached to a glutathione molecule either spontaneously or via glutathione-S-transferase and is subsequently shuttled down the gamma-glutamyl transpeptidase (GGT) xenobiotic metabolism pathway. There are three enzymatic steps involved and the final product is a toxic platinum thiol/mercapturic acid species that can lead to renal tubular cell death.[62] To protect the kidneys from this toxicity, cisplatin is administered with an intravenous diuresis protocol involving 0.9% to 3% NaCl to increase the chloride content in the renal tubules by elimination of the excess chloride. The elevation in chloride content mitigates the aquation and hydrolysis of the cisplatin and reduces the renal toxicity.[61]

Tavocept distributes well to the kidney given its water soluble nature. It has been demonstrated that it and its mixed disulfide metabolites interacts with cisplatin and the GGT xenobiotic metabolism pathway to prevent and mitigate cisplatin renal toxicity.[57,62-66] As previously described, when Tavocept enters the reductive intracellular

environment, it undergoes a non-enzymatic thiol transfer which produces mesna and mesna-disulfide heteroconjugates.[44] Mesna can directly bind to aquated cisplatin preventing it from becoming a substrate for the GGT xenobiotic metabolism pathway.[62] Additionally, many of the mesna-disulfide heteroconjugates can inhibit the enzymes of the GGT pathway at different steps. Mesna-glutathione and mesna-cysteinyl-glutamate have been shown to inhibit the initial enzyme of the pathway, gamma-glutamyl transpeptidase (GGT), in a dose-dependent fashion. This is true for both human and porcine GGT.[67] Interestingly, two of the heteroconjugates, Mesna-cysteine and mesna-cysteinyl-glycine, had a dose dependent and dose independent enhancement of GGT respectively.[67] The second enzyme of the GGT xenobiotic metabolism pathway is aminopeptidase N (APN). In a study evaluating the effects of Tavocept, mesna, and the mesna-disulfide heteroconjugates on the human APN enzyme, mesna-glutathione, mesna-cysteine, and mesna-cysteinyl-glycine were all shown to have an inhibitory effect.[68] It is also believed that all mesna-disulfide heteroconjugates will inhibit the final cisplatin-glutathione toxification step that is mediated by cysteine-conjugate β-lyase (CCBL).[62] In summary, Tavocept is postulated to prevent and mitigate cisplatin nephrotoxicity by both inhibiting the GGT mediated cisplatin toxification pathway which results in a toxic cisplatin thiol species, as well as preventing cisplatin from becoming a substrate for the GGT pathway by direct conjugation of mesna to the aquo and hydroxyl species of cisplatin that are formed or excreted in the renal tubular epithilium.

Nephroprotection by Tavocept has been demonstrated in both animal and human studies. In a rat model, creatinine levels and blinded Good Laboratory Practice (GLP) histopathological scores were evaluated and compared for different treatment and control

groups including no therapy, saline therapy plus 6 mg/kg cisplatin, 1000 mg/kg Tavocept only, 6 mg/kg of cisplatin, and the combination of different doses of Tavocept given with 6 mg/kg cisplatin. The doses of Tavocept were 37 mg/kg, 111 mg/kg, 333 mg/kg, and 1000 mg/kg which correspond to Tavocept: cisplatin molar ratios of 6:1, 17:1, 51:1, and 154:1 respectively. Significantly increased renal toxicity measured by increased creatinine levels and worsening blinded histopathology scores were observed in the 6 mg/kg cisplatin alone group and in the 6:1 and 17:1 molar ratio group compared to the other groups that received higher doses of Tavocept. In the higher molar ratio groups of Tavocept: cisplatin (51:1 and 154:1), both creatinine levels and blinded histopathological scores were not significantly different from no treatment, saline treatment, and Tavocept alone, demonstrating substantial nephroprotection at molar ratios of greater than 50:1.[69]

Similar GLP study results in rats were observed in a study that evaluated the timing of Tavocept and cisplatin with two different molar ratios of Tavocept to cisplatin. The study demonstrated that the lower molar ratio of Tavocept to cisplatin of 38:1 was not protective if given at or before the administration of cisplatin, and Tavocept had no nephroprotective effect when administered after cisplatin. Delayed administration of Tavocept also demonstrated an increase in nephrotoxicity. The higher molar ratios of Tavocept to cisplatin (308:1) confirmed nephroprotection when given prior to, concurrently, or if Tavocept was given five or fifteen minutes after cisplatin. This latter effect is significant in that it demonstrates a clear toxicokinetic effect of Tavocept on preventing and mitigating cisplatin renal toxicity even with delayed administration. It was expected that renal protection would be lost to a degree when Tavocept was given either five or fifteen minutes after the cisplatin, however there is a clear protective effect

at the higher molar ration, even with delayed administration of Tavocept after cisplatin.[69] The toxicity of low molar ratios of Tavocept: cisplatin was also evaluated in the canine model. When delivered at a molar ratio of 38:1, more toxicity was observed when compared to 3 mg/kg of cisplatin alone or Tavocept: cisplatin molar ratios of 154:1 and 615:1.[70] Low concentrations of Tavocept may actually stimulate the GGT portion of the GGT xenobiotic metabolism pathway and increase the nephrotoxicity of the drug. Higher doses of Tavocept appear to overwhelm this potential mechanism. As noted previously, mesna-cysteine and mesna-cysteinyl-glycine had a dose dependent and dose independent enhancement of GGT.[67] At low doses, these stimulatory effects may predominate, and at higher doses the inhibitory effects of the other heteroconjugates appear to be more pronounced and complete. Additionally, giving Tavocept after cisplatin could inhibit the GGT xenobiotic pathway after cisplatin has started being toxified by it, which may precede and outrun the protective effect of Tavocept, thereby making it more toxic. For these reasons, it is recommended that Tavocept be given at molar ratios of at least 50:1 and at least 15 minutes prior to cisplatin administration.

In human dose escalation studies involving three separate Phase I studies, Tavocept at doses of 4.1, 8.2, 12.3 18.4, 27.6, and 41.0 g/m^2 were given to assess toxicity. No dose limiting toxicities were observed from Tavocept even at the highest dosing group. However, a grade 2 skin rash, transient reversible local intravenous site discomfort, nausea, and thirst at two highest dose levels suggested that 18.4 g/m^2 was appropriate.[45,46,66] In a second study, 18.4 g/m^2 of Tavocept was used with either a combination of cisplatin/paclitaxel or single agent cisplatin in a protocol that tested the hydration requirements usually used to prevent nephrotoxicity. Even with no

supplemental hydration with 0.9% NaCl, a 75 mg/m^2 dose of cisplatin (molar ratio of 245:1) resulted in no clinically significant nephrotoxicity after three to nine treatment cycles.[46] This is remarkable, as nephrotoxicity would be expected at doses as low as 15-20 mg/m^2 without supplemental hydration and the absolute minimum of fluid supplementation recommended is 1 L with lower dosages (50-70 mg/m^2).[61,71] In the Japanese Phase III trial of non-small cell lung cancer using cisplatin and paclitaxel, Tavocept at a dose of 18.4 mg/m^2 versus placebo resulted in significantly lower creatinine and nephropathy scores, but not blood urea nitrogen.[39] The combined results of these studies demonstrate both the safety and nephroprotective benefits of Tavocept in the human patient.

2.4 Tavocept Mechanism of Action- Neuroprotection, Antiemesis, and Bone Marrow Protection

In addition to nephrotoxicity, cisplatin can cause peripheral neuropathy, acute emesis, and myelosuppression.[1] Tavocept may mitigate some of these effects as a result of its tissue distribution and intracellular metabolism. The uptake of Tavocept is limited primarily to the epithelial cells lining the tubular brush border of the kidneys, the intestines, the bone marrow, and the dorsal root ganglia.[57,59,62-66,72] Chemotherapy induced peripheral neuropathy (CIPN) is a serious and prevalent side effect with the administration of both taxane and platinum drugs.[73,74] CIPN is thought to occur from drug induced aberrant microtubule protein polymerization.[75] Tavocept distributes well to the dorsal root ganglia and in an in vitro experiment, Tavocept was able to prevent cisplatin-induced aberrant microtubule protein polymerization. The mesna that is produced from the intracellular metabolism of Tavocept can bind to cisplatin which

inhibits the platinum from attaching to the microtubular proteins, and preventing aberrant polymerization.[76] Clinically, neuroproection has been documented in a Phase I study.[46] CIPN is a clinically relevant adverse effect in people, but it may be under recognized in the veterinary patient.

Soon after administration of cisplatin, most human and veterinary patients experience nausea and vomiting if not treated with antiemetics. This is thought to be mediated by peripheral mechanisms, with the chemoreceptor trigger zone playing a minor role.[77] Tavocept is selectively taken up in the gut and is thought to reduce this side effect, but mesna inactivation of cisplatin does not appear to be the mechanism of protection.[63,78] In the dog, Tavocept appeared to reduce cisplatin-mediated emesis. When compared to a dog that received 3.0 mg/kg of cisplatin alone, dogs that received Tavocept at molar ratios of 38:1, 154:1, and 615:1 experienced a 47%, 27%, and 74% reduction in the number of vomiting episodes and a 55%, 42%, and 89% reduction in the duration of vomiting respectively.[70] In the previously described phase III Japanese trial of Tavocept versus placebo, the Tavocept group had a significantly lower vomiting score. Nausea and anorexia scores were not significantly different according to clinical reports.[39] However, in the patient reported scores of quality of life assessments, nausea/vomiting and anorexia were significantly lowered compared to placebo, but diarrhea was not.[39]

Myelosuppression is also a potential toxicity of cisplatin. Like most chemotherapy agents, cisplatin is more toxic to rapidly dividing cells, including the highly proliferative, immature bone marrow precursor cells. As previously mentioned, cisplatin causes intrastrand DNA crosslinks specifically at two adjacent purine bases,

which creates a road block to proliferation for molecules like DNA polymerase.[1] This can disrupt the normal expansion and differentiation of these cells and cause a drop in the white blood cell count.[1] Tavocept distributes well to the bone marrow. It is proposed that intracellular production of mesna leads to binding and inactivation of cisplatin, thus preventing the drug from cross-linking DNA. [59] In previous studies, 3.0 mg/kg cisplatin was given with or without Tavocept and white blood cell (WBC) counts were checked 3 days before drug and four days after drug. Cisplatin was given alone and at molar ratios of 38:1, 154:1, and 615:1 of Tavocept to cisplatin, and caused a decrease in the WBC count of 66%, 77%, 41%, and 44% respectively. The low molar ratio group showed decreased WBC counts compared to cisplatin alone, whereas at higher molar ratios values, the degree of myelosuppression was decreased.[70] Platelet counts were also less suppressed in the two higher molar ratio groups as compared to the cisplatin alone and 38:1 group. These findings suggest that higher molar ratios may provide myeloprotection when given with cisplatin.[70] In the Phase III Japanese trial, patients treated with cisplatin and Tavocept or placebo had significantly less suppression of hemoglobin and hematocrit when pretreated with Tavocept, although red blood cell decreases were not significantly different.[39] The combination of these results suggests that both the myeloid and erythroid cell lines may experience protection from Tavocept.

In summary, the different studies discussed show that Tavocept may be safely administered in high concentrations and that it acts both as a chemosensitizer and a chemoprotector. Both of these mechanisms increase the therapeutic index of antineoplastic agents like cisplatin. Adenocarcinomas, and potentially other tumors, are known to overexpress TRX/GRX. Tavocept disrupts this pathway, restoring the

neoplastic cell's sensitivity to chemotherapy. It is believed that this upregulation accounts for the differential effects between cancer and normal cells. Tavocept also protects the kidney, bone marrow, and peripheral nerves through mesna binding and inactivation of cisplatin. Mesna and mesna-disulfide heteroconjugates form intracellularly from Tavocept via a non-enzymatic thiol transfer and Tavocept does not interact with cisplatin in the plasma due to the predominance of the relatively stable disulfide form, and the highly oxidative environment and lack of Tavocept metabolism. Mesna disulfide heteroconjugates also play a role in preventing the toxification of cisplatin in the kidney. Finally, Tavocept is taken up by the intestinal enterocytes and clinically seems to reduce cisplatin mediated emesis, but the mechanisms of this are not well understood.

2.5 Hypothesis

Studies evaluating the effects of Tavocept and cisplatin have been performed in rats, mice, dogs, and humans in vitro and in vivo, looking at both normal and tumor tissue. However, it is currently unknown how Tavocept will interact with radiation. The goals of this project were to examine the effects of Tavocept on irradiated a nasal cancer cell line, with and without cisplatin. The hypotheses are that 1) Tavocept alone will not cause any cytotoxicity, 2) cisplatin with Tavocept will increase cytotoxicity compared to Tavocept alone, 3) radiation with cisplatin will increase cytotoxicity compared to radiation alone, 4) radiation with Tavocept will increase cytotoxicity compared to radiation alone, and 5) radiation, cisplatin, and Tavocept will increase cytotoxicity compared to radiation and cisplatin alone.

CHAPTER 3: Assessing Tavocept, Cisplatin, and Radiation Therapy Interactions in a Nasal Carcinoma Cell Line

3.1 Media, Reagent, and Cell Preparation

Five hundred milliliter (mL) bottles of Minimum Essential Medium (MEM)[a] were purchased commercially and stored at 4ºC when not in use. Fetal bovine serum (FBS)[b] and L-glutamine (L-glut)[c] were purchased commercially and stored at -20ºC. Prior to media preparation, each component was warmed to room temperature. A 500 mL, 0.2µm pore size MF75 Tissue Culture Filter[d] was used to mix and filter the media. 50 mL of FBS and 5 mL of L-glut was added to 445 mL of MEM to a total volume of 500 mL and concentrations of 10% FBS and 1% L-glut. All media was stored at 4ºC after preparation.

100% glacial acetic acid[e] was purchased commercially and was stored at room temperature prior to use. The acetic acid was diluted to a 1% concentration with distilled water and stored at room temperature prior to use. Trichloroacetic acid (TCA)[f] was purchased commercially and was stored at room temperature until ready for dilution. The TCA was diluted with distilled water until a 10% solution was obtained. The 10% TCA solution was stored at 4ºC at all times. Sulforhodamine B (SRB)[g] was purchased commercially and was stored at room temperature until ready for dilution. SRB was mixed with 1% acetic acid and dissolved over a stir plate for three hours at room temperature to a final concentration of 0.4%. The solution was protected from light at all times with aluminum foil. The SRB solution was stored at 4ºC until ready to use at which time it was placed back on the stir plate at room temperature for two hours.

