

Application of thermal therapies and remotely targeted drug delivery systems to treat cancer

by

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B.S., Kansas State University, 2022

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Veterinary Biomedical Sciences
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

Approved by:

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Abstract

Applied cancer research has been heavily focused on novel treatment methods to improve the current standard of care and subsequently ameliorate quality of life. This thesis describes developments in two projects focused on the treatment of aldosterone-producing adenomas and pancreatic ductal adenocarcinoma.

The first project characterizes a mouse model for utilization in thermal ablation studies for aldosterone-producing adenomas. A previous study demonstrating tumor response to MRI-targeted microwave ablation in mice had promising results but had challenges associated with an ill-defined mouse model. To address these challenges, a three-part study was executed. The first study tested three different inoculation protocols and determined that injecting 20 million HAC-15 cells suspended in Matrigel resulted in the most homogenous and efficient tumor growth. The second study sampled various intervals following an angiotensin II injection to determine when peak stimulation of aldosterone secretion occurs. The third study utilized tumors of various sizes and recorded aldosterone production to determine if there is correlation between tumor size and aldosterone production. No correlation was detected. From this study, we further characterized a mouse model of primary aldosteronism using the HAC-15 cell line. The defined inoculation protocol resulted in homogenous and efficient growth. The defined aldosterone peak post-angiotensin II injection can be utilized to determine residual tumor function post-treatment.

The second project characterized the efficiency and release of encapsulated gemcitabine via triggering the liposomal phase transition temperature (T_m). Future integration of liposomal chemotherapy and radiofrequency ablation into cancer treatment plans has the potential to reduce systemic effects by initiating targeted release of the anti-cancer agent in the tumor vasculature. Liposomes synthesized with a T_m of 41°C are remotely loaded with gemcitabine via an ion

gradient created with ammonium sulfate. The liposomes were applied to 96-well plates seeded with KPC (mouse) and BXPC-3 (human) pancreatic adenocarcinoma cell lines. Heat was applied via a hot water bath at various temperatures for various amounts of time. MTT reagents were added 24 and 48 hours post-heat treatment. The formazan absorbance was quantified spectrophotometrically and used to demonstrate cell viability. Overall, temperature-triggered release of gemcitabine has had a negative impact on cell viability as shown by the significantly lower viability of the treatment groups as compared to control groups at 24 hours post-heat treatment of both cell lines.

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Acknowledgements

First, I would like to thank my mentor, Dr. Matthew Basel, for taking a chance on a first-year veterinary student and allowing me into his lab. His unwavering support and guidance has been crucial for completion of this thesis.

Next, I would like to thank my other committee members, Dr. Bruce Schultz, Dr. Punit Prakash, and Dr. Tej Shrestha for their continued show of support throughout my time as a graduate student. I would also like to express great appreciation to Marla Pyle for teaching me lab protocols and the fundamentals of cell culture. Your help and suggestions were invaluable during my time in the lab.

I would like to give a special thanks to my friend, Jamison, for his kind words of encouragement. And lastly, to my parents, Christie and Tim, my grandparents, Dee and Don, and my husband, Sam. Thank you for being supportive of all of my endeavors, no matter where they take me. You all have been the backbone of my success and I couldn't have done it without you.

Chapter 1 - An introduction to aldosterone-producing adenomas

An aldosterone-producing adenoma (APA) is a functional benign growth of the zona glomerulosa of the adrenal gland. APA is one mechanism of primary hyperaldosteronism, with other conditions including primary unilateral hyperplasia and aldosterone-producing carcinomas. Of all patients referred for hypertension, 6.4-22.8% was reported to have an increased aldosterone to renin ratio indicating primary aldosteronism (PA).¹⁻⁶ About half of those PA patients had an aldosterone-producing adenoma. It is now the most common cause of endocrine-related hypertension.⁷

Major physiological regulators of aldosterone production include high plasma potassium and angiotensin II (upregulated with low mean arterial pressure, low plasma sodium, or plasma acidosis). Aldosterone acts on the distal nephron causing sodium, chloride, and water retention and hydrogen and potassium excretion. Nearly half of the naturally-occurring aldosterone-producing adenomas contain somatic gene mutations that encode for various channel proteins expressed in the zona glomerulosa cells. For example, potassium channels involving mutated KCNJ5 proteins have altered selectivity allowing for sodium influx and membrane depolarization resulting in activation of a voltage-gated calcium channel. The influx of calcium activates a calcium-dependent signal transduction pathway that upregulates aldosterone synthase, CYP11B2, leading to hypersecretion of aldosterone independent of its primary regulators.⁸⁻¹³

While this KCNJ5 gene mutation is commonly implicated in many human APAs, the gene itself is not expressed in the rat. Similarly, a TASK-3 gene that, when deleted, resembles human low-renin high-aldosterone hypertension, is expressed in very low levels in the human zona glomerulosa.¹⁴ Previous mouse models have utilized non-mouse systems like primary bovine adrenocortical cells xenografted into mice to further study the KCNJ5 gene.¹⁵ Other models of mouse potassium channel inactivation (KCNK3, KCNK9) closely resemble human

KCNJ5 mutations with minor physiological differences. Nevertheless, the culmination of mutations in various genes coding for K⁺ channel subunits results in some form of abnormal calcium mobilization from either the mitochondria or the plasma membrane, resulting in increased aldosterone synthesis.

While mouse models featuring some of the genetic alterations mentioned above could mimic human somatic mutations, the resulting neoplasia may not be similar enough to the human genotype to predict external validity. Low animal-to-human translational success rates can partially be attributed to low predictability of human outcomes based on animal physiology, genetics, epigenetics, and molecular biology.^{16,17} Xenograft models are being implemented into research methods more frequently to increase translational success.

The first established adrenocortical carcinoma cell line to be utilized in a xenograft model is termed the NCI-H295 cell line. This cell line was derived from an adrenocortical carcinoma in a female patient with metastasis.¹⁸ This cell line produces all adrenal steroids but is only shown to have some responsiveness to cAMP and no response to ACTH.^{19,20} In addition, a study published by Kurlbaum et al. in 2020 demonstrated that the NCI-H295 cell line had heterogeneous steroid production that was affected by both passage number and strain.²¹ From this first cell line multiple NCI-H295R strains were produced. These strains, like the parent cell line, produce all adrenal steroids, but in addition have been proven to have a steroidogenic response to angiotensin II, potassium, and cAMP.^{19,22–24} There are variable reports of the responsiveness to ACTH.²⁵ The derivative cell line's variable responsiveness to ACTH and the parent cell line's heterogeneous steroid production makes this cell line an unreliable model for functional aldosteronism.

