**Hyperthermia and Chemotherapy using Fe(Salen) Nanoparticles Might Impact Glioblastoma Treatment in Canine Models**

**Background**

 Cancer is a major global health problem, even though there have been unprecedented advances in the diagnosis and treatment of cancer. The majority of cancers still present an overwhelming challenge for the current medical system (Hui et al. 2016). Recent data collected shows that for the past decade, the rate of new cancer diagnoses decreased by roughly 2% per year in men, but has stayed about the same for women (American Cancer Society). Many of the cancer treatment therapies are insufficient due to multidrug resistance, adverse effects and reoccurrence due to insufficient removal (Nie 2016). Chemotherapy is frequently carried out to treat malignant cancer rather than surgery or radiotherapy. However, anticancer drugs are associated with an overabundance of side effects. Each of these therapies have its own set of adverse reactions which may cause patient incompliance and a major deterioration of the quality of life (Singh et al. 2018). For example, chemotherapy is known to be associated with significant cardiotoxicity, which is very unideal for a patient already fighting against cancer (Curigliano 2012). In response to the negative effects of chemotherapy on patients, the need for new anticancer methods that provide a more effective treatment with little toxicity on patient health, is of high focus in the scientific community.

 Chemotherapy uses certain drugs to kill cancer cells at the original tumor site or those that have spread throughout parts of the body, with the goal of either curing the cancer, controlling growth, or easing the symptoms of cancer at an advanced stage (American Cancer Society). The negative side effects of chemotherapy occur because of the drugs not only being toxic towards cancer cells but also healthy cells. This is the problem trying to be solved, by finding a treatment that is able to successfully identify and kill cancer cells while not harming the surrounding healthy tissue. Radiation therapy exposes areas where cancer cells are present to toxic levels of radiation, a similar problem with chemotherapy is accurately killing cancer cells and no healthy ones. One study proposes the assembly of a program that will increase the statistical confidence of treatments to allow the trade-off between sufficient tumor coverage and sparing healthy tissues under uncertainty (Zaghian 2018).

 The introduction of nanoparticles for cancer treatment has caught the attention of many researchers, because they have multiple useful characteristics like the ability to cross the blood-brain barrier. Nanoparticles have been tailored for many uses such as imaging, drug delivery vehicles, and most recently as a therapeutic component in initiating tumor cell death in magnetic and photonic ablation therapies (Revia 2016). One study proposed the potential use of nanoparticles called quantum dots that possess special fluorescent properties that could be used for image-guided tumor resection to allow visualization of cancer cells (Radenkovic 2016). This is useful because of the difficulties in defining tumor margins when performing treatment that results in cancer cells often remaining, leading to reoccurrences (Radenkovic 2016). Because nanoparticles possess a myriad of functional groups that can create targeted treatments, it could become possible to kill cancer cells without the expense of healthy cells (Ung 2015). A treatment method of interest is magnetic hyperthermia, designed for producing enough heat to kill cancer cells of solid, inaccessible human tumors. However, the main challenge of this technology is increasing the local temperature with minimal side effects on the surrounding healthy tissue (Sanz 2017). This research proposal will focus specifically on using μ-oxo N,N'-bis(salicylidene)ethylenediamine iron [Fe(Salen)] nanoparticles for a possible hyperthermia treatment.

 The nanoparticle Fe(Salen) is a magnetic organic compound, that has direct anti-tumor activity, and generates heat in an alternating magnetic field (Ohtake 2017). Because these nanoparticles have magnetic properties, a research team was able to successfully deliver them, by a magnet, to a desired location, as an anti-cancer drug, in cultured cells and in various animal cancer models (Eguchi 2015). With conventional hyperthermia, the past particles used are usually made of iron oxide, which does not exhibit anticancer activity in the absence of an alternating magnetic field (Sato 2016). A study on Fe(salen) nanoparticles found that they induced apoptosis in cultured cancer cells, and that exposure to an alternating magnetic field enhanced the apoptotic effect (Sato 2016). The study then went on to test the combined strategy of chemotherapy with Fe(Salen) nanoparticles, magnetically guided delivery of the nanoparticles to the tumor, and AMF-induced heating of the nanoparticles to induce local hyperthermia, in a rabbit model of tongue cancer. The results showed that the tumor masses were dramatically reduced, indicating that the combined strategy of hyperthermia-chemotherapy using Fe(Salen) nanoparticles specifically delivered with magnetic guidance, was a promising and had the potential of becoming a powerful new approach for cancer treatment (Sato 2016).