Tris(hydroxymethyl)aminomethane (Tris)[h] was purchased commercially and was stored at room temperature until ready for dilution. A 10mM solution was achieved by dissolving Tris with distilled water. The 10mM solution was then stored at 4ºC until ready for use. Prior to use, Tris was allowed to reach room temperature.

The RPMI 2650 cell line (nasal septum squamous cell carcinoma)[i] was purchased commercially and immediately placed and stored in liquid nitrogen at -80°C for 1 week. After one week, the cryovial[j] containing the cells was removed from liquid nitrogen and thawed by placing it in a warm water bath at 37ºC. The cryovial was then washed with isopropyl alcohol and dried, and the thawed cells were pipetted out and placed into a T-75 flask[k] with 20 mL of fresh media (MEM 10% FBS, 1% L-glut, no antibiotics). Cells were maintained in a water-jacketed cell culture incubator[l] at 37°C in a humidified atmosphere containing 5% CO_2. The cells were passaged five times (P5) after which TrypLE™[m] was used to dissociate the cells from the flask, and the cells were suspended in MEM with 10% DMSO[n] and aliquotted to cryovials. The cryovials were placed into a Cryo 1ºC "Mr. Frosty" freezing container[o] using 100% isopropyl alcohol and then placed in a -80°C freezer to allow for a 1°C/minute cooling rate. The cells were removed after 24 hours and directly placed into liquid nitrogen for storage.

3.2 The SRB Assay

The SRB assay was performed to assess the clonogenic survival and cytoxicity of the treatments used in this study. The SRB assay was run on cells plated on 96 well plates[p] that had undergone five doubling times in the incubator after a treatment or series of treatments. After the five doubling times, the cells were removed from the incubator, the media discarded, and 100µl of 10% TCA was pipetted into each well to kill

and fix the cells to the plates. The plates were placed in a sealed plastic bag to prevent evaporation of reagents, and stored at 4ºC for 1.5 hours. The plates were then removed from the bag, and the TCA was discarded. The plates were washed five times with distilled water and then allowed to air dry for 30 minutes at which time 100µl of room temperature SRB (0.4%, 1.0% acetic acid) was added to each well. The SRB was allowed to stain the fixed cellular proteins for 15 minutes and then discarded. The plates were then washed five times with 1% acetic acid and allowed to air dry for 30 minutes. Next, 150µl of Tris buffer was added to each well and the plates were shaken for 5 minutes using the Titer plate shaker[q]. The plates were then read using a Spectramax Plus 384 UV plate reader[r] with a UV light at 570 nm and the optical density (OD) of each of the wells was obtained.

3.3.1 Cell Doubling Time Experiment Methods and Materials

A P5 cryovial was defrosted and passaged two additional times in T-75 flasks. After the second passage became 80% confluent, the cells were trypsinized for 7 minutes, washed with 10 mL of fresh media, collected, and counted with a standard hemocytometer[s] using trypan blue[t] to exclude non-viable cells. For counting, a 20 µl volume of the cell/media solution was placed into a microcentrifuge tube[u] and then 10 µl of trypan blue was added. This was thoroughly mixed and 10 µl of the cell/media/trypan blue mixture was removed from the tube and placed on a hemocytometer for counting. Cell counts were repeated. The cell concentrate was diluted using fresh media until a concentration of 2 $x10^5$ cells/mL was achieved. One mL was added to each of five T-25 flasks[v]. Seven additional mL of fresh media were also added to each flask resulting in a total of 8 mL/flask.

In order to obtain a doubling time for the cell line RPMI 2650, an experiment was performed that serially counted the cells every 24 hours for 5 consecutive days. Each day, one of the T-25 flasks was removed, the media discarded, and the cells trypsinized for 10 minutes to ensure complete detachment. The cells were vigorously washed with 2 mL of fresh media to further detach any remaining cells from the bottom of the flask. The cells were counted on a hemocytometer in the manner previously described. This process was repeated 5 days in a row using one T-25 flask each day and obtaining counts for 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours from seeding. The 24 hour value was not used in the calculation of the doubling time in order to allow the cells to become seated to the flask. Two formulas were used to calculate the doubling time and averages of the doubling times were calculated to ensure repeatability.

BioNumerik Pharmaceuticals (BNPI) Formula: Cell Count @time X = 2 x 10^5 cells x $2^{(t/to)}$ where t = 24, 48, 72, 96 or 120 hours and t0 = doubling time

Knapp Lab Formula: [dt = [t] x [ln2/ln(Ct/Co)] where dt= doubling time, t=time between cell counts Ct and Co, Co=initial count, Ct=count after time t and ln=natural log)

3.3.2 Cell Doubling Time Experiment Results

The doubling time values obtained using the BNPI formula were 28.2 h, 30.1 h, 24.5 h, and 24.4 h respectively for the 48, 72, 96, and 120 hour time frames. The average result was 26.8 hours (standard deviation ±2.8 h). The doubling time values using the Knapp laboratory formula was only slightly different with 28.2 hours, 30.1 hours, 24.5 hours, and 24.5 hours respectively for the 48, 72, 96, and 120 hour time frames. The results of these calculations are presented in Figure 3.3.2A. A graphical

representation of the cell growth is presented in Figure 3.3.2B. Based on these results, the value of five doubling times was calculated to be 134 h, or roughly 5.6 days. 6 days was chosen as the interval to allow five doubling times due to laboratory use time constraints.

3.4.1 Cells/100µl Experiment

In order to determine the ideal number of cells to plate per well for optimal assessment after five doubling times, a cell per well experiment was performed. A P5 cryovial was defrosted and passaged an additional two times in T-75 flasks. After the second passage became 80% confluent, the cells were trypsinized for 7 minutes, washed with 10 mL of fresh media, collected, and counted using a standard hemocytometer using trypan blue to exclude non-viable cells. The cell and media mixture was diluted to 1000, 1200, 1400, and 1600 cells/100µl and 100µl of each concentration was added to a 96 well plate according to the plate design in Figure 3.4.1A. 100µl of fresh media was then added to each of these wells for final concentrations of 500, 600, 700, and 800 cells/100µl and these numbers were chosen from previous experience with the cell line.

After plating, the cells were allowed to remain in the incubator for seven days (24 hours to adhere to the plate, and six days for five doubling times). After seven days, the cells were removed from the incubator, the media was removed, and the SRB assay was performed.

3.4.2 Cells/100µl Results

Results for the cells/100µl experiment are shown in Figure 3.4.2A. The linear range of the Spectramax spectrometer is displayed in Figure 3.4.2B. At values greater than 2.0, the linear relationship between OD and cell number is lost. The OD for 500

cells was 1.64 (1.44-1.85), for 600 cells was 2.09 (1.90-2.28), for 700 cells was 2.40 (2.26-2.54) and for 800 cells was 2.55 (2.48-2.624). 500 cells was the only OD value in which the median value did not exceed the OD of 2.0 and therefore was chosen for future experiments.

3.5.1 Inhibitory Concentration 50% (IC-50) Experiment

To determine the ideal cisplatin concentration for the final experiment, a study was performed to find the concentration of cisplatin that inhibited 50% of cellular growth (IC_{50}). Based on the previous experiment, 100µl of the 500 cells/100µL solution was plated per well onto a 96-well plate excluding the blank wells. The plates were placed in the incubator for 24 hours to allow for cellular attachment. In order to simulate the final experiment, the plates were handled as if they were receiving Tavocept and radiation as well. After the 24 hour incubation, the old media was discarded, and 200µl/well of fresh media was added. This was performed to simulate the Tavocept drugging step. The plates were placed back in the incubator for one hour, removed, and then had the media discarded. The plates were washed with 200µl/well to simulate washing out the Tavocept. Cisplatin[w] was diluted with fresh media into 8 separate tubes to obtain concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56 µM . The wash media in the plates was discarded and 100µl of cisplatin and media solution of the various concentrations was added to the appropriate wells. This was immediately followed by an additional 100µL of fresh media cutting the final cisplatin concentration in half according to Figure 3.5.1A. Non-drugged wells had 200µL of fresh media added. Once drugged, the plates were placed in an incubator for one hour. After the incubation, the cisplatin and media mixture was discarded and 200µl of wash media was added to each well and

discarded. Another 200µl of fresh media was added and the cells were transported manually to the University of Missouri linear accelerator (LINAC)[x] to simulate the radiation treatment. After 10 minutes the cells were returned to the incubator and were allowed to incubate for 5 doubling times, at which time the SRB assay was performed. Inconsistent results were obtained with the 500 cells/100µL concentration, so this experiment was repeated with 750 cells/100µL and 1000 cells/100µL. The 1000 cells/100µL was repeated for confirmation of consistency. The same methods were used in the repeat experiments.

3.5.2 Inhibitory Concentration 50% (IC-50) Results

The control wells of the IC-50 plates at 500 cells/100µL had an average OD below 1.0 at 0.44 (0.30-0.55) and the data was deemed unacceptable for use. Therefore, the experiment was repeated with 500 cells/100µL and the higher cell concentrations of 750 cells/100µL and 1000 cells/100µL as well. The OD of the 500, 750, and 1000 cells/100µL were and 0.61 (0.47-0.75), 1.47 (1.33-1.61) and 1.45 (1.27-1.63). This data is represented in Figure 3.5.2A. The 1000 cells/100µL experiment was repeated and yielded an average OD of 1.89 (1.45-2.33). The IC-50 values were calculated using Origin software program[y] and are demonstrated on the curves in Figure 3.5.2B. The values were 5.4 µM, 9.0 µM, and 13 µM for the three different concentrations (500, 750 and 1000 cells/100µL respectively). The SF2 experiments were run concurrently with the IC-50 experiments, and based off of the combined results of the two experiments, 1000 cells/ 100µL was chosen as the new cell seeding concentration.

3.6.1 Survival Fraction 2Gy (SF2) Experiment

To determine the amount of cell kill caused by 2 Gy of radiation, the survival fraction 2 Gy (SF2) experiment was performed. The SF2 experiment was performed concurrently with the IC-50 experiment and 500 cells/100µL were seeded per well onto a 96 well plate excluding the blank wells. The plate layout is shown in Figure 3.6.1A. The plates were placed in the incubator for 24 hours to allow for cellular attachment. In order to simulate the final experiment, the plates were handled as if they were receiving Tavocept and cisplatin. After 24 h incubation, media was discarded, and 200µL/well of fresh media was added in. This was performed to simulate the Tavocept drugging step. The plates were placed back in the incubator for one hour and then the media was discarded. The plates were washed with 200µL/well to simulate washing out the Tavocept. The wash media in the plates was discarded and 200µL of fresh media was added to the wells and the plates were incubated for one hour. This was to simulate the cisplatin drugging step. After the incubation, the media was discarded and 200µL of wash media was added to each well and discarded. Another 200µl of fresh media was added and the cells were transported manually to the University of Missouri 8 MV LINAC and a total dose of 2 Gy of x-irradiation was delivered to the plates from parallel opposed fields to ensure dose homogeneity. The plates were placed at a source to surface distance (SSD) of 100 cm and 2.5 cm of bolus was placed under and on top of the plates to allow for maximum dose build up. Bolus was also placed around the plates to promote side scatter. The dose and set up were simulated with 3D radiation planning equipment $(Xio)^{Z}$ prior to treatment to ensure homogenous dose. After the treatment, the cells were returned to the incubator and allowed to incubate for 5 doubling times, at which time the SRB assay was performed. As with the IC_{50} experiment, the SF2 experiment was

repeated with 750 cells/100µL and 1000 cells/100µL. The SF2 was also confirmed again with 1000 cells/100µL. The same methods were used in these repeat experiments.

3.6.2 Survival Fraction 2Gy (SF2) Results

The results of the SF2 experiment from the two separate 500 cells/100µl groups are shown in figure 3.6.2A. As mentioned previously, the average OD of the first and second 500 cells/100µL experiments were 0.44 (0.30-0.55) and 0.61 (0.47-0.75) respectively. The OD of the irradiated cells were 0.14 (0.10-0.18) and 0.11 (0.06-0.16) resulting in SF2 values of 30.6% and 17.9%. Since both of the control ODs were below 1.0, these data were considered inaccurate. Figure 3.6.2B demonstrates the SF2 of the higher cell concentrations. The average ODs of the controls were 1.47 (1.33-1.61) and 1.45 (1.27-1.63), 1.89 (1.45-2.33) for 750, 1000 cells/100µL, and the repeat 1000 cells/100µL experiment respectively. The average ODs of the irradiated cells were 0.53 (0.41-.66), 0.29 (0.19-0.38), and 0.75 (0.58-0.92) which resulted in a SF2 values of 36.1%, 20.0%, and 39.7% for three respective groups. The results from the two separate 1000 cells/100µL groups produced a fairly repeatable SF2 and so this was chosen as the seeding concentration.

3.7 IC-50/SF2 Combination Experiment and Results

The radiation plates from the SF2 experiment contained cisplatin treated cells as well. The IC-50 of cisplatin for radiation treated cells was obtained using the same methods as the cells that were not irradiated. The IC-50 of radiation/cisplatin cells at 1000cells/100µL was lower than the cisplatin only cells at the same cell concentration (6.6 µM versus 10.9 µM). This is demonstrated in Figure 3.7A. The IC-50 for the repeat

1000 cells/100µL group was 7.4 µM. The average IC-50 from the two irradiated plates was 7.0 µM of cisplatin, and this was chosen as the final treatment dose.

3.8.1 Interaction of Tavocept in Combination with Cisplatin and Radiation Experiment

The final experiment to test the interaction of radiation, cisplatin and Tavocept involved using 2 Gy of radiation therapy, 7µM of cisplatin and 1 mM and 10mM of Tavocept[aa] as seen in Figure 3.8.1A and 3.8.1B. 10 mM of Tavocept was chosen because it represents the serum concentration of Tavocept after the clinical dose of 18.4 mg/kg was given intravenously to human patients.[45-47] 1 mM of Tavocept was chosen to evaluate any dose dependent effects. 1000 cells/100µl were seeded per well onto a 96 well plate excluding the blank wells. The plates were incubated for 24 hours to allow for cellular attachment. After the 24 hour incubation, media was discarded and 100µl of 20 mM and 2 mM Tavocept were added into the appropriate wells, followed by 100µL of fresh media resulting in final Tavocept concentrations of half (10 and 1 mM). 200µl of media was added to remaining control wells. The plates were placed back in the incubator for one hour and then removed and media was discarded. The plates were washed with 200µL/well of fresh media and the wash media was discarded. 100µL of 14 µM cisplatin and media was added to the appropriate wells, which was followed by an additional 100µL of fresh media, cutting the concentration in half. 200µL of fresh media was added into the control wells and the plates were placed in an incubator for one hour. After incubation, the media was discarded and 200µl of wash media was added to each well and discarded. Another 200µl of fresh media was added and the cells were transported to the LINAC and a total dose of 2 Gy of x-irradiation was delivered as

previously described to the appropriate plates. After the treatment, the cells were returned to the incubator and allowed to incubate for 5 doubling times, after which time the SRB assay was performed. This experiment was repeated a second time for confirmation of the data.