The HAC-15 cell line can provide adequate replication of the pathophysiology of human aldosterone-producing adenomas. The HAC-15 cell line was thought to be a novel cell line, but

after analysis it was found that the clones were isolated from a culture contaminated with the NCI-H295R cell line. As seen in the table below, when compared to the NCI-H295 cell line, the HAC-15 cell line has better responsiveness to angiotensin II and potassium, and when compared to its parent strain (NCI-H295R), it has a significant ACTH response via increased cortisol production.²⁶ Lastly, the HAC-15 model, like its parent strain, can produce all 5 forms of the cytochrome P450 enzyme that are involved in adrenal steroidogenesis.^{24,26-28}

Table 1.1 Summary of Human Adrenocortical Cell Lines.Table adapted from Wong and Rainey, 2011 ²⁹

Cell Line Name	Ang II	K ⁺	ACTH	cAMP	Steroids produced	Reference
NCI-H295	ND	ND	–	+	Mineralocorticoids Glucocorticoids Adrenal Androgens	(Gazdar et al., 1990 ; Staels et al., 1993 ; Rainey et al., 1994)
NCI-H295A	–	ND	–	+	Mineralocorticoid Glucocorticoids Adrenal Androgens	(Rodriguez et al., 1997 ; Huang et al., 2005)
NCI-H295R Strains	+	+	–/+	+	Mineralocorticoids Glucocorticoids Adrenal Androgens	(Bird et al., 1993 ; Rainey et al., 1994 ; Bird et al., 1995 ; Clark et al., 1995 ; Denner et al., 1996)
ACT-1	ND	ND	ND	ND	None reported	(Ueno et al., 2001)
Pediatric adrenocortical adenoma derived cell line	ND	ND	ND	ND	Mineralocorticoids Glucocorticoids	(Almeida et al., 2008)
HAC-15	+	+	+	+	Mineralocorticoids Glucocorticoids Adrenal Androgens	(Parmar et al., 2008)
PRKAR1A inactivated cell line	ND	ND	–	–	None reported	(Nesterova et al., 2008)

Clinical manifestations of primary aldosteronism result from the retention of sodium and chloride and the excretion of potassium. Patients are often in a chronic hypertensive and hypokalemic state. Chronic hypertension can have many impacts on patient health. However, patients with chronic hypertension as a direct result of hyperaldosteronism are at a greater risk of developing severe cardiovascular pathologies such as left ventricular hypertrophy and cardiac fibrosis, as compared to those that have hypertension from another disease process.^{30,31} Prolonged elevation of aldosterone can cause diabetes, metabolic syndrome, and stroke.^{32–35} In addition, mineralocorticoid receptors are suspected to play a role in memory, anxiety, and depression.^{36–38}

For unilateral disease, adrenalectomy is the current standard of care with most patients becoming normokalemic and 30-60% of patients becoming normotensive post-surgery.^{39–42} Adrenocortical tumors have been considered radioresistant, eliminating radiation therapy as a definitive treatment.^{43–45} Mitotane, an adrenal-targeted chemotherapy agent, is controversial in its usage as a primary therapy with a low therapeutic index and side effects including adrenal insufficiency and alterations in thyroid hormone and testosterone metabolism.^{46,47} Current standard of care for bilateral disease or patients that cannot undergo surgery is conservative management consisting of a mineralocorticoid receptor antagonist, namely spironolactone.⁴⁸ Common side effects of spironolactone in females include polyuria, abnormal menstruation, lightheadedness, and gastrointestinal upset. Males often experience hair loss, loss of libido, gynecomastia, and general feminization.^{49,50} In addition, recent studies have shown that despite spironolactone treatment, patients with primary aldosteronism have a two-fold increased risk of developing heart conditions as compared to patients who are undergoing medical management for essential hypertension.⁵¹ Therefore, there is a growing demand for treatments that will

completely resolve the primary aldosteronism while sparing healthy adrenal tissue with minimal long-term effects. Other therapies that have been applied to adrenal neoplasms include chemical ablation, cryoablation, radiofrequency ablation, and microwave ablation. Chemical ablation involves image-guided percutaneous injection of acetic acid or ethanol. Tumor volume can take at least two years to have a significant decrease, and many patients had to be maintained on oral antihypertensives until enough of the tumor regressed.^{52,53} Standard cryoablation involves two 10-minute freeze cycles reaching temperatures from -80°C to -150°C. There are limited clinical experiences reported in literature, with one case description of cryoablation of an adrenal carcinoma metastasis that resulted in malignant hypertension⁵⁴, and a study involving successful cryoablation of the normal adrenal gland in dogs.⁵⁵ Radiofrequency ablation utilizes an electrical current delivered through a needle electrode to cause ionic agitation within the surrounding tissues causing frictional heat and damage.^{56,57} Microwave ablation is similar to radiofrequency ablation in that it agitates water molecules producing friction and heat.⁵⁸⁻⁶⁰ Microwave ablation, however, can target a larger volume, has a more rapid increase in temperature, can achieve an overall higher temperature, and does not need grounding pads.⁶¹ One example of microwave ablation from a 2016 study utilized a 15-gauge cooled shaft antenna and a 21-gauge thermocouple on a unilateral aldosterone-producing adenoma. The antenna emitted a frequency of 2450 MHz with an outpower ranging from 20W at the beginning to 50W once the patient's blood pressure was stabilized. Post-ablation, the patient's aldosterone, renin, and potassium levels normalized. Six months later the patient's CT scan showed a focal area of necrosis where the adenoma had been.⁶² This study shows promise for future utility of microwave ablation.

Chapter 2 - Characterization of a mouse model of an aldosterone-producing adenoma

A manuscript published by Sebek et al. (2022)⁶³ defined a system for delivering microwave ablation to subcutaneous tumors in small animals under high-field MRI thermometry guidance. The authors strove to improve upon a previously developed hyperthermia system with the addition of real-time MRI thermometry.⁶³ Thermal therapies for use in cancer treatment can have challenges associated with the variability of tissue temperature. The presence of large blood vessels and variability of tissue characteristics such as electrical conductivity and perfusion rate can all affect tumor temperature. A previous study noted variability of heat generation across a tumor up to 2-3 fold when the same energy settings were used.⁶⁴ Tracking the thermal profile of various regions within the tumor can allow for further interpretation of results and can limit off-target heating. Real-time MRI thermometry is preferred to thermocouples due to its ability to construct maps of in vivo tissue temperature with enhanced spatial and temporal resolution.⁶⁵

Sebek and colleagues have utilized this MRI thermometry-guided device to deliver mild hyperthermia (40-45°C) to subcutaneous tumors in mice.⁶⁶ This system had yet to be assessed at ablative temperatures against implanted tumors. The objectives of this experiment included: (1) ascertaining the extent of the ablation zone in both targeted and non-targeted tissue to determine thermometry accuracy and compare it to that of mild hyperthermia in previous experiments; (2) comparing the volumetric real-time MRI temperature readings with data from fiber optic sensors; and (3) validate that there is a decline a tumor viability post-ablation in mice with a subcutaneous HAC-15 tumor.