 The research team continued momentum with this promising study of the Fe(salen) nanoparticles by testing them on human glioblastoma. Glioblastoma, also referred to as glioblastoma multiforme (GBM), is a highly aggressive type of brain tumor with an extremely poor prognosis (John 2017). This kind of cancer is the fastest growing grade of astrocytoma, tumors that start in cells called astrocytes and can spread widely throughout the brain, blending with the normal brain tissue, and also can be found on cerebral spinal fluid pathways, which can make them very hard to remove by surgery (American Cancer Society). Glioblastoma is one of the most challenging tumors with difficult treatment options, the poor results of traditional chemotherapies for glioblastoma are mainly attributed to the insufficient and nonspecific drug delivery into the brain tumors as well as the incomplete drug release at the tumor sites (Zimiao 2017). The scientific community testing many different approaches of way to potentially cure this aggressive cancer, one study focuses on the reactivation of defective cell death programs that the glioblastoma cells have effectively turned off to allow for tumor growth, is currently considered as a promising approach for treatment (Simone 2018).

 The second study performed multiple tests on how effective the three part strategy with the Fe(salen) nanoparticles is on treating the challenging glioblastoma cancer cells (Ohtake 2017). The first test involved exposing the Fe(salen) nanoparticles to several glioblastoma cell lines (Ohtake 2017). The in vitro study results showed anti-tumor activity towards several of the cell lines, the nanoparticles inhibited cell proliferation, and its apoptosis-inducing activity was greater than that of clinically used drugs (Ohtake 2017). Similar results were observed with the second tests performed, the results showed in vivo anti-tumor activity in mouse models infected with glioblastoma tumors located in their leg (Ohtake 2017). To avoid the possibility of injuring normal cells, the researchers tested the treatment with normal human astrocytes. The results showed that hyperthermia alone at 43 °C was enough to kill the glioblastoma cells, but had little effect on the viability of the normal astrocytes (Ohtake 2017). However, at 47 °C, the number of dead normal astrocyte cells increased significantly, supporting that the combination of hyperthermia and Fe(salen) at 43 °C is selectively cytotoxic to glioblastoma cells only (Ohtake 2017). The researchers from the previous study mentioned discussed the limitations they faced during its completion and what would be the next steps for further research with the Fe(salen) hyperthermia combination. The next section will discuss what the next steps from the previous are and how this proposal will attempt to achieve these steps and add to the research already done so far.

**Specific Aim**

From multiple past studies, the Fe(salen) nanoparticle shows promising results for the use in cancer treatment. A recent study had demonstrated the anti-cancer properties of the nanoparticles and when combined with chemotherapy and hyperthermia, the treatment effectiveness in killing glioblastoma cells increased (Ohtake 2017). However, the study was unable to test the hyperthermia treatment on the mouse brain, this was because the small mouse skull was unable to hold enough Fe(salen) particles inside to produce sufficient heat to cause cytotoxicity to the glioblastoma cells (Ohtake 2017). The research team determined that more than 500 μl Fe(Salen) (10 mM) should be needed to reach 43.0 °C, and because the sufficient volume for hyperthermia was permitted in the leg, the study used a leg tumor model to evaluate the dual strategy in vivo (Ohtake 2017). The study then stated that a large animal model, which is permitted higher volume injection into the brain, would be needed in order to examine the dual strategy in the brain tumor model (Ohtake 2017). The specific aim of this proposal will focus on moving forward from the previous study, in that a larger animal model will be used to allow for a higher volume of Fe(Salen) to be injected, so that the optimal heat needed for hyperthermia treatment can be reached and studied further on glioblastoma in the brain. The new animal model proposed will be canine models diagnosed with spontaneous cancer, because of their closer evolutionary relationship to humans than rats and the larger cranium size is more ideal. The potential results from this proposed study could provide more supporting data essential for the eventual leap to applying this study on actual glioblastoma diagnosed humans.

**Methodology**

**Reagents**

 The Fe(Salen) nanoparticles will be purchased from Tokyo Chemical Industry Co. Ltd. The nanoparticles will then be sonicated for 30minutes and suspended in normal saline before use (Ohtake 2017). The chemotherapy drug, Carmustine (BCNU), will be purchased from Sigma (Ohtake 2017).

**Electric Devices**

 The alternating magnetic field (AMF) will be driven by a transistor inverter (Hot Shot, Ameritherm Inc., New York, USA) and generated by a solenoid copper coil (resistivity: 1.673× 10−8Ωm) (Ohtake 2017). The experiments will be performed at a frequency of 280 kHz and a current of 335 (Ohtake 2017). A thermograph (InfraRed camera, Nippon Avionics Co., Ltd, Tokyo, Japan) will be used to determine temperature in vivo (Ohtake 2017). The distance between the tumor and the edge of the coil will be 5mm (Ohtake 2017). 5 of the 10 Fe(salen) injected canines will be exposed to an AMF for 20 minutes, twice a week (Ohtake 2017).

**Patient cohort**

20 companion dogs of various breeds and sizes will be studied from the Bush Veterinary Neurology Service in Rockville, Maryland (Coleman 2017). All participating dogs will have been diagnosed with a glioma tumor and have shown no anti-tumor response to traditional treatment, prior to this study. Participation in the study will be offered to the dogs’ owners if they have declined euthanasia. However, participation will be declined to dogs that are not expected to survive for the length of the study period based on clinical progression of tumor

Uncontrolled seizures (MacDiarmid 2016). This study will be carried out in strict accordance with the guidelines for the acceptance of animals for research or experimentation, under Title 3.2. Agriculture, Animal Care, and Food. Subtitle V. Domestic Animals of VA law (Va. Code Ann). Signed informed consent will be obtained from all owners (MacDiarmid 2016). Refer to Table 1. under the figures and tables section for a more detailed visual of the proposed groups for this study.