3.8.2 Statistical Analysis

The analyzed data were ODs from 2 different radiation treatment (RT) groups; the plates that received radiation (+), and the plates that did not receive radiation (-). One 96-well plate was in each RT group, and this design was replicated in a second experiment, creating a total of two plates per RT group. There were 7 different drug groups within each plate: Control (8 reps per plate), 1 mM Tavocept (12 reps per plate), 10 mM Tavocept (12 reps per plate), 7 μM cisplatin (24 reps per plate), 7 μM cisplatin + 1 mM Tavocept (12 reps per plate), 7 μM cisplatin + 10 mM Tavocept (12 reps per plate), and blank wells (8 reps per plate). The data were assessed for normality via graphically and statistically via Shapiro-Wilks test and normality assumptions were not met, even after attempts to transform the data. Therefore non-parametric methods were used.

Overall differences in median OD between plates were tested via Kruskal-Wallace rank sum test (two-sided, alpha = 0.05). If a significant difference was detected, multiple comparison procedures were used to identify significantly differing pairs of plates. If significant differences were detected between different RT groups but not within each RT group (i.e. the two experiments were similar but the two RT groups were not), the intra-group variability was considered a non-factor and the plates with the same RT group were analyzed together. If no difference was detected between the RT groups,

experiment and RT group were both considered non-factors and all plates were combined and analyzed as one set. Otherwise each plate was analyzed separately.

Statistical interactions between RT group and drug treatment group were assessed graphically and tested for a difference in median OD using the Kruskal-Wallis rank sum test (two-sided, alpha = 0.05). If significant interactions were suspected, differences in median OD between drug groups were tested within each RT group and differences between RT groups were tested within each drug treatment group (see below). If no interactions were detected RT and drug groups were each tested separately for differences in median OD.

Overall differences in median OD between RT and/or drug groups were tested using Kruskal-Wallis rank sum test (two-sided, alpha = 0.05). Planned comparisons included 7 μM cisplatin versus control, 7 μM cisplatin versus 7 μM cisplatin + Tavocept(1mM), and 7 μM cisplatin versus 7 μM cisplatin + Tavocept(10mM). If a significant overall chemotherapy difference was found, multiple comparison procedures were used to identify the pairs of chemotherapy groups that differed (two-sided, alpha = 0.05). Multiple comparisons were not necessary if significant RT effects were detected since there were only 2 groups. All statistical analyses were performed using R: A language and environment for statistical computing[bb].

3.8.3 Interaction of Tavocept in Combination With Cisplatin and Radiation Results

No graphical or statistical differences were observed in the median OD between the two experiments within the same treatment group (RT (-) or RT (+)), therefore, all plates were combined and analyzed as one set. Significant differences were noted between the RT (+) plate and the RT (–) plate with a p-value $< 2.2 \times 10^{-16}$. This is

summarized in Figure 3.8.3A. The median OD for each individual treatment group is demonstrated in Figure 3.8.3B. There is no apparent difference between the control wells and the Tavocept only wells at either concentration (median OD (interquartile range) 2.54 (2.46-2.56), 2.54 (2.51-2.56), and 2.53 (2.35-2.54) for the control, 1mM Tavocept, and 10mM Tavocept, respectively. It must be noted that the ODs are above the linear range of the UV spectrometer creating artificially low values and a tighter range of numbers in the RT (-) control and Tavocept alone groups. The cisplatin and radiation treatment groups were both statistically different from the control group demonstrating the expected cell kill with these treatment modalities. 7 μM cisplatin resulted in a median OD of 1.83 (1.51-2.27) and 2 Gy of radiation therapy caused a drop in the OD to 1.27 (1.24-1.38) and these were statistically different that the control group ($p<0.005$ and $p<6.212 \times 10^{-12}$ respectively). When Tavocept was added to the cisplatin group, an additional statistically significant cell kill was seen as compared to cisplatin alone. The median OD declined from 1.83 (1.51-2.27) to 1.28 (1.17-1.54) for the 1 mM group and 1.23 (1.05-1.57) for the 10 mM group ($p<0.005$ for both). There was no visual difference noted between the two Tavocept concentrations but this was not statistically analyzed. When Tavocept was added to irradiated cells, no graphical difference was seen compared to radiation alone at either concentration. The OD of the radiation group was 1.27 (1.24-1.38) compared to 1.23 (1.10-1.39) and 1.22 (1.12-1.42) for the 1 mM and 10 mM Tavocept with radiation groups respectively. Adding 7 μM of cisplatin to the irradiated cells lowered the OD to 0.63 (0.52-0.68) which was significantly different from radiation alone 1.27 (1.24-1.38) with a p value <0.01. The drop in OD was very close to 50% and this confirmed our IC-50 dose of cisplatin (7 μM) in irradiated cells. The addition of either concentration of

Tavocept to radiation/cisplatin treated cells increased the cell kill significantly. The OD of the 1 mM group was 0.46 (0.39-0.52) and 0.46 (0.33-0.50) for the 10 mM group ($p<0.01$ for both). The blank wells for both the irradiated and non-irradiated plates resulted in ODs of 0.001 (-0.003-0.003) and -0.001 (-0.003-0.003) respectively suggesting that no non-specific stain uptake was occurring.

CHAPTER 4: Discussion

Tavocept has been shown to be a safe and effective drug that both prevents and mitigates cisplatin's toxicities as well as increases its effectiveness against carcinomas.[39] Cisplatin is a documented radiosensitizer and can potentially increase control rates of head and neck cancers without increasing toxicity.[23] Cisplatin has also been used safely as a radiosensitizer in veterinary patients.[37,38] There is little information at this time regarding the interactions of Tavocept and radiation therapy with or without the addition of cisplatin.

Theoretically, Tavocept could act as both a radiosensitizer and a radioprotector. Tavocept has previously been shown to restore apoptotic sensitivity to cisplatin in cancer cells that upregulate the TRX/GRX pathways like adenocarcinomas.[39] Activation of TRX/GRX stimulates downstream pathways that are important in DNA synthesis, anti-apoptosis, VEGF production, and increased glutathione production could make tumor cells more resistant to radiation therapy. Tavocept could shut these downstream pathways off by inactivating TRX/GRX signaling and restoring sensitivity to ionizing radiation.[50,54] Particularly of interest is the inhibition of glutathione production since radiation can cause a majority of its damage through free radical formation and

glutathione is a potent radical scavenger.[24] Since the TRX/GRX pathways are not up-regulated to the same degree in non-malignant cells, combining Tavocept with radiation could increase the therapeutic index when compared to radiation therapy alone.[51,54]

Tavocept may also act as a radioprotector. The major mechanism of Tavocept's chemoprotective activity when used with cisplatin is the binding of intracellular mesna to the aquated cisplatin and thus preventing its interactions with key targets such as the DNA of the bone marrow stem cells or the GGT metabolism pathway in the kidney.[59,62] Mesna may also act as a protective agent by binding to free radicals created by radiation therapy. Mesna is a free thiol much like glutathione, and could potentially bind up reactive oxygen species that are formed after radiation therapy.[79] As mentioned previously, free radicals are responsible for a majority of x-irradiation therapy damage and Tavocept may minimize this damage.[24] Glutathione is the most abundant free thiol inside the cell and it can be found at 1mM concentrations.[40-42] After delivering a dose of 18.4 mg/m^2 of Tavocept, 10 mM of drug can be found in the plasma. The low percentage uptake by tumor cells and greater distribution to the kidneys, intestine, dorsal root ganglia, and bone marrow may allow for a large increase in intracellular free thiol concentration in the normal tissues compared to the neoplasm, increasing the therapeutic index.[57,62,64-66,80]

The fact that most canine nasal tumor patients die due to local disease and not metastatic disease illustrates that more intense local therapy is required.[35] Median survival times of patients treated with total doses below 56 Gy of megavoltage external beam radiation therapy have been around 12-14 months.[30-35] It has been suggested that more effective tumor control could be gained with larger total doses, but an increase in

normal tissue toxicity would also be expected. The recent ability to employ advanced radiation techniques such as intensity modulated radiation therapy (IMRT) allows for better tumor targeting and normal tissue sparing, making safely increasing the total dose more feasible.[81] Repeating radiation protocols in the face of recurrence months after the initial protocol has also shown promise in gaining longer local tumor control.[82] The addition of other local or systemic therapies in combination with radiation therapy can also increase the local control rate. Surgery prior to radiation therapy was historically performed with lower energy orthovoltage radiation units and it provided a survival benefit as compared to radiation alone.[83] With the use of higher energy megavoltage radiation machines, post-operative radiation has lost the survival advantage and instead gained an increase in patient morbidity as compared to megavoltage radiation alone.[83,84] Surgery following megavoltage radiation therapy has been met with greater success. The median survival time for patients treated with an accelerated protocol followed by nasal exenteration was 47.7 months. Surgery was only performed in patients that had a good initial response to the radiation. Most patients did experience late side effects such as chronic nasal discharge, but the increased survival time and local control rate was dramatic.[31]

Increasing local control using systemic chemotherapy as a radiosensitizer has not been as successful. Cisplatin used either as a low dose intravenous treatment or in the form of implantable beads was safe and did not increase local radiation side effect. However, it did not increase patient survival time or local control rates when compared to historical controls.[36,38] Theoretically, further increasing the chemotherapy dose would allow for better tumor radiosensitization and tumor control, but enhanced

radiosensitization could also be seen in the normal tissues. Furthermore, higher doses of chemotherapy can be expected to increase systemic toxicities. Ideally, a drug acting as a chemo/radiosensitizer on the neoplastic tissue and as a chemo/radioprotector on the normal tissue would allow for a safe increase in the radiation and chemotherapy dose, in addition to an increase in local tumor control.

A large percentage of canine nasal tumors are adenocarcinomas, therefore it is likely that most canine nasal tumors upregulate TRX/GRX.[85] As previously described, TRX/GRX upregulation promotes survival and growth signaling and prevents apoptosis.[50-55] This signaling may play an important role in the relative therapeutic resistance seen in canine nasal adenocarcinomas. Restoration of apoptotic sensitivity through inhibition of TRX/GRX would allow for greater local tumor control with radiation or chemotherapy using the current doses, and potentially obviate the need for aggressive surgery after radiation.

Radiation side effects are usually characterized as early or late, and the total dose of radiation is typically limited by late side effects.[24] Early toxicities usually do not limit the total dose, but they can be severe and adversely affect the quality of the patient's life.[24] The major tissues of concern in patients being treated for nasal tumors are the skin, mucus membranes, eyes, bone, and brain. The use of a radioprotector would help reduce toxicity in both acute and late responding tissues and allow larger total doses of radiation to be used. Although Tavocept has not been shown to distribute highly to the skin, mucous membranes, eyes, bones, or brain specifically, the ability to achieve very high concentrations in the plasma safely may still allow it to function as a protector in these tissues.[80] One potential concern, however, is the possibility of radioprotection within the

tumor tissues as well. If the protection provided outweighs the sensitization gained by inhibition of TRX/GRX, then some of the benefits to the therapeutic index may be lost.

As a radiosensitizer, chemotherapy dose can be limited by the increase in normal tissue toxicity within the radiation field as well as by systemic toxicity.[86] Tavocept is theorized to exert a chemoprotective effect when used with cisplatin by binding to the platinum intracellularly by its metabolite mesna.[59,62] This prevents the interaction of the chemotherapy with the DNA and therefore decreases toxicity. This may limit the platinum drug's ability to radiosensitize the normal tissue, allowing for larger doses to be used. Tavocept would also be expected to limit any myelosuppression or nephrotoxicity cisplatin may have. Once again, it is possible that mesna could bind to the platinum inside the tumor cells and decrease cisplatin's ability to act as a radiosensitizer. However, this offset would have to be weighed against the gain in apoptotic sensitivity of the tumor as well as by the increased doses of radiation and chemotherapy that could be used. In summary, the use of Tavocept for the treatment of canine nasal tumors would hypothetically allow for larger doses of either radiation therapy or chemotherapy to be employed safely, as well as increase the sensitivity of the tumor to the therapies allowing for more durable local tumor control.

In this study, Tavocept by itself apparently lacked cytotoxicity, but cell death was enhanced when it was combined with cisplatin as expected. However, there was no apparent enhancement or protection when Tavocept was given with radiation in this cell line. When given alone, Tavocept at either the low or high concentration did not cause any obvious deviation in survival from the control cells. This is expected as very high levels of the drug can be given safely without adverse effects. Radiation given alone or

cisplatin given alone also demonstrated the anticipated results with a decrease in survival in both as compared to controls. The use of radiation and cisplatin in combination also showed an additive effect in cell kill compared to either treatment used alone. When cisplatin was administered after the Tavocept, increased cell kill was documented in both the high and low Tavocept concentration groups. The RPMI 2650 cell line is a metastatic squamous cell carcinoma which has not been evaluated for TRX/GRX upregulation, so the enhanced kill with the addition of Tavocept would support that cellular uptake of Tavocept is occuring in the presence of upregulation of TRX/GRX in the cell line. When Tavocept and radiation therapy were combined, no graphical difference was obvious when compared to radiation alone. When Tavocept at either concentration was added into the cisplatin and radiation group, there was significantly more cytotoxicity than without Tavocept. This effect was most likely due to the cisplatin and Tavocept interactions since Tavocept didn't seem to alter radiation's effects.

Hypotheses for the absence of any obvious radioprotection or sensitization with Tavocept include a lack of interaction between Tavocept and radiation therapy, a lack of sufficient overexpression of TRX/GRX in the cell line to see an effect with radiation, a nullification of sensitizing and protective effects, too much kill with radiation to observe sensitization, or a flaw in the experimental design. It appears that Tavocept is being taken up by the cells and that TRX/GRX is a pathway that is important in this cell line due to the enhanced kill from the combination of Tavocept and cisplatin. Radiation may induce different stress pathways important in survival so inhibition of TRX/GRX was not helpful and sensitization did not occur. It is also possible that the lack of sensitization is because the RPMI 2650 cell line does not overexpress TRX/GRX enough to make

Tavocept a radiosensitizer, but expresses it enough to make it a chemosensitizer. On the other hand, the lack of radioprotection may be due to ineffective free radical scavenging by mesna or a re-equilibration in the free thiol balance by the time the radiation was delivered. These different factors could explain why the expected results were seen on the remainder of the plate, and a lack of difference was seen between radiation alone and radiation with Tavocept.