Material specifics included an antenna that radiates 2.45 GHz microwave power coupled with a copper reflector to aim the radiation and a closed-loop coolant flow circulated through the

reflector tube. Transmitted and reflected power was monitored. The MRI scanner was utilized for external positioning of the probe adjacent to the subcutaneous tumor. The MRI also provided phase images that demonstrated relative change in temperature. A fiber optic probe obtained the baseline body temperature of the mouse prior to treatment for the in vivo study.

The equipment was first tested ex vivo on agar models and chicken breast to demonstrate MRI thermometry (MRIT) accuracy prior to in vivo studies. The accuracy of MRIT with the 14.1 tesla high-field scanner in the mild hyperthermia range of 40-44°C was within 0.4°C. Animal experiments utilized athymic nude (Nu/nu) mice injected with 6 million HAC-15 cells subcutaneously. Tumor measurements were obtained every other day starting at day 7 post-injection. Mice were treated when the tumor reached 5 millimeters in greatest dimension. Twelve mice were inoculated and then divided into 6 pairs. Within each pair, one mouse was exposed to microwave radiation while the other was also anesthetized but not treated with radiation exposure. Anesthesia was chamber induced with 5% and oxygen and maintained on 1-2% isoflurane and oxygen via a nose cone for treatment. Once induced, the mice were placed in an acrylic fixture and attached to the temperature sensor. The microwave probe was adhered to the mouse with tape in the direction of the heating pattern. The acrylic fixture with the mouse was loaded into the MRI and initial scans confirmed the position of the probe. Tissue temperature in regions of interest were monitored continuously during the exposure. Heating was discontinued when 55°C was reached in all regions of interest. Following the procedure, animals were recovered from anesthesia and returned to housing. Twenty-four hours later, angiotensin II was administered intra-peritoneally and blood was collected 4 hours later via a cardiac puncture post-euthanasia. The tumor was harvested and stained with tetrazolium chloride (TTC) and hematoxylin and eosin (H&E) before blind analysis by pathologists.

My contribution to the Sebek et al.⁶³ project included cell culture, mice inoculation, tumor monitoring, participating in monitoring mice during ablative treatments, and tumor and blood collection post-treatment. HAC-15 cells were stored in a cryopreservation medium consisting of 10% DMSO, 40% complete growth medium, and 50% cosmic calf serum in liquid nitrogen vapor. Frozen vials were thawed via a slight agitation in a hot water bath keeping the cap out of the water. The vial was spun at 1,000 rpm for 3 minutes before the supernatant was removed and the pellet was resuspended with culture medium consisting of 88% DMEM-F12 (Dulbecco's Modified Eagle Medium) (Gibco; Waltham, MA), 1% Insulin-Transferrin-Selenium (ITS) (Corning Life Sciences; Corning, NY), 1% penicillin-streptomycin (Gibco; Waltham, MA), and 10% cosmic calf serum (Corning Life Sciences; Corning, NY). Cells were cultured to 80% confluency and split in a 1:2-1:3 density reduction. This is a slow-growing cell line with incomplete adherence. To speed up population growth, the old media removed from the plate during media changes was spun down so non-adhered cells could be added back to the plate. To subculture, PBS (phosphate buffered solution) (Corning Life Sciences; Corning, NY) was used as a plate rinse and trypsin-EDTA (Gibco; Waltham, MA) was used for lifting.

To prepare for injections, cells were lifted with 0.1% trypsin-EDTA and stopped with medium after approximately 5 minutes. The total volume was centrifuged at 1000 rpm for 3 minutes and the cell pellet was resuspended in 0°C saline. The cells were counted using a Nexcelom Cellometer on a BXPC-3 cell template with trypan blue. Approximately six million HAC-15 cells were delivered in each inoculation in a volume of 250 ul or less. Twelve male athymic nude (nu/nu) mice approximately 4-6 months old were injected subcutaneously with HAC-15 cell suspension in the right hind flank under isoflurane anesthesia in dorsal recumbency. Mice were chamber-induced with 4% isoflurane anesthesia and povidone-iodine was applied to

the right flank to reduce microorganism load prior to injection. Tumor size was measured using calipers approximately every other day starting at seven days post-inoculation.

Once tumors reached 5 mm in diameter, tumors were ablated with an MRI image-guided, directional, radiofrequency ablative probe. Tumors were heated to a target temperature of 55°C as measured by MRI thermometry feedback. After tumor treatment, mice were recovered in cages and euthanized via a CO₂ chamber twenty-four hours later. Four hours prior to euthanasia, mice were injected with angiotensin II (1 mg/kg). Blood was collected immediately post-euthanasia via cardiac puncture for aldosterone and other steroid hormone analysis. Tumors were also harvested to assess ablative efficiency.

Tumors were stained with tetrazolium chloride (TTC) immediately post-collection to determine the viability of the tumor tissue post ablation. TTC viability stain suggested that 5/6 mice had tumors that were successfully ablated. Tumors were then fixed in buffered neutral formalin (BNF) and submitted for histological evaluation. Blinded verification by H&E indicated that of the 6 mice that underwent heat treatment, 4 of them had tumors that were successfully ablated, 1 was had a tumor that was partially ablated, and 1 had a tumor that was not successfully ablated (Figure 2.1). One of the mice with a successfully ablated tumor has unusable data due to excessive movement during treatment. Thermal dose, based on tumor coverage, was in agreement with viability assessment based on TTC staining (Table 2.1).

Table 2.1. Achieved coverage of tumor, maximum estimated temperature, and tumor ablation status for 5 out of 6 mice exposed to treatment. Table adapted from Sebek et al., 2022

Mouse #	TTC	H&E	Notes:	Volume tumor [mm ³]	Volume of coverage [mm ³]	Vcov/Vtumor [%]	Maximum Temperature [°C]	Heating time [s]
1	Not ablated	Not ablated	Likely mistargeted	9.06	0	0	50.5	259
2	Ablated	Ablated		12.46	11.81	94.71	70	103
3	Ablated	Ablated		35.92	22.02	61.31	66.5	150
4	Not ablated	Ablated	Only 80% of tumor was ablated, not enough to show full ablation of TTC stain	8.29	7.52	90.73	59	134
5	Ablated	Ablated		5.43	5.32	97.98	67.5	135