**Magnet-guided delivery of Fe(Salen) particles in canine models**

10 canines will be injected with Fe(salen), 5 with BCNU, and 5 with saline. Injection of Fe(Salen), BCNU, or saline (control) will be administered via an aseptically placed peripheral vein catheter (left cephalic; 1 ml/min) as a weekly dose(injection volume will be one-third of the tumor volume), for the duration of the study (MacDiarmid 2016). A bar magnet (630 mT) will be used to generate a magnetic field for drug delivery (Eguchi 2015). The circular top of the magnet will be gently placed in contact with the top of the tumor mass and held in place for three hours (Eguchi 2015). This process will be repeated every day for 14 days (Eguchi 2015).

**Evaluation of anti-tumor and hyperthermia effects in canine brain tumor model.**

 Responses to the treatments will be assessed by MRI scans approximately every 8 weeks during the course of the treatment (MacDiarmid 2016). The MRI scans will be performed with a 1.5T Phillips Achieva Scanner (MacDiarmid 2016). The sequences that will be obtained are Sagittal T1, axial T2, coronal gradient echo, axial diffusion pre-contrast, coronal flair, axial T1 and sagittal T1 post-contrast (MacDiarmid 2016). The maximum dimensions (mm) of the tumor (M/L, dorsal to ventral, craniocaudal) in each dog will be provided by the veterinary surgeon based on MRI (MacDiarmid 2016). Brain tumor volume will be assessed by using the formula: length x width x height x (π/6) (MacDiarmid 2016).

**Date analysis and statistics**

The average regression rate of change in tumor volume will compared between the groups in response to their individual treatments (Ohtake 2017). Statistical comparisons among groups will be performed using Students’ *t*-test or one-factor analysis of variance (ANOVA) with the Bonferroni post hoc test (Ohtake 2017). A *p* value of less than 0.05 will be considered statistically significant.

**Possible Pitfalls**

 The possible pitfalls for this experiment would be that not enough canine models will be available to participate in the study. If sufficient canine models are unobtainable, another alternative could be to use feline models instead. Because companion animals are living longer, the occurrences of cancers have also risen (Yoshihiro 2013). Feline models with glioblastoma could potentially be found based on, just like canines, they frequently demonstrate the same clinical signs and pathophysiology as the diseases in humans (Saafan 2012). One other possible pitfall is initiating and maintaining the consent of the owners throughout the study for their own ethical reasons. The uncertain survivability of the tested canines will be a factor, in that it is unknown for how long the models will be alive to continue the experiment.

**Potential Conclusions**

 The main conclusion that hopefully will be shown from the data collected, is that the combination of hyperthermia therapy and the Fe(salen) nanoparticles resulted in an significant increase of tumor depletion compared to the control or chemotherapy drug groups. This study would give additional information of how effective the nanoparticles are at achieving hyperthermia on glioblastoma cells in a living organism model. The results would either support or not support the previous study involving glioblastoma tumors in the legs of rats, in that they provide input of this treatment when implemented on a larger model in the actual brain area. This proposed study will succeed in taking the next step determined by the previous research team, in that a larger model organism would need to be used to possibly achieve hyperthermia in the brain.

 Figure 1 under the figures and tables section shows the results from the previous study on the glioblastoma cells injected into the mice legs (Ohtake 2017). The top half visually shows the changes in the tumor sizes from the beginning to the end of the study. A similar figure could be utilized for this proposed study, using the MRI data collected on the tumor models throughout the duration of the study. The graph in the lower half shows the regression rate of the tumor volume in the tested mice. A similar graph could be made to display the change in the tumor volumes possibly made during the proposed study.

 With these possible conclusions from this proposed study, the next question is what would be the next step from here. Because of their closer evolutionary connection to humans, the canine model could be a more suitable candidate for preclinical drug screening as well as clinical trials for this cancer treatment than the previous rodent trials (Chen 2013). Bringing the results to a major scientific conference such as the From bench to first in-human trials’ conference, would provide a unique opportunity to expose the finding with experts worldwide to hopefully gain attention and support for further research in the public eye (Al-Hujaily 2018).

**Figures and Tables**

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Fig 1. (h) IVIS images of mouse leg tumor in each treatment group at Day 0 *(upper*) and Day 28 (*lower*). (j) Regression rate of tumor volume changes

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| **Groups (N=5)** | **Treatment** |
| 1 (control)  | Saline |
| 2 | BCNU (chemotherapy) |
| 3 | Fe(Salen) |
| 4 | Fe(Salen) + AMF |

Table 1. A visual representation of how the canines will be group and what treatment methods will be assigned to each group.

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