Another theory for the apparent lack of sensitizing or protective effects is that both were occurring, but that they were cancelling each other out. As mentioned previously, Tavocept creates an intracellular oxidative environment that causes an inhibition of TRX/GRX pathways, but it can also create a large amount of intra-cellular free thiol in the form of mesna. These two factors may have balanced each other out at both the low and high concentration resulting in a lack of obvious sensitizing or protecting effect. A different theory is that the radiation dose was high enough that little sensitization could occur. Tumor response curves like that presented in Figure 1A are sigmoidal and if our radiation dose was near the plateau region, either sensitization or protection could be missed. This could be easily dealt with by trying different doses of radiation with Tavocept to see if there is a different effect.

Finally, the design of the study may have been inappropriate to adequately detect the difference. Tavocept was given two hours before radiation therapy and only one hour before the cisplatin. The cells were washed twice between Tavocept and radiation and only once prior to receiving the cisplatin. By the time radiation was performed, it is possible that very little Tavocept was remaining inside the cells. The diluted concentration of Tavocept could have been too low to cause an effect and no interactions

were observed when combined with radiation therapy. OD may also be an inadequate measure of apoptosis or necrosis in this cell line. Assays such as H2AX, the Comet assay, and PARP have been used with radiation therapy to assess DNA damage and lethality in different cell lines. It is possible that one of these other assays would be more accurate and could identify a protective or sensitizing interaction that was missed with the SRB assay.[1] Additionally, the Tavocept metabolites that are important to radioprotection and/or sensitization may not be formed well in vitro due the unnatural environment (high oxygen concentration), and in vivo tests such as tumor xenografts may be a more accurate model.

The concentration of 7μM of cisplatin chosen in this experiment was relatively low. After the administration of 1 mg/kg of cisplatin to adult female beagles (8-10 kg), peak plasma concentrations of cisplatin were around 4.0 μg/mL, or 13.2 μM.[87] Therapeutic dosages in canine patients range from 50-70 mg/m^{2}which would correlate with a dose of 2.4-3.4 mg/kg in these beagle dogs.[88] In previous canine nasal tumor sensitization studies, dogs received either 7.5 mg/m^2 prior to every other radiation treatment or 60 mg/m^2 as an implantable slow release form.[36,38] The dose of cisplatin was determined by the IC-50 values obtained with radiation therapy. If higher concentrations were used in this study, cell kill would have been too high, especially in the combination radiation/cisplatin/Tavocept groups. Interquartile ranges were wide amongst the different treatment groups, and if OD readings went much lower, it would have been difficult to distinguish survival differences from variability between the wells. Although it may be clinically feasible to safely increase the concentration of cisplatin in

the plasma, it may be technically challenging if using the SRB assay and a spectrometer with a linear range up to 2.0.

The radiation dose of 2 Gy was selected based on previous experimentation using the predictive assay of radiosensitivity called SF2. Additionally, 2 Gy is a very clinically applicable dose per fraction.[89] Veterinary patients are often treated with larger doses of radiation per fraction due to the requirement of repeat anesthesia but finer fractionation schemes have been gaining in popularity. In general, carcinomas are intermediate in radiosensitivityand adenocarcinomas tend to be being more sensitive than squamous cell carcinomas.[89] This has been occasionally noted with canine nasal tumors with squamous cell carcinoma carrying a poorer prognosis.[32,83] Typical SF2 values for carcinomas are between 40-50%, which correlates moderately with the average value of approximately 38% obtained in this study.[89]

Shortcomings of this study include the large amount of variability and lack of repeatability in the cell per well, IC-50, and SF2 experiments, using control data that was out of the linear OD range in the RT (-) plates in the final experiment, and the use of a non-adenocarcinoma cell line. The initial cell concentration assay suggested that 500 cells/100µL was ideal but upon repeat experimentation it was obvious that it was far too low. 1000 cells/100µL also resulted in control OD values that were highly variable. The median OD from the first experiment was 1.47 and 1.89 from the repeat experiment. The hypothesis for this lack of consistency was the adherent nature of the cell line in use. The RPMI 2650 cell line took an average of 7 minutes of trypsinization and still required vigorous pipetting to separate the cells. Despite these steps, clumps of cells were still occasionally visualized when examine on the hemocytometer making counting

inaccurate. The long trypsinization time and forceful washing to detach the cells from the T-75 flasks may have also damaged the cells, causing a falsely elevated cell count. Trypan blue exclusion was used when counting the cells and it was noted that delayed counting resulted in higher percentages of dead cells. This corroborated the idea that the efforts to detach and separate the cells damaged them and eventually lead to their death. It is also possible that during the counting procedure, some of the recently detached cells were reattaching to the conical tube that they were stored in. In the final experiment, the duplicate RT (+) and RT (-) plates lacked graphical or statistical differences within the treatment groups suggesting that repeatability was achieved. Amongst the different individual drug treatment groups, large statistical differences were able to be obtained using fairly conservative two-sided non-parametric multiple comparisons procedures demonstrating a low level of variability in the data. Experience gained by working with the cell line allowed for better understanding of its limitations and development of better and more efficient techniques to be used in the final experiment. However, in future studies, devices like a cell culture spatula for easier cell detachment and an automated cell counter for more rapid results may help eliminate some of these problems and allow for even better results.

Another weakness of the study was the use of control data that was out of the linear range in the RT (-) group. Initially, identifying a control sample that resulted in an OD just below 2.0 was the goal. However, the large amount of kill achieved with the combination treatments resulted in values that were near 0 with these controls. It was decided to use the current data because the interactions between the drugs and radiation could still be observed, and statistical differences were still detected. Possible differences

between Tavocept and the control group may have been missed, however. Using a different clonogenic assay or a different spectrometer that could facilitate the accurate detection of wide degree of cytotoxicity would abolish the need to use control samples that are out of the linear range.

Finally, the cell line chosen for experimentation was collected from the pleural effusion of a human patient with metastatic nasal septum squamous cell carcinoma. Canine patients develop nasal adenocarcinomas more frequently than squamous cell carcinomas and the fact the RPMI 2650 was derived from cells in pleural effusion could make the cells genetically and phenotypically different than a primary nasal tumor.[85] The sensitizing effects of Tavocept rely upon the inhibition of TRX/GRX which is known to occur in adenocarcinomas, although this study provides evidence that RPMI 2650 overexpresses TRX/GRX, as well. Ideally, a canine nasal adenocarcinoma cell line would have been used, but none were available through ATCC at the time the study was performed.

To further evaluate the interaction of Tavocept and radiation, Tavocept could be delivered at different times before radiation therapy. Tavocept could be present in the media at the time of radiation, 30 minutes, an hour, an hour and 30 minutes, and two hours before radiation. This would help determine if the timing of the drug is important as well as if the extra washings in this experiment had any effect on Tavocept's ability to modulate radiation damage. After Tavocept enters the cell, the cytoplasm will have a large increase in disulfides creating an oxidized environment. In cells that upregulate TRX/GRX, this would initially lead to radiosensitization. As the non-enzymatic thiol transfer progressed, more mesna would be produce, the total free thiol concentration

would be elevated, and the availability to scavenge free radicals would be increased. This may create a radioprotective environment. This dynamic process may occur to different degrees in various cell types and protection and sensitization may occur at different times. Cells that do not over express TRX/GRX would not be expected to have radiosensitization with Tavocept and some cell types may experience more protection with Tavocept than others. Future studies could also be performed to evaluate the differences between normal cells and tumor cells and the differences in protection and sensitization as well as xenografts to evaluate the effects in a more natural environment.

Other potential applications for Tavocept with radiation therapy in veterinary patients include bone marrow transplantation for the treatment of canine multicentric lymphoma and gastro-intestinal lymphoma in cats. Bone marrow transplantation employs the use of both 500-650 mg/m^2 of cyclophosphamide and total body radiation to sterilize the cancer as well as the normal hematopoietic cells.[90] The hematopoietic cells are part of the most sensitive organ system of the body to whole body radiation therapy, and the patient would succumb to sepsis if not rescued by a bone marrow transplant.[24] The second most sensitive system to total body radiation is the gastro-intestinal tract, and becomes the dose limiting body system when bone marrow transplantation is performed. Patients will die of malabsorption, fluid and electrolyte loss, and bacterial translocation if doses too high for the intestine to handle are used.[24] Tavocept has been shown to accumulate in the enterocytes and if it acts as a radioprotector as hypothesized, gastro-intestinal side effects may be minimized.[59] White cells such as lymphocytes do not appear to uptake Tavocept, so the therapy would most likely have no effect on them.[91]

Tavocept in this setting may allow for increased doses of radiation and increase the success at completely sterilizing all of the cancer cells.

Feline gastro-intestinal lymphoma is the most common form of lymphoma in older, vaccinated cats.[92] Recently, fractionated radiation therapy was delivered after an induction chemotherapy protocol to several cats with moderate success.[93] The dose limiting structures in the abdomen in the fractioned radiation setting are the kidneys. This is complicated by the fact that many older feline patients can have some level of underlying chronic kidney disease.[93] A vast majority of injected Tavocept ends up in the kidneys and therefore could be used to spare them from radiation damage when treated with abdominal radiation therapy. This could also allow the use of a higher total dose which may result in longer remission rates and survival times.

Conclusion

In this study, there was no obvious sensitization or protection seen when Tavocept was given with radiation. Given that Tavocept may cause radiosensitization in tumors and radioprotection in normal tissue, further evaluation is warranted. An increase in cell kill was noted with the addition of Tavocept to the cisplatin treatment groups suggesting that RPMI 2650 does indeed upregulate TRX/GRX and validating the success of the assay. The use of a clonogenic assay to evaluate radiation and Tavocept delivered at different times in an adenocarcinoma cell line may elucidate the proposed mechanisms of action.

APPENDIX

FIGURES

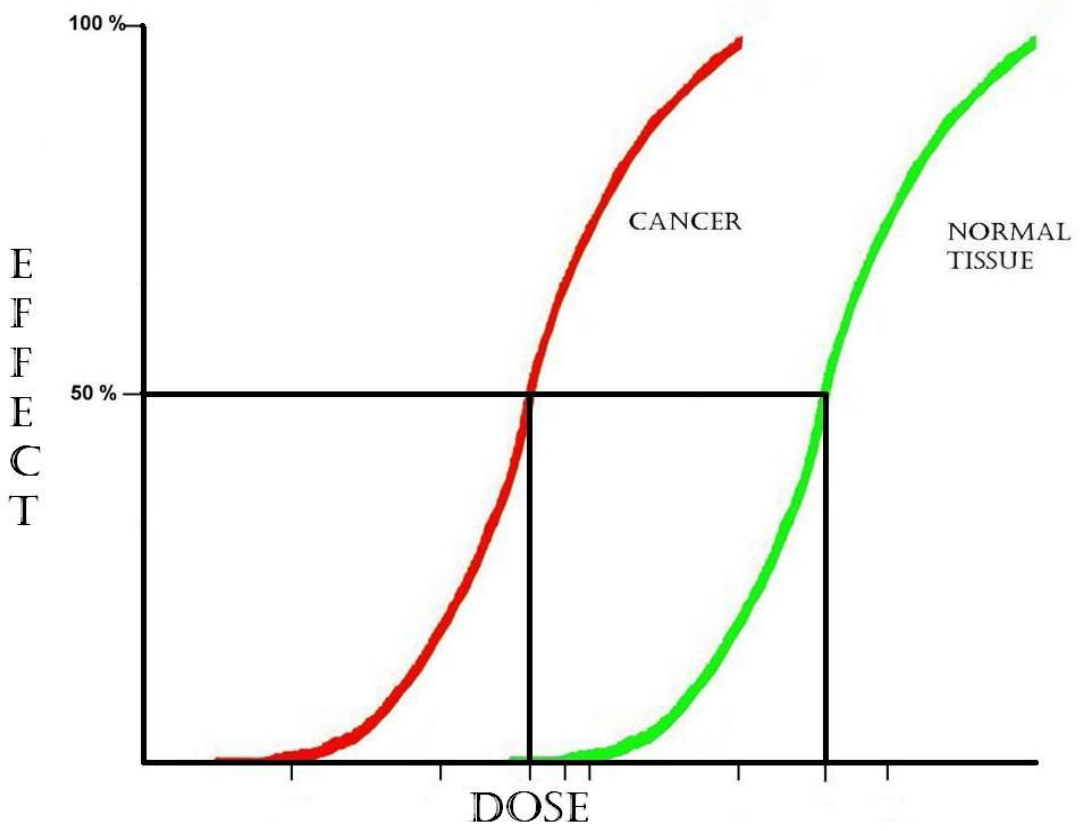

Figure 1A- Example of idealized response curves of cancer and normal tissue to treatment. The separation of the curves represents the therapeutic index of the treatment. In this situation, there is a positive therapeutic index when a dose that is 50% effective in treating a cancer causes minimal toxicity to the normal tissues. Chemo/radioprotecting agents would shift the normal tissue curve to the right and chemo/radiosensitizers would shift the cancer curve to the left. Either technique increases the therapeutic index.

Days	Hours	Cell Count	BNPI Calculation		Knapp Lab Calculation
0	0	200000	n/a		n/a
1	24	250000	n/a		n/a
2	48	650000	28.2		28.2
3	72	1050000	30.1		30.1
4	96	3000000	24.5		24.5
5	120	6000000	24.4		24.5
		Average Doubling Time	**26.8**		**26.8**
		Standard Deviation	**2.8**		**2.8**

Figure 3.3.2A- Cell counts and doubling time calculations performed in the doubling time experiment. Both the BNPI and Knapp formulas resulted in similar outcomes. It takes approximately 24 hours for the cells to adhere to the flasks so calculations were not performed after the first day. The average doubling time was used to calculate the five doubling time value needed to perform the SRB assay.