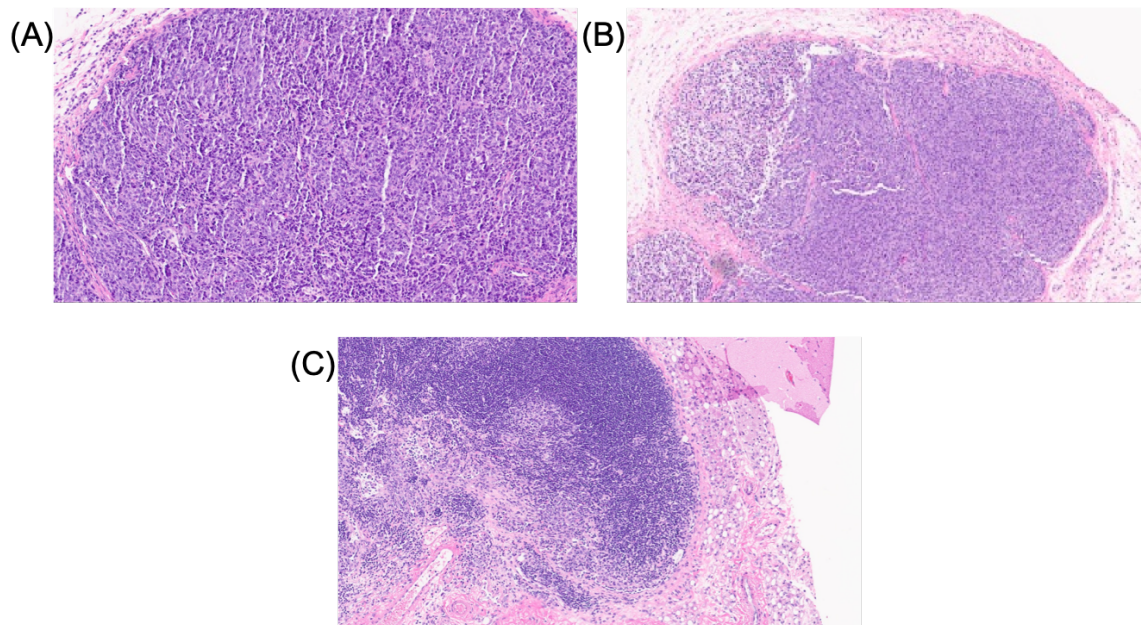


Figure 2.1 Representative H&E images demonstrating partial, full, or no ablation in subcutaneous HAC-15 tumors. (A) no ablation (B) partial ablation (C) full ablation.

Proposed challenges for the in vivo experiment included mistargeted microwave energy and animal movement. Future experiments may utilize motion compensation techniques and further adherence of the animal to the acrylic restraint within the imaging coil to prevent

movement artifacts. In addition, while 12 mice were ultimately used for this experiment, more than 12 were inoculated. Of the inoculated mice, one-third did not grow tumors, one-third developed tumors slowly, and one-third developed tumors rapidly. Heterogenous and unpredictable tumor growth made coordinating ablation experiments difficult because the mice were reaching the 5 mm tumor growth mark on different days post-injection. This prolonged the total experiment time and utilized excess lab resources. Most importantly, the plasma samples taken to assess tumor functionality post-treatment were of little value, likely due to missing the sampling window for peak aldosterone production post-angiotensin II stimulation. From this experiment, we determined that there is a need for a better defined HAC-15 mouse model.

To improve the efficiency of this model, a 3-part experiment was developed to address each of the challenges (Figure 2.2). The first experiment determined the most efficient inoculation protocol for rapid tumor growth. The second experiment determined appropriate sampling to obtain peak plasma aldosterone concentration post angiotensin II injection. The third experiment correlated aldosterone production to tumor size.

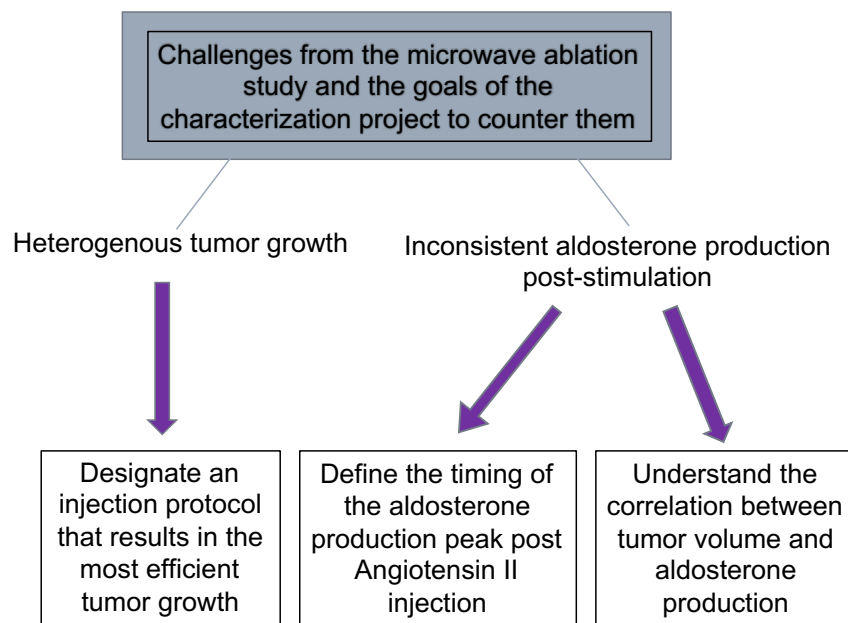


Figure 2.2 Flowchart for characterization of a model of aldosterone-producing adenomas project.

The protocol for the first experiment involved injecting male athymic nude mice approximately 4-6 months old with 250 μ l of various cell concentrations +/- Matrigel. Matrigel is a commercially available proprietary basement membrane preparation that contains growth factors, proteoglycans, laminin, type IV collagen, and heparan sulfate. Matrigel has been used in cell culture to increase cellular grafting and growth and was hypothesized to do the same for inoculated HAC-15 cells. For this procedure, fifteen mice were split into three experimental groups. The first group received 6 million HAC-15 cells suspended in saline, the second received 10 million HAC-15 cells suspended in Matrigel, and the third received 20 million HAC-15 cells suspended in Matrigel. HAC-15 injections were given in freezer-chilled 1 ml syringes into the right hind flank subcutaneously under isoflurane anesthesia laying in dorsal recumbency. Tumor size was measured approximately every other day starting on day 7 post-inoculation. Tumor size was recorded in millimeters and volume was calculated using a standard ellipsoid volume calculation with a long and short axis. Tumor size (Figure 2.3) demonstrated that both the 10 and 20 million cell groups suspended in Matrigel grew significantly faster (Figure 2.3) than the treatment group consisting of 6 million cells without Matrigel. The negative error bars indicate that the standard deviation was larger than the average growth. The tumor growth for both the 10 and 20 million cell groups suspended in Matrigel was more homogenous than the growth resulting from the 6 million cells without Matrigel treatment group, as demonstrated by the consistently elevated standard deviation percentage of the 6 million group cells without Matrigel treatment group (Figure 2.4). Statistical analysis was conducted on XLSTAT (2007), a Statistical Software for Excel, using a repeated measures test followed by Tukey's Honest Significant Difference post-hoc test. The repeated measures test yielded significant variance between all time points except 25- and 31-days post-injection. The Tukey post-hoc test demonstrated a significant ($P < 0.5$) difference between the mean tumor size of the 20 million cell group and the

mean tumor size of the 6 million cell group for every time point. The mean tumor size of the 10 million cells suspended in Matrigel treatment group was statistically significant when compared to the tumor size mean of the 6 million cells without Matrigel treatment group at time points 7, 11, 14, 16, 18, and 21 days post-inoculation. Lastly, the mean tumor size of the group containing 10 million cells suspended in Matrigel was statistically significant from the mean tumor size of the 20 million cells suspended in Matrigel group at time points 11, 14, 16, and 18-day post-injection, but caught up to the 20 million cell treatment group by day 21. At the end of the study, the mice within the 6 million HAC-15 cells without Matrigel treatment group were euthanized and the tumors (Figure 2.5) and major organs were excised. The organs collected were stained with H&E before being examined histology for evidence of metastases. No evidence for metastasis was identified in any of the following tissues: heart, lungs, liver, spleen, or kidneys. MRI images showed no evidence of local invasion.

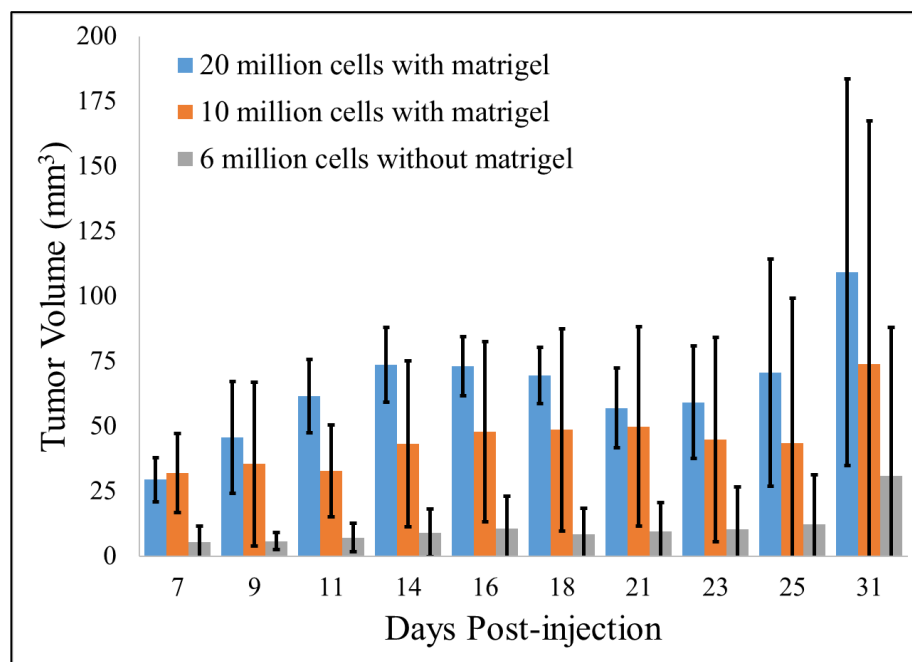


Figure 2.3 Tumor volume of neoplasms grown in the right hind of flank of immunodeficient mice injected subcutaneously with human HAC-15 adrenocortical carcinoma cells. Black bars represent standard deviation.

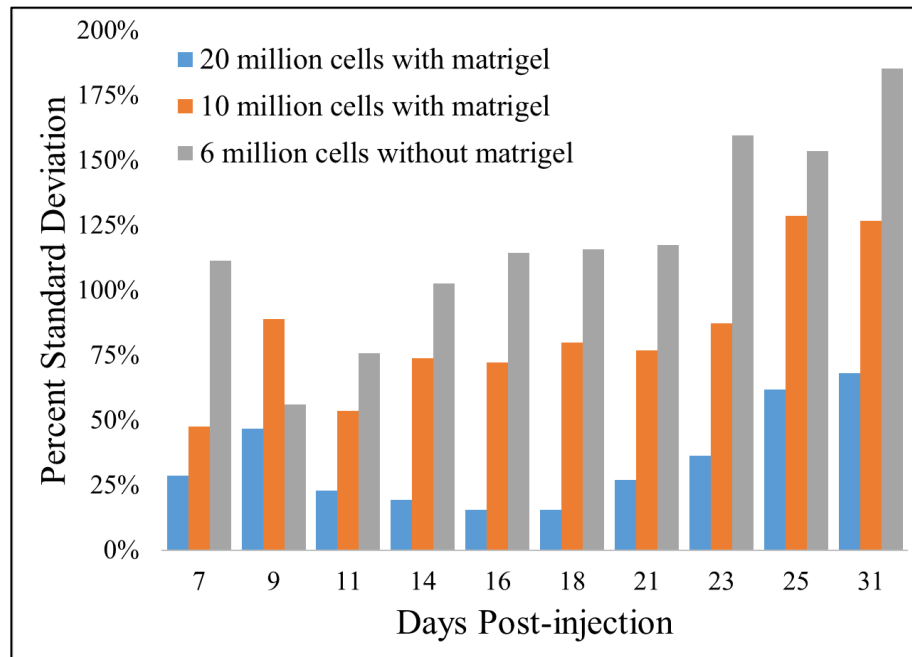


Figure 2.4 Percent standard deviation each day post injection.

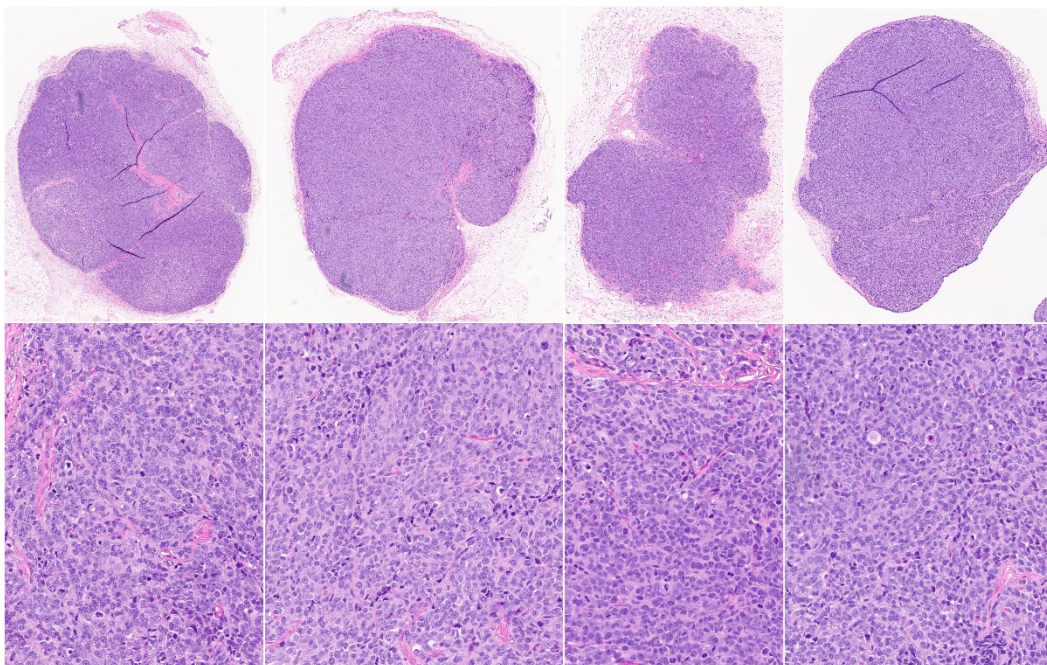


Figure 2.5 Histology of untreated HAC-15 tumor. Neoplasms were grown in the right hind flank of immunodeficient mice injected subcutaneously with human HAC-15 adrenocortical carcinoma cells. Tumors were excised and stained with hematoxylin and eosin (H&E) to demonstrate tumor structure.