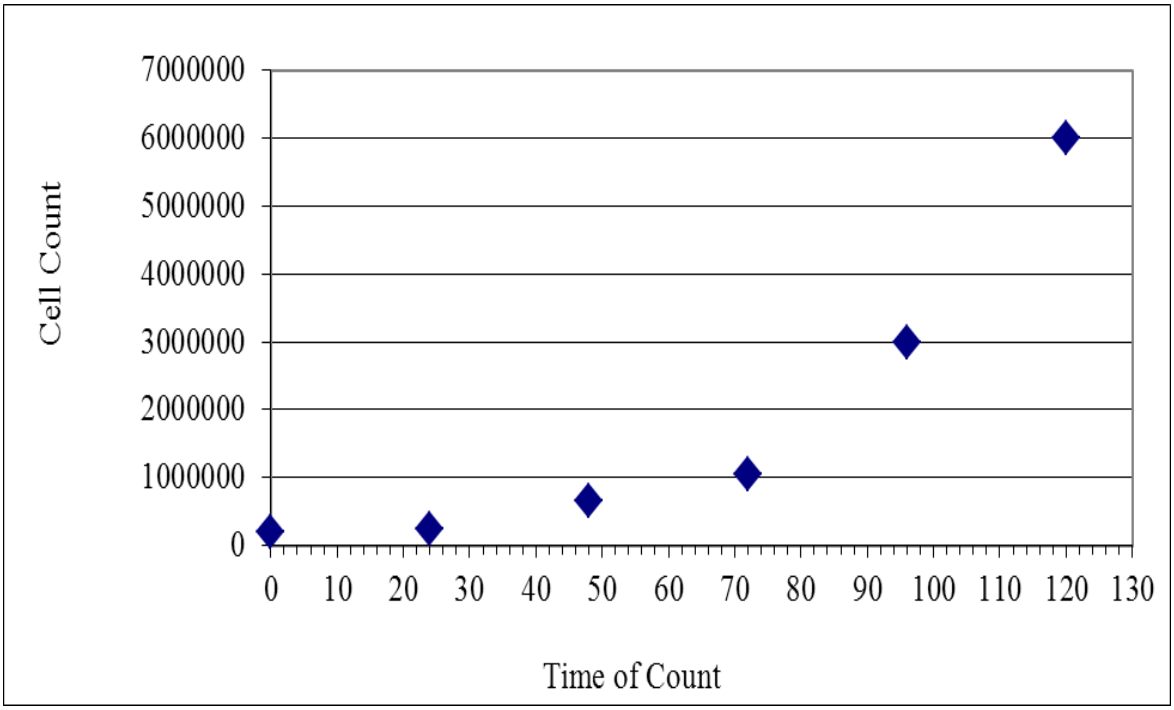

Figure 3.3.2B- Graphical representation of the doubling time experiment. Very little growth was noted after the first 24 hours and then the growth appears to be exponential.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	500 Cells/100 µL			600 Cells/100 µL			700 Cells/100 µL			800 Cells/ 100 µL	
B												
C												
D												
E												
F												
G												
H												

Figure 3.4.1A- Layout of the 96 well plates in the Cells/100 µL experiment. Blank controls were performed to assess non-protein stain uptake.

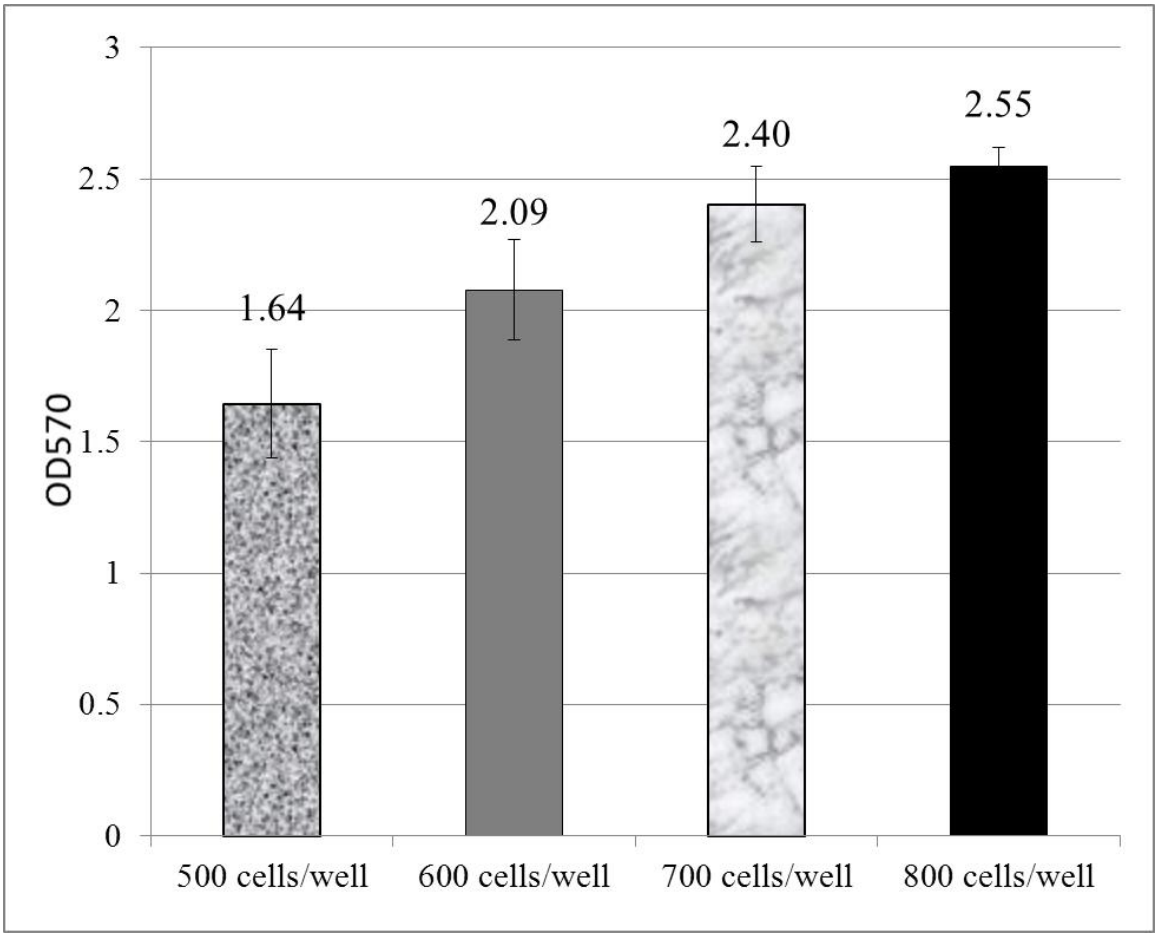

Figure 3.4.2A- Results of the Cells/100 µL experiment. The linear range of the UV plate reader goes up to 2.0, as a result, 500 cells/100 µL was chosen as the seeding value.

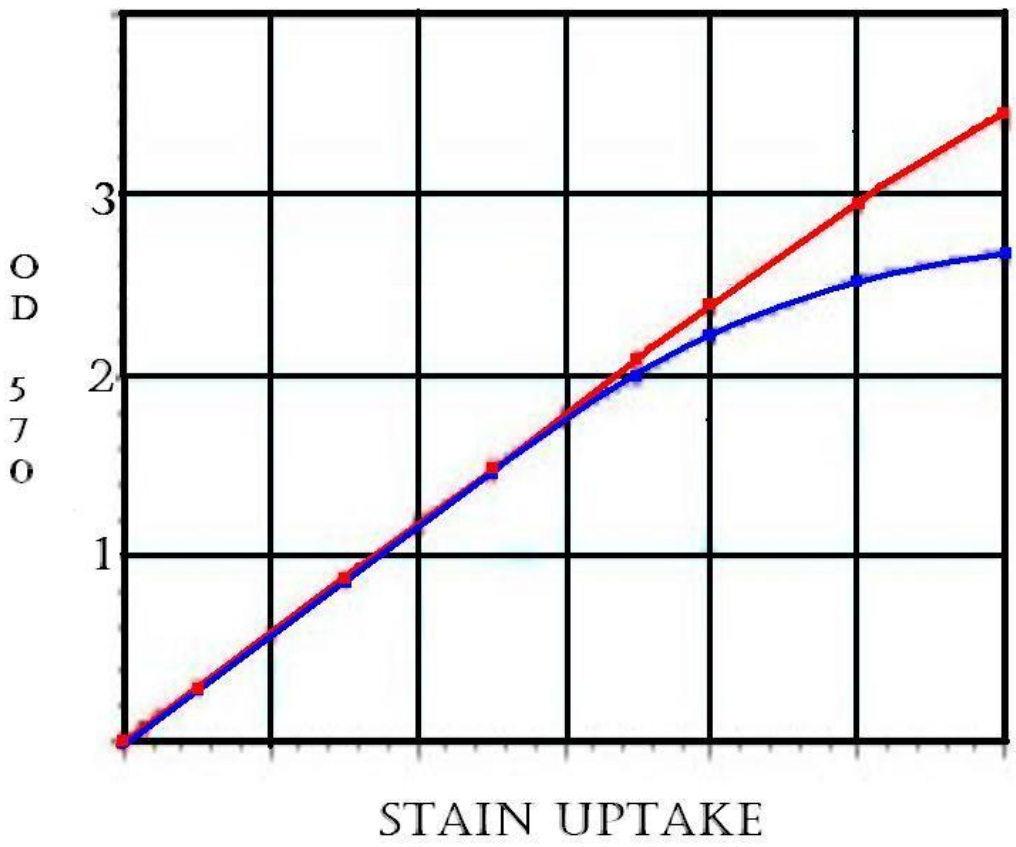

Figure 3.4.2B- Representative graph of the linear OD range for the Spectramax Plus 384 UV plate reader. The straight line represents a linear relationship and the curved line represents the observed relationship. Past an OD of 2.0, the stain uptake is no longer linearly related to the OD.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	Cells Only			0.78 µM Cis	1.56 µM Cis	3.12 µM Cis	6.25 µM Cis	12.5 µM Cis	25 µM Cis	50 µM Cis	100 µM Cis
B												
C												
D												
E												
F												
G												
H												

Figure 3.5.1A- Layout of the 96 well plates in the IC-50 Cisplatin experiment. Concentrations of cisplatin were serially diluted in half to treat each subsequent column and compared to the cells in the control wells.

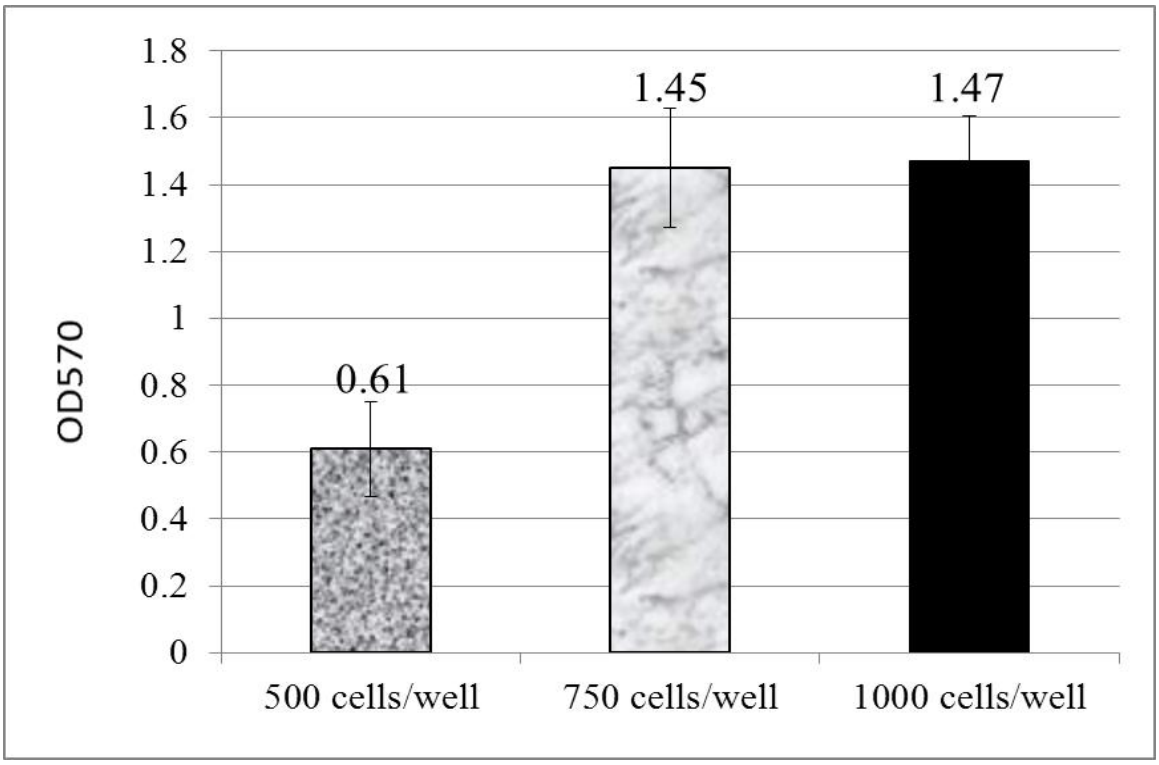

Figure 3.5.2A- Results from the control wells of the IC-50 Cisplatin experiments. The 500 cells/100µL is less than 1.0 which is not an ideal value for a control in experiments that are expected to have low survival fractions. Based on this data and data from the SF2 experiments, 1000 cells/100µL was chosen.

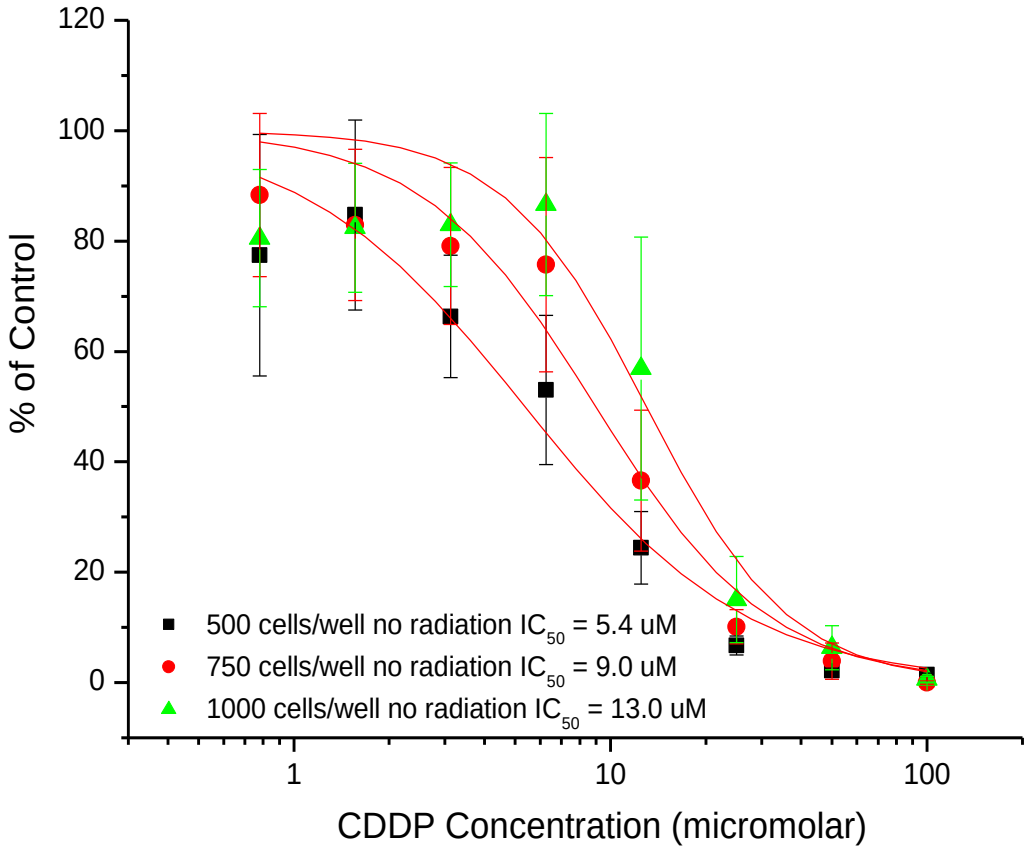

Figure 3.5.2B- IC-50 curve produced by Origin software for the various concentrations of cells. The control wells for the 500 cells/100µL were below an OD of 1.0 so the results were considered unreliable.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	Radiation Only			0.78 µM Cis + Rad	1.56 µM Cis + Rad	3.12 µM Cis + Rad	6.25 µM Cis + Rad	12.5 µM Cis + Rad	25 µM Cis + Rad	50 µM Cis + Rad	100 µM Cis + Rad
B												
C												
D												
E												
F												
G												
H												

Figure 3.6.1A- Layout of the 96 well plates in the IC-50 Cisplatin/SF2 experiment. The control cells were used to calculate the SF2 based on the control cells from the IC-50 Cisplatin experiment. The control cells were also used to calculate the IC-50 Cisplatin/radiation value.