The protocol for the second experiment involved inoculating male athymic nude mice approximately 4-6 months old with 20 million HAC-15 cells suspended in Matrigel following protocol from the tumor size experiment. Tumor size was measured approximately every other day. The resulting adenomas were grown until they reached at least 5 mm in one dimension, at which point angiotensin II (5 μ g/gram) was administered intraperitoneally. Mice were held in dorsal recumbency for the procedure. Two mice with APAs served as untreated controls. Following the angiotensin II injections, mice were assigned to a waiting interval of either 0 minutes, 30 minutes, 1 hour, 2 hours, or 4 hours with 3 mice per interval. At the end of the waiting interval the mice were euthanized via a carbon dioxide chamber and a cardiac puncture was performed with 1 ml heparinized syringes. Blood samples were centrifuged at 2,000 g for 15 minutes. Plasma was removed and sent to Birmingham University, UK for quantification of both aldosterone and its precursors. Statistical analysis completed on XLSTAT involved an ANOVA of aldosterone and its precursors for each set of mice. For comparison between the sets of mice with and without tumors a repeated measures test was utilized. Both the repeated measures test and ANOVA were followed by Tukey's Honest Significant Difference post-hoc test. Analysis of the following precursors was used to determine tumor aldosterone production time following angiotensin II injection: progesterone, deoxycorticosterone, and corticosterone. When looking solely at the set of mice with tumors, all of the precursors had a mean value for baseline that was statistically significant ($P < 0.5$) when compared to the mean value of the 120-minute treatment group (Figure 2.6A). The post-hoc for aldosterone produced a significant p-value when comparing baseline to 120 minutes as well (Figure 2.6B). The set of mice without tumors has a peak plasma aldosterone concentration occurring anywhere between 0 and 30 minutes (Figure 3.6E) with the physiologic response to the angiotensin II stimulation being much smaller. Additionally, when comparing the mean aldosterone production of mice with and without tumors

side by side in a repeated measures test, the only significant difference is at the 120-minute time point.

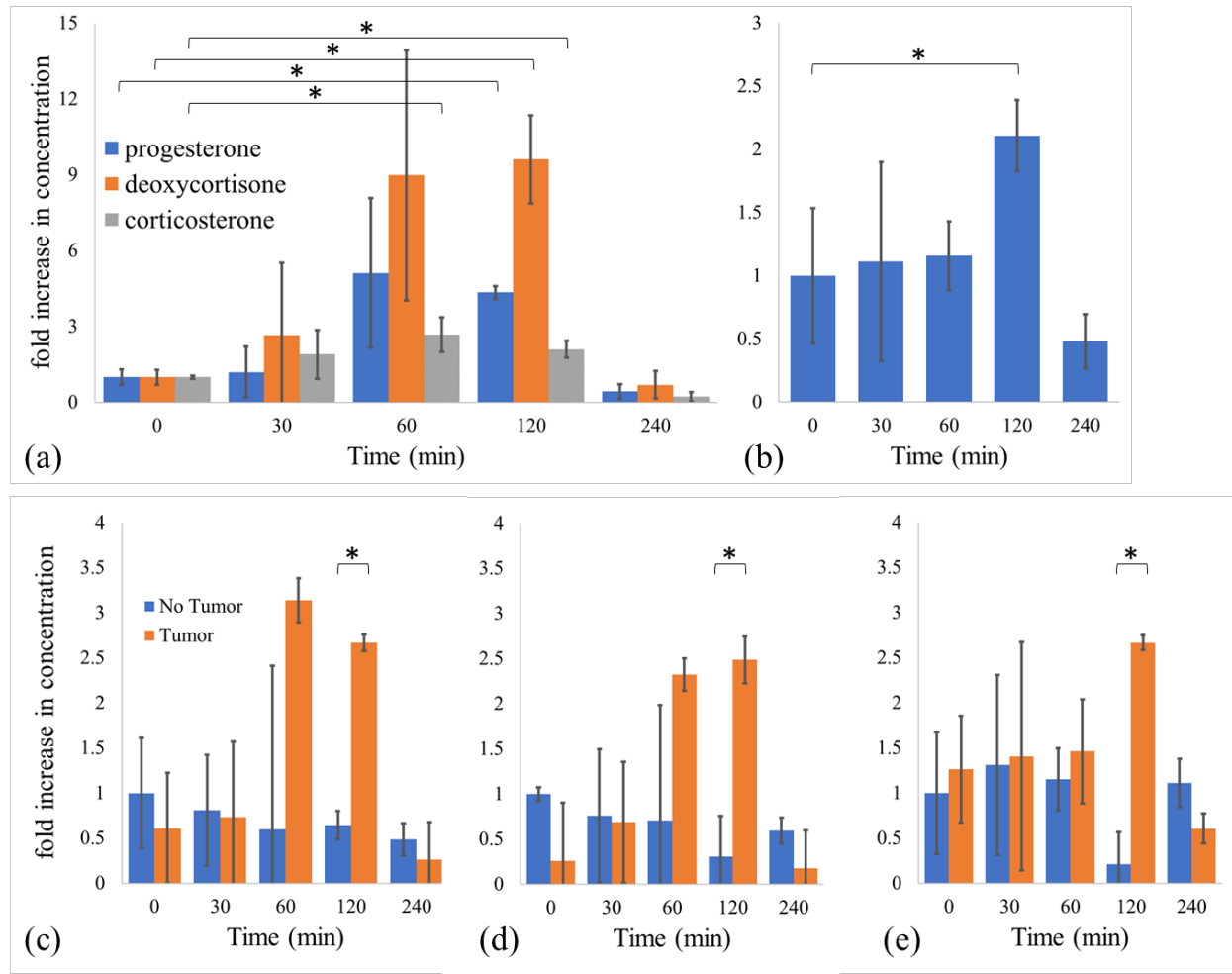


Figure 2.6 Plasma aldosterone and aldosterone precursor concentration post-angiotensin II injection with and without tumors. Mice with and without tumors underwent an angiotensin II injection and a subsequent waiting interval before plasma collection. (A) - Time after ANGII for precursors. (B) - Time after ANGII for aldosterone. (C) - Progesterone w+w/o tumors. (D) - Deoxycorticosterone w+w/o tumors. (E) - Aldosterone w+w/o tumors. Asterisks = $p < 0.05$. Black bars indicate standard deviation.

The goal for the third experiment was to determine if there is any correlation between tumor size and aldosterone production. The protocol involved male athymic nude mice

approximately 4-6 months old injected with 20 million HAC-15 cells suspended in Matrigel following protocol from the tumor size experiment. Tumor size was measured every other day. A total of 21 mice were allocated into 7 groups of 3 for assessment. On days 3, 6, 10, 15, 20, and 25 post HAC-15 inoculation, assessment was initiated with intraperitoneal injections of angiotensin II (1 mg/ml) at a dose of 5 μ l/gram. Two hours post-injection mice were euthanized via a carbon dioxide chamber and a cardiac puncture was performed with 1 ml heparinized syringes. Blood samples were centrifuged at 2,000 g for 15 minutes. Plasma was frozen for future analysis. Tumors were excised, measured, flash frozen with liquid nitrogen, and stored at -80°C. Plasma samples and excised tumors were sent to Birmingham University, UK for analysis. The post-excisional tumor volume was graphed over aldosterone concentration relative to an established baseline aldosterone level (Figure 2.7). No significant correlation between tumor size and aldosterone concentration was noted (Pearson Correlation = -0.1303).

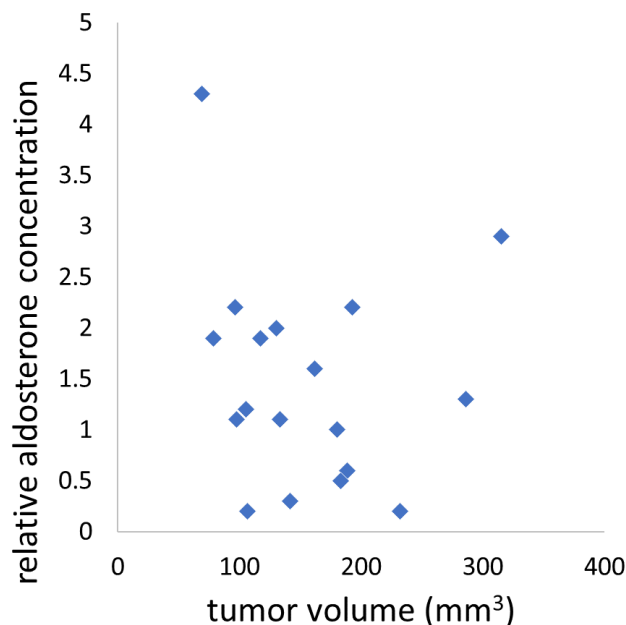


Figure 2.7 Tumor size and correlating aldosterone production post-angiotensin II injection. Neoplasms grown in the right hind of flank of immunodeficient mice inoculated subcutaneously with human HAC-15 adrenocortical carcinoma cells. The mice were euthanized on specific intervals post-inoculation and the aldosterone concentration was correlated to the post-excisional tumor volume.

This study demonstrates the development and characterization of a mouse model of human primary neoplastic aldosteronism that is useful for testing ablative therapies on APAs. To be optimal for testing thermal therapies, a model must replicate the basic features of human pathophysiology and have quantifiable and consistent changes in blood biochemical markers that can be used to identify treatment success. Multiple mouse models of primary aldosteronism currently exist, although their utility has been questioned due to differences between the pathophysiology of mouse tumors and human tumors and the impact animal models can have on translational success. A xenograft mouse model of human metastatic adrenocortical carcinoma was developed and characterized using the human NCI-H295R cell line. The HAC-15 cell line is a derivative of the NCI-H295R cell line that was selected for responsiveness to both angiotensin II and ACTH. When compared to NCI-H295R, HAC-15 demonstrates a broader spectrum of steroidogenic ability, sensitivity to ACTH and angiotensin, and a more stable steroidogenic phenotype, as the NCI-H295R steroidogenesis has recently been shown to be impacted negatively by both passage and strain. These observations suggest that the HAC-15 cell line is more likely to provide a more stable and consistent xenograft mouse model of primary aldosteronism.

True APA should be non-malignant. Metastatic aldosteronism would be classified as aldosterone-secreting adrenocortical carcinomas (ACC). The parent NCI-H295R line was derived from a naturally occurring ACC, but subcutaneous models using NCI-H295R cells did not demonstrate any malignant ability. Similarly, during the time course of this study, the HAC-15 model did not demonstrate any metastasis to major organs evaluated by a pathologist or local infiltration on MRI suggesting that this model's behavior more closely resembles a functional adenoma. Assessment with longer mouse survival would be required to bolster this conclusion.

One surprising result from this study was the apparent disconnect between aldosterone production and tumor size in the model. This may not be a weakness of the model, though, as currently the literature is significantly split on whether tumor size and aldosterone production correlate in APAs. Several studies have suggested that tumor size correlates with aldosterone production and symptoms^{67,68}, while other studies suggest that there is no correlation between size, aldosterone production, and symptoms in human APA patients.^{69–71}

The model reported here can also be useful for analyzing the biochemical response to treatments. The HAC-15 cell line is sensitive to angiotensin II and produces aldosterone in response. The xenograft model replicates this; it was sensitive to angiotensin II and serum aldosterone increased at a defined time-point after angiotensin II exposure. This should allow rapid and defined interrogation of the biochemical status of the mice in response to a treatment.

In conclusion, this HAC-15 xenograft model of primary aldosteronism will be useful for characterizing the effects of ablative thermal treatments on APA and may have utility in assessing other treatments as well.

Chapter 3 - Background on liposomes

After more than 60 years of development, the liposomal drug delivery system has been utilized for a wide variety of therapies including cancer^{72,73}, microbial infections^{74,75}, hormone and enzyme deficiencies⁷⁶, metal detoxification⁷⁷, gene deficiency/malfunction^{78,79}, and vaccines.⁸⁰ Key properties of liposomes include the ability to contain both water soluble and insoluble drugs, protection from physiological degradation extending the plasma half-life, and good biocompatibility and safety. Targeting of the payload can be achieved with passive accumulation via the permeation and retention effect or active targeting with triggered release to reduce systemic side effects and increase the maximum tolerated dose.⁸¹

Basic lipid structures ultimately affect the overall liposome structure and function. Lipids with single chains will form spherical micelles that are smaller than liposomes and have a single layer of lipids with a non-polar core. Double-chain amphipathic molecules will form bilayers similar to biological membranes with hydrophobic tails pointing into the membrane and inwardly facing polar head groups lining an internal polar core.

Liposomes typically are composed of phospholipids, specifically glycerophospholipids and sphingomyelins. Glycerophospholipids have glycerol as the backbone with a hydrophilic head group and two hydrophobic side chains. Variations in types of glycerophospholipids are achieved with different head groups, length of non-polar chains, and types of bonds.^{82,83} Liposomes can carry different net surface charges in conjunction with carbohydrates integrated into the membrane to further stabilize the bilayer.⁸⁴⁻⁸⁶ Liposomes with this structure are often opsonized and removed by the reticuloendothelial system and have instability in plasma resulting in short circulation half-life.⁸⁷ A second generation of liposomes were created with an added structural hydrophilic polymer that coated the membrane surface, polyethylene glycol (PEG).

PEGylated liposomes have the potential to evade certain components of the immune system, thus increasing circulating half-life.