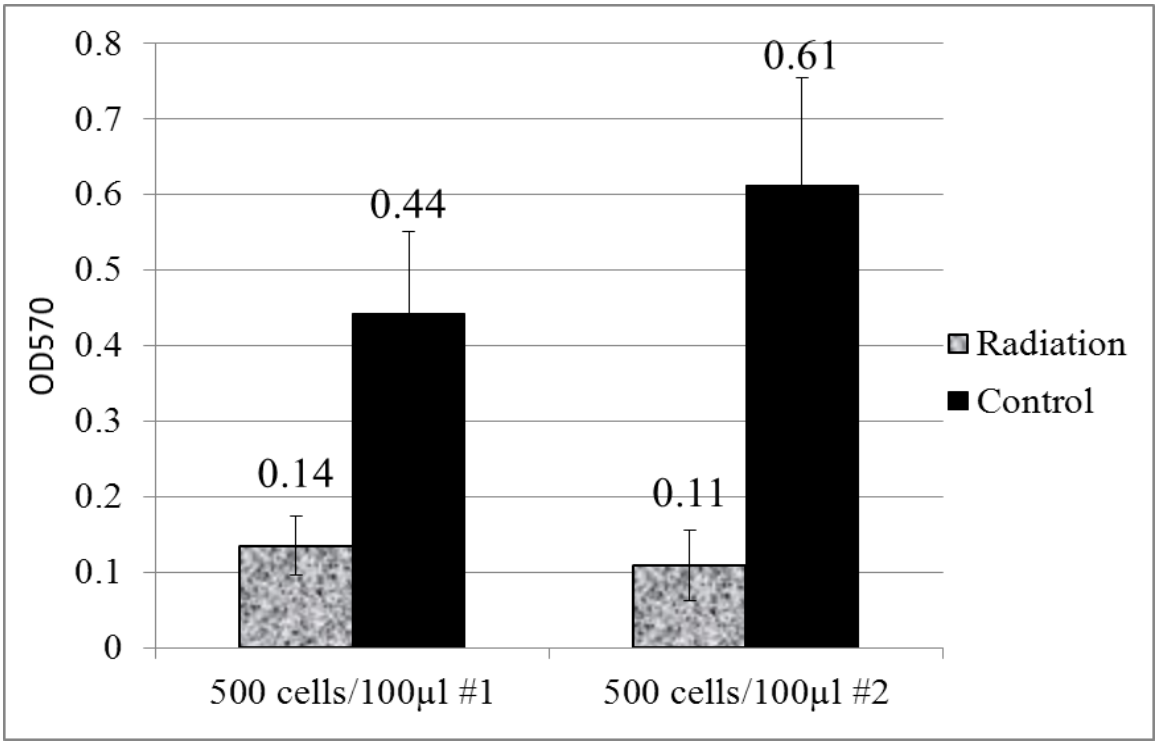

Figure 3.6.2A- Results of the SF2 experiment for the two separate 500 cells/100µL groups. The initial experiment's SF2 was 31.8% and the second experiment's SF2 was 18.0%. These results were considered unreliable based on the OD being less than 1.0 for the control wells.

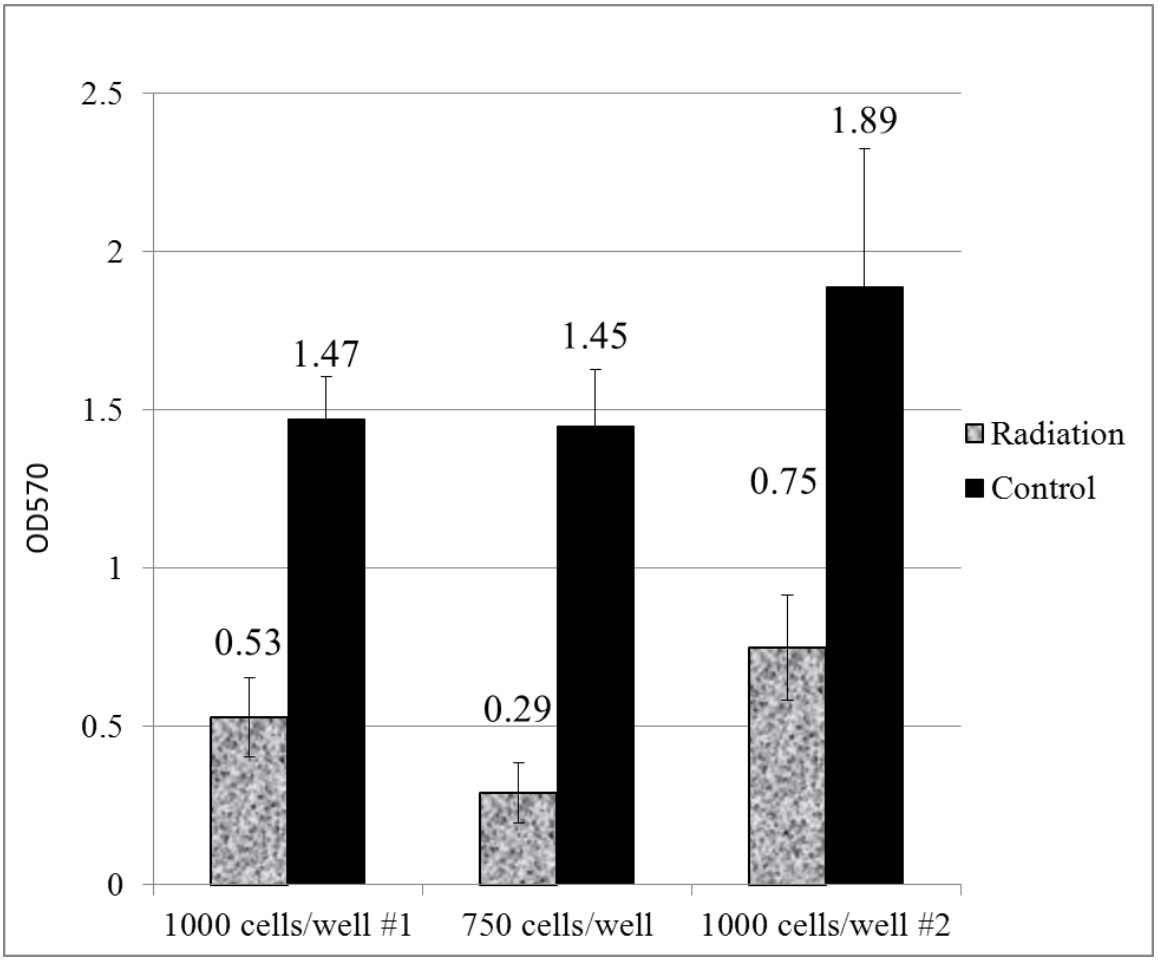

Figure 3.6.2B- Results of the SF2 experiment for the 1000cells/100µL, 750cells/100µL, and repeat 1000cells/100µL groups. The SF2 values were 36.1%, 20.0%, and 39.7% respectively. 1000cells/100µL was chosen as the seeding concentration because of the repeatability in results.

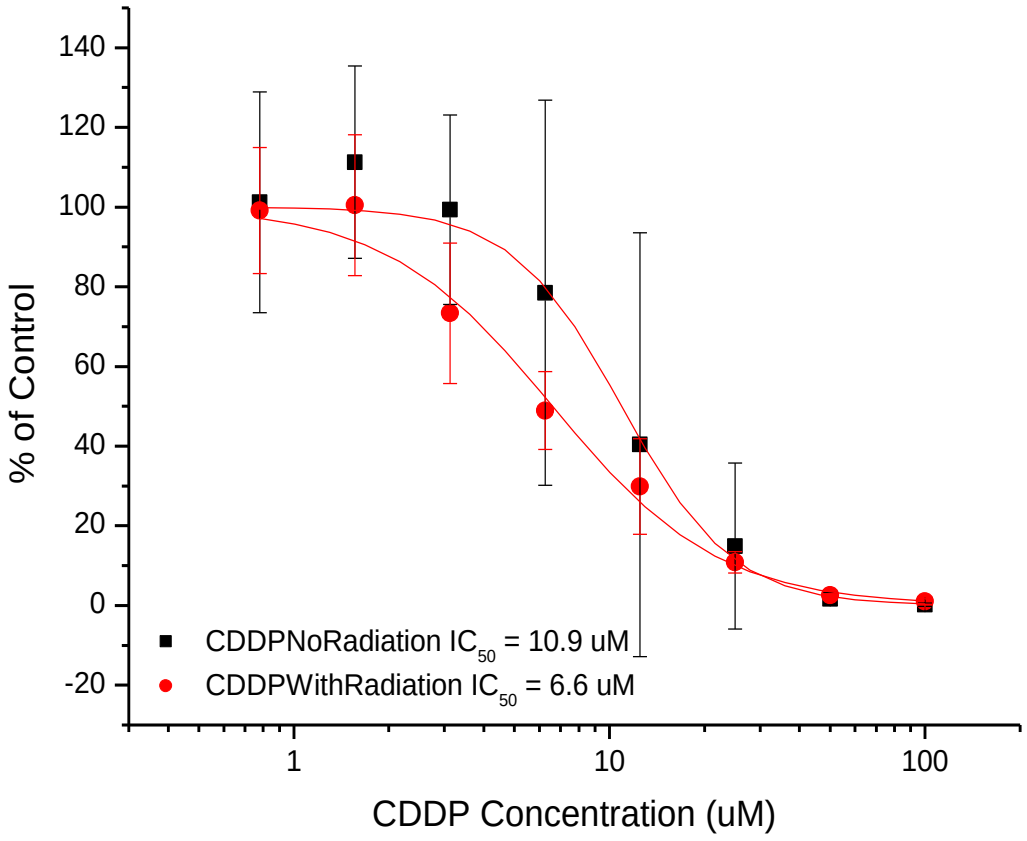

Figure 3.7A- IC-50 curve produced by Origin software for 1000 cells/100µL with and without radiation. Radiation appears to decrease the IC-50. An IC-50 with radiation value of 7.4 µM was obtained for the second 1000 cells/100µL experiment and the two values were averaged to obtain a final IC50 with radiation value of 7.0 µM.

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td rowspan="8">BLANK</td><td rowspan="8">Rad Only</td><td colspan="3" rowspan="8">7 μM Cis+ Rad</td><td colspan="3" rowspan="4">1 mM Tav+ Rad</td><td colspan="3" rowspan="4">1 mM Tav+ 7 μM Cis+ Rad</td></tr>
<tr><td>B</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td><td colspan="3" rowspan="4">10 mM Tav+ Rad</td><td colspan="3" rowspan="4">10 mM Tav+ 7 μM Cis+ Rad</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td></tr>
</table>

Figure 3.8.1A- Layout of the 96 well plates for the final Radiation/Cisplatin/Tavocept plate. Two different concentrations of Tavocept were chosen to see if there was a dose dependent effect.

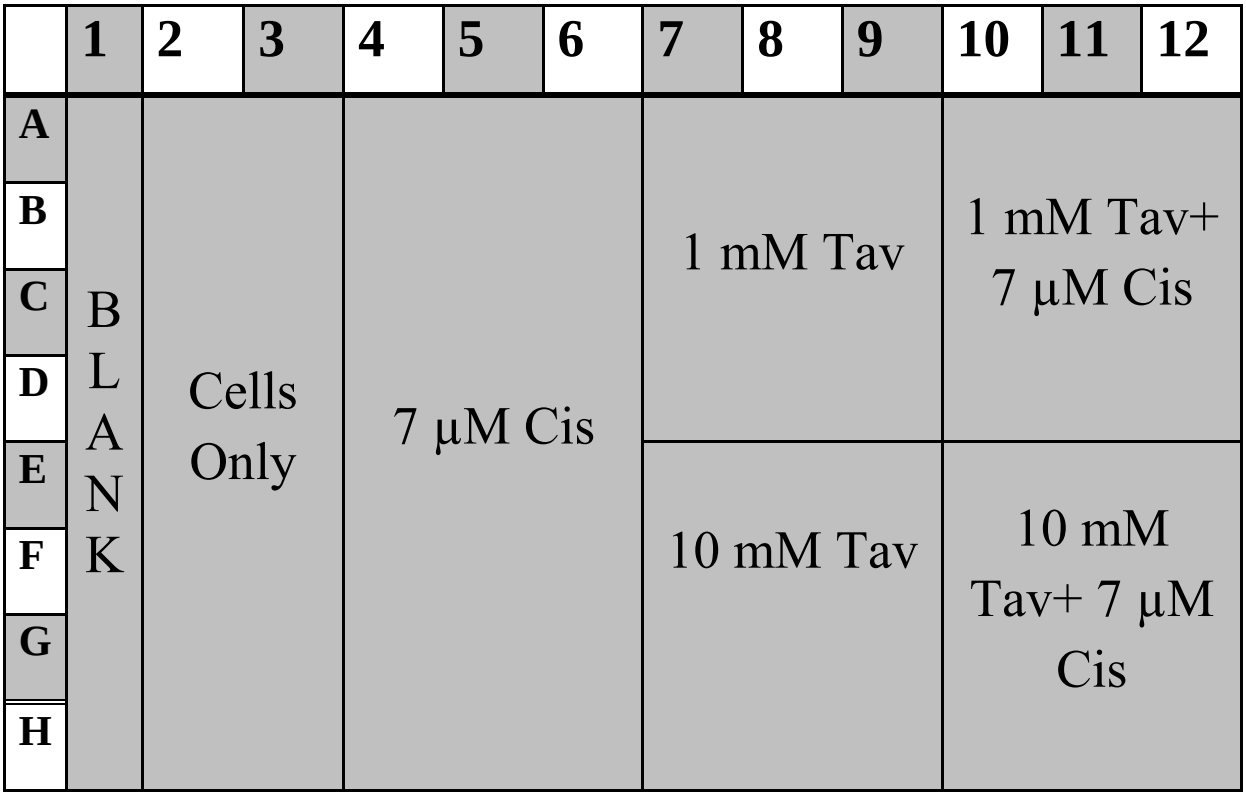

Figure 3.8.1B- Layout of the 96 well plates for the final Cisplatin/Tavocept plate without radiation.

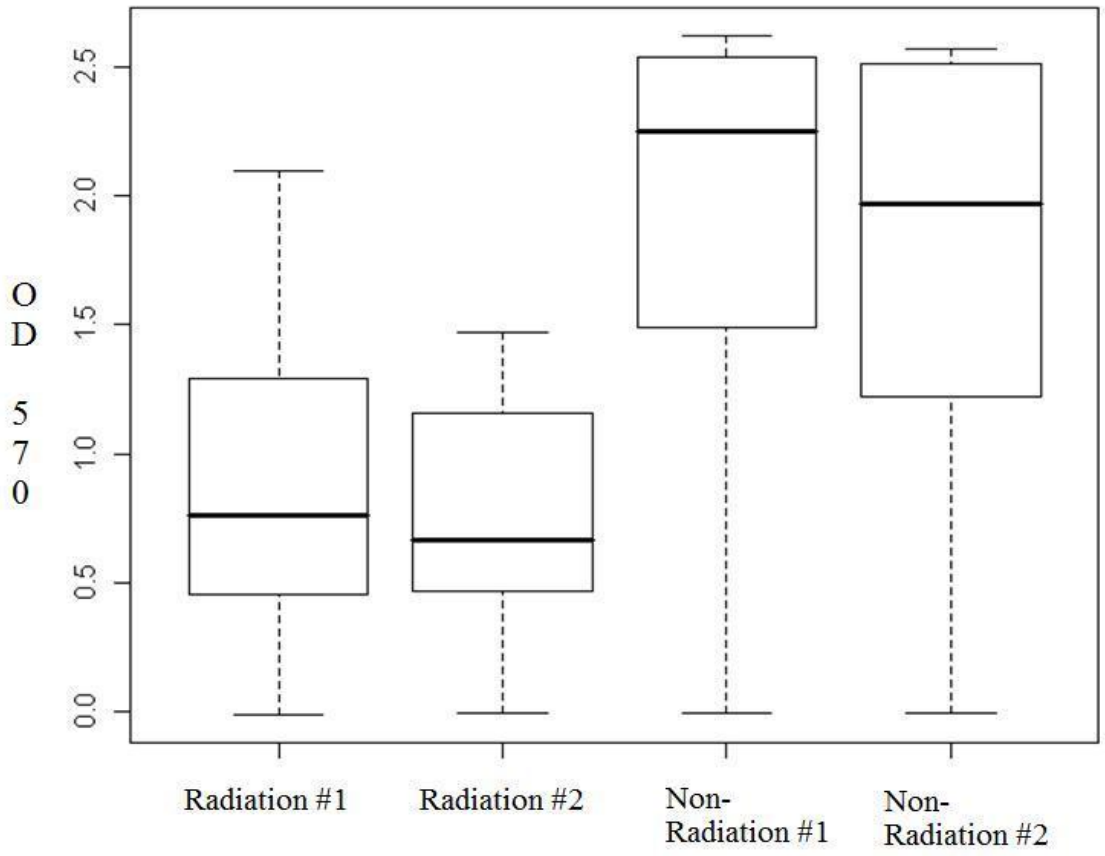

Figure 3.8.3A- Box and whiskers plot comparing the treatment groups (RT (+) and RT (-)) between the repeat experiments #1 and #2. Visually little difference was seen and statistically no difference was noted within groups, but highly significant differences were noted between groups ($p<2.2e^{-16}$). Based on the similarity of the results between the experiments, data from the two experiments were pooled for the final analysis.

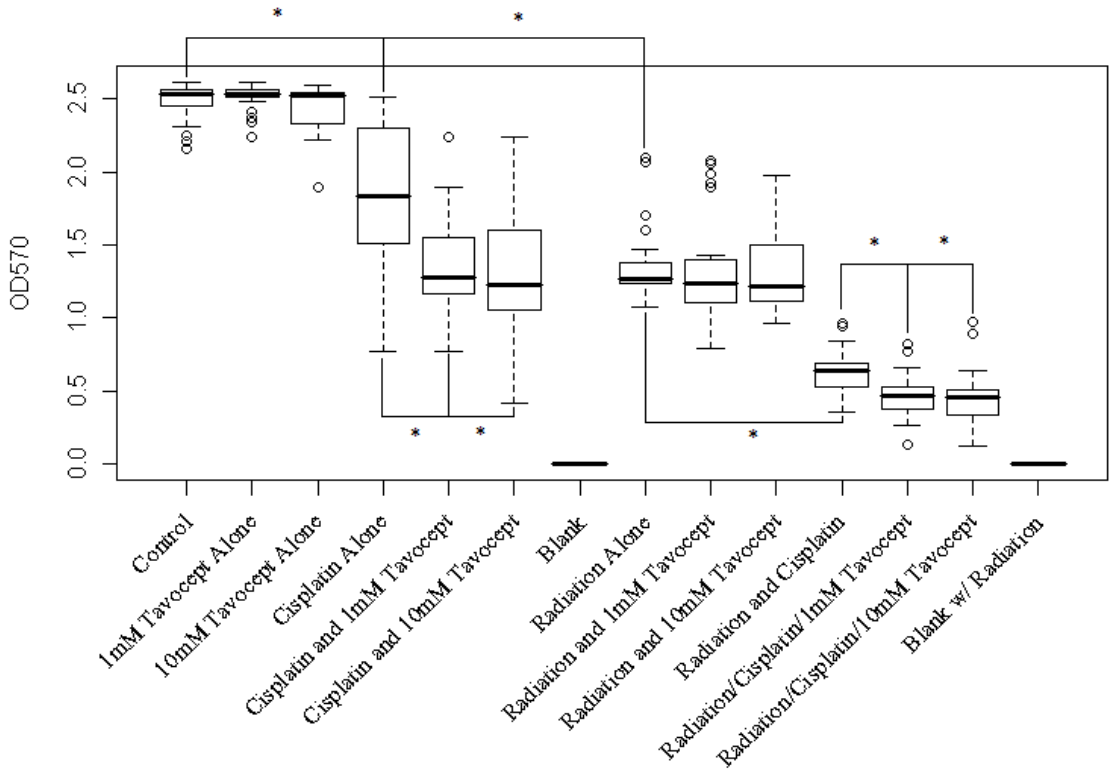

Figure 3.8.3B- Box and whiskers plot comparing the individual treatment groups from the pooled RT (+) and RT (-) plates. Significant differences are represented by an asterix and they were between the control versus radiation alone ($p<6.212 \times 10^{-12}$), control versus cisplatin alone ($p<0.005$), cisplatin versus either concentration of Tavocept ($p<0.005$), radiation alone versus radiation and cisplatin ($p<0.01$), and radiation and cisplatin versus radiation and cisplatin and either concentration of Tavocept ($p<0.01$). No visual differences were seen between control versus either Tavocept alone at either concentration, radiation alone versus radiation and Tavocept at either concentration, or between any of the two Tavocept concentrations with any other treatment group. The blanks in either RT group were essentially 0 demonstrating a lack of non-specific stain uptake.

FOOTNOTES

a. Minimum Essential Medium Invitrogen, Carlsbad, CA, USA

b. Fetal bovine serum, Invitrogen, Carlsbad, CA, USA

c. L-glutamine, Invitrogen, Carlsbad, CA, USA

d. MF75 Tissue Culture Filter with a 0.2µm pore and a 50 mm membrane diameter, Thermo Fisher Scientific, Waltham, MA, USA

e. 100% Glacial Acetic Acid Reagent Grade, J.T. Baker, Phillipsburg, NJ, USA

f. Trichloroacetic acid, Cole-Parmer, Vernon Hills, IL, USA

g. Sulforhodamine b dye, Sigma-Aldrich, St. Louis, MO, USA

h. Tris (hydroxymethyl) aminomethane, Alfa Aesar, Ward Hill, MA, USA

i. RPMI 2650, ATCC, Manassas, VA, USA

j. Cryogenic Vials, Thermo Fisher Scientific, Waltham, MA, USA

k. T-75 Flasks, Sigma-Aldrich, St. Louis, MO, USA

l. Incubator, Heracell 150, Thermo Fisher Scientific, Waltham, MA, USA

m. TrypLE™ Express (Trypsin), Invitrogen, Carlsbad, CA, USA

n. Dimethy sulfoxide (DMSO), Sigma-Aldrich, St. Louis, MO, USA

o. Cryo 1C “Mr Frosty” Freezing Container using 100% isopropyl alcohol, Thermo Fisher Scientific, Waltham, MA, USA

p. 96 Well Plates, Thermo Fisher Scientific, Waltham, MA, USA

q. Plate Shaker, Titer Plate Shaker, Thermo Fisher Scientific, Waltham, MA, USA

r. Spectramax Plus 384 UV Plate Reader, Molecular Devices, Sunnyvale, CA, USA

s. Hemacytometer, standard- Hausser Scientific, Horsham, PA, USA

t. Trypan Blue, Sigma-Aldrich, St. Louis, MO, USA

u. Microcentrifuge Tube, Corning, Lowell MA, USA

v. T-25 Flasks, Sigma-Aldrich, St. Louis, MO, USA

w. Cisplatin, APP Pharmaceuticals Schaumburg, IL, USA

x. Linear Accelerator (LINAC) Mevatron, Siemens, Washington DC, USA

y. Origin version 7.0552, OriginLab, Northampton, MA, USA

z. Xio Planning Software, Elekta, Stockholm, Sweden

aa. Tavocept, BioNumerik Pharmaceuticals, San Antonio TX, USA

bb. R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria

BIBLIOGRAPHY

1. Tannock I. *The basic science of oncology*. 4th ed. New York: McGraw-Hill, Medical Pub. Division, 2005.

2. Gupta PB, Chaffer CL, Weinberg RA. Cancer stem cells: mirage or reality? *Nat Med* 2009;15:1010-1012.

3. Brizel DM, Albers ME, Fisher SR, et al. Hyperfractionated Irradiation with or without Concurrent Chemotherapy for Locally Advanced Head and Neck Cancer. *New England Journal of Medicine* 1998;338:1798-1804.

4. Vokes EE, Weichselbaum RR, Lippman SM, et al. Head and Neck Cancer. *New England Journal of Medicine* 1993;328:184-194.

5. Cvetkovic RS, Scott LJ. Dexrazoxane: A Review of its Use for Cardioprotection During Anthracycline Chemotherapy. *Drugs*: ADIS International Limited, 2005;1005-1024.

6. Singal PK, Iliskovic N. Doxorubicin-Induced Cardiomyopathy. *New England Journal of Medicine* 1998;339:900-905.

7. Sorenmo KU, Baez JL, Clifford CA, et al. Efficacy and Toxicity of a Dose-Intensified Doxorubicin Protocol in Canine Hemangiosarcoma. *Journal of Veterinary Internal Medicine* 2004;18:209-213.

8. Swain SM, Vici P. The current and future role of dexrazoxane as a cardioprotectant in anthracycline treatment: expert panel review. *Journal of Cancer Research and Clinical Oncology* 2004;130:1-7.

9. Yoshiya T, Masayoshi O, Mohammed AK. A canine model of dilated cardiomyopathy induced by repetitive intracoronary doxorubicin administration. *The Journal of thoracic and cardiovascular surgery* 1998;115:1367-1373.

10. Cox PJ. Cyclophosphamide cystitis--Identification of acrolein as the causative agent. *Biochemical Pharmacology* 1979;28:2045-2049.

11. Mace JR, Keohan ML, Bernardy H, et al. Crossover Randomized Comparison of Intravenous versus Intravenous/Oral Mesna in Soft Tissue Sarcoma Treated with High-Dose Ifosfamide. *Clinical Cancer Research* 2003;9:5829-5834.

12. Spencer CM, Goa KL. Amifostine. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential as a radioprotector and cytotoxic chemoprotector. *Drugs* 1995;50:1001-1031.

13. Kane RC, Bross PF, Farrell AT, et al. Velcade(R): U.S. FDA Approval for the Treatment of Multiple Myeloma Progressing on Prior Therapy. *Oncologist* 2003;8:508-513.

14. Mortenson MM, Schlieman MG, Virudachalam S, et al. Effects of the proteasome inhibitor bortezomib alone and in combination with chemotherapy in the A549 non-small-cell lung cancer cell line. *Cancer Chemotherapy and Pharmacology* 2004;54:343-353.

15. Jansen B, Schlagbauer-Wadl H, Brown BD, et al. bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. *Nat Med* 1998;4:232-234.

16. Webb A, Cunningham D, Cotter F, et al. BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. *The Lancet* 1997;349:1137-1141.

17. Sharom FJ. The P-Glycoprotein Efflux Pump: How Does it Transport Drugs? *Journal of Membrane Biology* 1997;160:161-175.

18. Dalton W, Grogan T, Meltzer P, et al. Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. *Journal of Clinical Oncology* 1989;7:415-424.

19. Saeki T, Ueda K, Tanigawara Y, et al. Human P-glycoprotein transports cyclosporin A and FK506. *Journal of Biological Chemistry* 1993;268:6077-6080.

20. McGinn CJ, Shewach DS, Lawrence TS. Radiosensitizing Nucleosides. *Journal of the National Cancer Institute* 1996;88:1193-1203.

21. Theodore SL, Jonathan M. Fluoropyrimidines as radiation sensitizers. *Seminars in radiation oncology* 1993;3:20-28.

22. Theodore SL, Joel ET, Arthur WB. Fluoropyrimidine-radiation interactions in cells and tumors. *Seminars in radiation oncology* 1997;7:260-266.

23. Begg AC. Cisplatin and radiation: Interaction probabilities and therapeutic possibilities. *International journal of radiation oncology, biology, physics* 1990;19:1183-1189.

24. Hall EJ, Giaccia AJ. Radiobiology for the radiologist. 6th ed. Philadelphia: Lippincott Williams & Wilkins, 2006;1 online resource (ix, 546 p.).

25. Dische S. Chemical sensitizers for hypoxic cells: A decade of experience in clinical radiotherapy. *Radiotherapy and Oncology* 1985;3:97-115.

26. Brown JM. SR 4233 (Tirapazamine): a new anticancer drug exploiting hypoxia in solid tumours. *Br J Cancer* 1993;67:1163-1170.

27. Bruce GH, Yung HS, Clarence TS, et al. Mitomycin C as an adjunct to postoperative radiation therapy in squamous cell carcinoma of the head and neck: Results from two randomized clinical trials. *International journal of radiation oncology, biology, physics* 1993;27:241-250.

28. McEntee MC. A survey of veterinary radiation facilities in the United States during 2001. *Veterinary Radiology and Ultrasound* 2004;45:476-479.

29. MacEwen EG, Withrow SJ, Patnaik AK. Nasal tumors in the dog: Retrospective evaluation of diagnosis, prognosis and treatment. *Journal of the American Veterinary Medical Association* 1977;170:45-48.

30. Northrup NC, Etue SM, Ruslander DM, et al. Retrospective study of orthovoltage radiation therapy for nasal tumors in 42 dogs. *Journal of Veterinary Internal Medicine* 2001;15:183-189.

31. Adams WM, Bjorling DE, McAnulty JF, et al. Outcome of accelerated radiotherapy alone or accelerated radiotheraphy followed by exenteration of the nasal cavity in dogs with intranasal neoplasia: 53 Cases (1990-2002). *Journal of the American Veterinary Medical Association* 2005;227:936-941.

32. Theon AP, Madewell BR, Harb MF, et al. Megavoltage irradiation of neoplasms of the nasal and paranasal cavities in 77 dogs. *Journal of the American Veterinary Medical Association* 1993;202:1469-1475.

33. McEntee MC, Page RL, Heidner GL. A retrospective study of 27 dogs with intranasal neoplasms treated with cobalt radiation. *Vet Radiol* 1991;32:135-139.

34. Adams WM, Miller PE, Vail DM, et al. An accelerated technique for irradiation of malignant canine nasal and paranasal sinus tumors. *Veterinary Radiology and Ultrasound* 1998;39:475-481.

35. Thrall D, Heidner G, Novotney C. Failure patterns following cobalt irradiation in dogs with nasal carcinoma. *Vet Radiol Ultrasound* 1993;34:1133-1226.

36. Nadeau ME, Kitchell BE, Rooks RL, et al. Cobalt radiation with or without low-dose cisplatin for treatment of canine naso-sinus carcinomas. *Veterinary Radiology and Ultrasound* 2004;45:362-367.

37. Lana SE, Dernell WS, Larue SM, et al. Slow release cisplatin combined with radiation for the treatment of canine nasal tumors. *Veterinary Radiology and Ultrasound* 1997;38:474-478.

38. Lana SE, Dernell WS, Lafferty MH, et al. Use of radiation and a slow-release cisplatin formulation for treatment of canine nasal tumors. *Veterinary Radiology and Ultrasound* 2004;45:577-581.

39. Hausheer F, Bain S, Perry M, et al. Comprehensive Meta-Analysis of Survival Outcomes from Two Randomized Multicenter Trials in First-Line Advanced Non-Small Cell Lung Cancer in Patients Treated with the Novel Investigational Antitumor-enhancing and Chemoprotective Agent Tavocept. *The Asia-Pacific Journal of Oncology & Hematology* 2010;2:1-13.

40. Andersson A, Isaksson A, Brattstrom L, et al. Homocysteine and other thiols determined in plasma by HPLC and thiol- specific postcolumn derivatization. *Clin Chem* 1993;39:1590-1597.

41. Mansoor MA, Svardal AM, Ueland PM. Determination of the in vivo redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. *Analytical Biochemistry* 1992;200:218-229.

42. Mårtensson J. Method for determination of free and total glutathione and [gamma]-glutamylcysteine concentrations in human leukocytes and plasma. *Journal of Chromatography: Biomedical Applications* 1987;420:152-157.

43. Svardal AM, Mansoor MA, Ueland PM. Determination of reduced, oxidized, and protein-bound glutathione in human plasma with precolumn derivatization with monobromobimane and liquid chromatography. *Analytical Biochemistry* 1990;184:338-346.

44. Hausheer FH, Kochat H, Parker AR, et al. New approaches to drug discovery and development: a mechanism-based approach to pharmaceutical research and its application to BNP7787, a novel chemoprotective agent. *Cancer Chemotherapy and Pharmacology* 2003;52:3-15.

45. Masuda N, Negoro S, Hausheer F, et al. Phase I and pharmacologic study of BNP7787, a novel chemoprotector in patients with advanced non-small cell lung cancer. *Cancer Chemotherapy and Pharmacology* 2010:1-10.

46. Boven E, Westerman M, Van Groeningen CJ, et al. Phase I and pharmacokinetic study of the novel chemoprotector BNP7787 in combination with cisplatin and attempt to eliminate the hydration schedule. *British Journal of Cancer* 2005;92:1636-1643.

47. Verschraagen M, Boven E, Zegers I, et al. Pharmacokinetics of BNP7787 and its metabolite mesna in plasma and ascites: a case report. *Cancer Chemother Pharmacol* 2003;51:525-529.

48. Hausheer FH, Kanter P, Cao S, et al. Modulation of platinum-induced toxicities and therapeutic index: mechanistic insights and first- and second-generation protecting agents. *Semin Oncol* 1998;25:584-599.

49. Brock N, Pohl J, Stekar J, et al. Studies on the urotoxicity of oxazaphosphorine cytostatics and its prevention--III. Profile of action of sodium 2-mercaptoethane sulfonate (mesna). *European Journal of Cancer and Clinical Oncology* 1982;18:1377-1379, 1381-1387.

50. Powis G, Mustacich D, Coon A. The role of the redox protein thioredoxin in cell growth and cancer. *Free Radical Biology and Medicine* 2000;29:312-322.

51. Powis G, Kirkpatrick DL. Thioredoxin signaling as a target for cancer therapy. *Current Opinion in Pharmacology* 2007;7:392-397.

52. Biaglow JE, Miller RA. The thioredoxin reductase/thioredoxin system: novel redox targets for cancer therapy. *Cancer Biol Ther* 2005;4:6-13.

53. Kakolyris S, Giatromanolaki A, Koukourakis M, et al. Thioredoxin Expression Is Associated with Lymph Node Status and Prognosis in Early Operable Non-Small Cell Lung Cancer. *Clinical Cancer Research* 2001;7:3087-3091.

54. Lonn ME, Hudemann C, Berndt C, et al. Expression pattern of human glutaredoxin 2 isoforms: identification and characterization of two testis/cancer cell-specific isoforms. *Antioxid Redox Signal* 2008;10:547-557.

55. Soini Y, Kahlos K, Napankangas U, et al. Widespread expression of thioredoxin and thioredoxin reductase in non-small cell lung carcinoma. *Clin Cancer Res* 2001;7:1750-1757.

56. Hausheer F. Anti-Cancer Activity Augmentation Compounds and Formulations and Methods of Use Thereof In: Office USP, ed. *United States Patent Application*. US: BioNumerik Pharmaceuticals, Inc., 2007.

57. Verschraagen M, Boven E, Torun E, et al. Pharmacokinetic behaviour of the chemoprotectants BNP7787 and mesna after an i.v. bolus injection in rats. *Br J Cancer* 2004;90:1654-1659.

58. BioNumerik Pharmaceuticals I. Confidential Unpublished Study Report, Modulation of the Toxicity and Anti-tumor Activity of Cisplatin (CDDP) by Dimesna (BNP7787) (Ward Colorectal Cancer Study).

59. BioNumerik Pharmaceuticals I. Unpublished Observations, Communicated by Dr. Frederick Hausheer, MD.

60. BioNumerik Pharmaceuticals I. Confidential Unpublished Study Report, Antineoplastic activity of paclitaxel (Taxol™ in combination with O-00042 (BNP7787) in Nude Mice Bearing HT-29 or KB Xenograft Tumors

61. Chabner B, Longo DL. Cancer chemotherapy and biotherapy principles and practice. 4th ed. Philadelphia: Lippincott Williams & Wilkins, 2006;1 online resource (xv, 879 p.).

62. Shanmugarajah D. Mechanism of action of BNP7787, a novel chemoprotective agent *Health Science*. San Antonio: Univeristy of Texas, 2008;293.

63. Ormstad K, Uehara N. Renal transport and disposition of Na-2-mercaptoethane sulfonate disulfide (Dimesna) in the rat. *FEBS Letters* 1982;150:354-358.

64. Verschraagen M, Boven E, Torun E, et al. Possible (enzymatic) routes and biological sites for metabolic reduction of BNP7787, a new protector against cisplatin-induced side-effects. *Biochem Pharmacol* 2004;68:493-502.

65. Ormstad K, Orrenius S, Lastbom T. Pharmacokinetics and metabolism of sodium 2-mercaptoethanesulfonate in the rat. *Cancer Research* 1983;43:333-338.

66. Verschraagen M, Boven E, Ruijter R, et al. Pharmacokinetics and preliminary clinical data of the novel chemoprotectant BNP7787 and cisplatin and their metabolites[ast]. *Clin Pharmacol Ther* 2003;74:157-169.

67. Hausheer FH, Shanmugarajah D, Leverett BD, et al. Mechanistic study of BNP7787-mediated cisplatin nephroprotection: modulation of gamma-glutamyl transpeptidase. *Cancer Chemother Pharmacol*;65:941-951.

68. Hausheer FH, Parker AR, Petluru PN, et al. Mechanistic study of BNP7787-mediated cisplatin nephroprotection: modulation of human aminopeptidase N. *Cancer Chemother Pharmacol*;67:381-391.

69. BioNumerik Pharmaceuticals I. Confidential Unpublished Study Reports A Toxicity Study of Single Dose Cisplatin and Single or Multi-Dose Dimesna Injected Intravenously into Rats

70. BioNumerik Pharmaceuticals I. Confidential Upublished Study Report, A single dose study of dimesna and cisplatin given intravenously to beagle dogs

71. Boven E, Verschraagen M, Hulscher TM, et al. BNP7787, a novel protector against platinum-related toxicities, does not affect the efficacy of cisplatin or carboplatin in human tumour xenografts. *Eur J Cancer* 2002;38:1148-1156.

72. Connelly E, Markman M, Kennedy A, et al. Paclitaxel delivered as a 3-hr infusion with cisplatin in patients with gynecologic cancers: unexpected incidence of neurotoxicity. *Gynecol Oncol* 1996;62:166-168.

73. Cavaletti G, Marzorati L, Bogliun G, et al. Cisplatin-induced peripheral neurotoxicity is dependent on total-dose intensity and single-dose intensity. *Cancer* 1992;69:203-207.

74. Rowinsky EK, Chaudhry V, Cornblath DR, et al. Neurotoxicity of Taxol. *J Natl Cancer Inst Monogr* 1993:107-115.

75. Lee JJ, Swain SM. Peripheral neuropathy induced by microtubule-stabilizing agents. *J Clin Oncol* 2006;24:1633-1642.

76. Parker AR, Petluru PN, Wu M, et al. BNP7787-mediated modulation of paclitaxel- and cisplatin-induced aberrant microtubule protein polymerization in vitro. *Mol Cancer Ther*;9:2558-2567.

77. Akwari OE. The gastrointestinal tract in chemotherapy-induced emesis. A final common pathway. *Drugs* 1983;25 Suppl 1:18-34.

78. Allan SG, Smyth JF, Hay FG, et al. Protective effect of sodium-2-mercaptoethanesulfonate on the gastrointestinal toxicity and lethality of cis-diamminedichloroplatinum. *Cancer Res* 1986;46:3569-3573.

79. Grdina DJ, Murley JS, Kataoka Y. Radioprotectants: current status and new directions. *Oncology* 2002;63 Suppl 2:2-10.

80. BioNumerik Pharmaceuticals I. Confidential Unpublished Study Report, Pharmacokinetic Study of BNP7787 in Rats and Monkeys.

81. Gutierrez AN, Deveau M, Forrest LJ, et al. Radiobiological and treatment planning study of a simultaneously integrated boost for canine nasal tumors using helical tomotherapy. *Vet Radiol Ultrasound* 2007;48:594-602.

82. Bommarito DA, Kent MS, Selting KA, et al. Reirradiation of recurrent canine nasal tumors. *Veterinary Radiology & Ultrasound*:in press.

83. Adams WM, Withrow SJ, Walshaw R, et al. Radiotherapy of malignant nasal tumors in 67 dogs. *Journal of the American Veterinary Medical Association* 1987;191:311-315.

84. LaDue TA, Dodge R, Page RL, et al. Factors influencing survival after radiotherapy of nasal tumors in 130 dogs. *Vet Radiol Ultrasound* 1999;40:312-317.

85. Reif JS, Bruns C, Lower KS. Cancer of the nasal cavity and paranasal sinuses and exposure to environmental tobacco smoke in pet dogs. *Am J Epidemiol* 1998;147:488-492.

86. LeBlanc AK, LaDue TA, Turrel JM, et al. Unexpected toxicity following use of gemcitabine as a radiosensitizer in head and neck carcinomas: a veterinary radiation therapy oncology group pilot study. *Vet Radiol Ultrasound* 2004;45:466-470.

87. Litterst CL, Gram TE, Dedrick RL, et al. Distribution and disposition of platinum following intravenous administration of cis-diamminedichloroplatinum(II) (NSC 119875) to dogs. *Cancer Res* 1976;36:2340-2344.

88. Withrow SJ, Vail DM. *Withrow & MacEwen's small animal clinical oncology*. 4th ed. St. Louis, Mo.: Saunders Elsevier, 2007.

89. Malaise EP, Fertil B, Chavaudra N, et al. Distribution of radiation sensitivities for human tumor cells of specific histological types: comparison of in vitro to in vivo data. *Int J Radiat Oncol Biol Phys* 1986;12:617-624.

90. Suter S. Update: Autologous Peripheral Blood Stem Cell Transplantation For The Treatment Of Canine B-Cell Lymphoma. Veterinary Cancer Society Meeting 2010.

91. Pendyala L, Schwartz G, Smith P, et al. Modulation of plasma thiols and mixed disulfides by BNP7787 in patients receiving paclitaxel/cisplatin therapy. *Cancer Chemother Pharmacol* 2003;51:376-384.

92. Grover S. Gastrointestinal Lymphoma in Cats. *Compendium* 2005:741-751.

93. Williams LE, Pruitt AF, Thrall DE. Chemotherapy followed by abominal cavity irradiation for feline lymphoblastic lymphoma. *Veterinary Radiology & Ultrasound*;51:681-687.