Characterization of liposomes is broadly based on size, stability, zeta potential, encapsulation, and release. Size and polydispersity (degree of homogeneity and dispersion of sample size) are the two most important factors when characterizing liposomes. The ideal size is between 50-200 nm and is affected by the lamellarity. Unilamellar vesicles have a single bilayer between the interior and exterior aqueous compartments and are further subdivided into small (20-100 nm), large (>100 nm), and giant (>1 μm) vesicles. If many bilayers surround the internal aqueous core, then it is considered multilamellar (>0.5 μm). If a single bilayer vesicle encapsulates many non-concentric bilayer vesicles then it is a multi-vesicular vesicle that can reach sizes greater than 1 μm .^{84,88,89}

Polydispersity index (PDI) ranges between 0 and 1, with less than 0.7 indicating size homogeneity and greater than 0.7 indicating sample size heterogeneity or possible aggregates.^{90,91} Zeta potential constitutes the liposomal surface electrical charge. During synthesis, high potential is useful because it generates strong repulsion forces that prevents aggregation. A surface potential greater than 30 mV or below -30 mV can maintain stability.^{92,93} Size, PDI, and zeta potential are all commonly measured via dynamic light scattering with a Zetasizer. Other less commonly used methods include visualizing morphology and size with an electron microscope and using gel permeation chromatography.^{94,95}

Encapsulation efficacy can be measured using a hydrophilic marker, commonly a fluorescent dye. Methods of quantification can include spectrophotometry, fluorescence spectroscopy, enzyme methods, or electrochemical techniques.⁹⁶⁻⁹⁸ Following synthesis and loading, methods of separation are employed to isolate liposomes from free drug in solution. Centrifugation and size exclusion column chromatography are two common methods.

Encapsulated drug/hydrophilic marker is released with a surfactant (e.g. Triton X100) and quantified.⁹⁴

Classical liposome models have incurred some challenges such as inefficient drug loading and release, release affected by exposure to serum proteins, low retention, and leaking of the drug preemptively. Many methods of synthesis have been developed to combat these challenges. Liposome synthesis typically employs a series of basic steps: lipid film layer produced by drying lipids dissolved in an organic solvent, dissolving the layer in an aqueous solvent, sonicating or extruding, purifying the liposomes, and analyzing the final product.⁹⁹

Formation of the bilayer is an intermediate stage and precedes liposome production.¹⁰⁰ The Bangham method of liposome synthesis involves dissolving different lipids in an organic solvent (usually chloroform) for even dispersion before evaporating the organic solvent to leave behind a lipid film. Next the lipid layer is hydrated with an aqueous solution creating thin-film hydration.⁸⁹ The formed planar bilayers are unstable due to hydrophobic hydrocarbon chains exposed to the aqueous solution. Hydrophobic interactions drive the lipids to reorient themselves to reduce exposure to the polar solvent, changing a flat layer into a disk. The “edge energy” of a disk is not in the most minimized state, so the disk will undergo more reorganization until it eventually forms a sphere. Reduction of the edge energy is a direct result of vesicle formation.¹⁰¹ You can use sonication to aid in the lipid reorganization. Generally, this basic method of formation results in multilamellar liposomes that can be reduced to large and small unilamellar vesicles via mechanical dispersion. A key component of liposome formation involves the synthesis temperature being maintained above the predicted phase transition temperature to ensure stability.

Loading the drug into the liposome can be accomplished by either a passive or active loading technique. Examples of passive loading include mechanical dispersion, solvent

dispersion, and detergent removal. Methods of mechanical dispersion include sonication, freeze-thaw, membrane extrusion, microemulsification, and dried reconstituted vesicles. Sonication for 5-10 minutes can disperse multilamellar vesicles into small unilamellar vesicles, though final size is dependent on the lipid composition.⁹⁸ Limitations of sonication involve potential contamination by the probe and heterogeneity of the sonication energy throughout the sample. Freeze-thaw cycles involve rapidly freezing and thawing the liposomes to create fusion of small unilamellar vesicles by dispersion of aggregates.¹⁰² Membrane extrusion consists of the liposomal solution being passed through a polycarbonate membrane filter, generally 0.2 μm or greater,¹⁰³ via a pump.¹⁰⁴ Micro-emulsification uses a microfluidizer to create strong shearing forces that produce small unilamellar vesicles with a maximum encapsulation efficiency of 75%.¹⁰⁵ Solvent dispersion can be completed with either ether or ethanol injections. Ether dispersion consists of dissolving the lipids in ether then injecting them into an aqueous solution at 55-65°C.¹⁰⁶ Ethanol injection is similar except it does not need the high temperature, rather, 15 minutes of stirring at room temperature.¹⁰⁷ With detergent loading, lipids are hydrated and solubilized with a detergent solution. The detergent will associate with the hydrophobic part of the phospholipids and allow mixed micelles to form. Removing the detergent causes the micelles to become unilamellar vesicles.^{108,109} The main limitation for passive loading is low efficiency, with the average encapsulation efficiency of water-soluble drugs usually at <30%. Active loading is commonly utilized for water-soluble drugs to increase trapping efficiency to close to 100%.¹¹⁰

Active loading involves using pH or ion gradients to drive the drug through the bilayer membrane. This procedure has to be completed following liposome formation using either a hypertonic aqueous solvent or an inorganic salt. The inorganic salt, like ammonium sulfate, will dissociate to ammonia and a proton. The inorganic portion (in this case ammonia) can diffuse out

of the liposome, leaving behind the proton to lower the interior pH. A weak-base drug should ionize with the free protons and become trapped inside the liposome.^{111,112}

There are three main problems associated with the classical liposome model. The first is inefficient drug loading and release. Drug loading has poor encapsulation efficiency and low retention (i.e. leaking). Loading above the solubility limit of the drug and using pH gradients can improve encapsulation efficiency. The benefits of pH loading are that it is done remotely, independent of the site and time of liposome production. Drug release can also be affected by exposure to serum proteins and lipoproteins. Phospholipid molecules are transferred to high-density lipoproteins (HDL) via signals from phospholipid transfer proteins in the serum. Interactions with these proteins can result in the release of liposomal contents. Lipid composition can be altered to prevent the transfer of phospholipids to HDL.^{113,114} Incorporation of cholesterol into the membrane reduces the number of phospholipids transferred by “tightening” the bilayer, prolonging the stability of the lipid membrane.^{115,116} Egg phosphatidylcholine (EPC) has a similar effect as cholesterol by preventing the transition of phospholipids into HDL. EPC also creates a high transition temperature making the vesicle more stable.¹¹⁷ Phospholipids with saturated fatty acyl chains like distearoylphosphatidylcholine (DSPC) prevent HDL transition, eliminating the solute release in plasma.^{118,119}

The second challenge with the classical liposome model is rapid clearance by the mononuclear phagocyte system (MPS), especially within the liver and spleen. Within the liver, liposomes escape the endothelium via fenestrations and are then entrapped by resident macrophages in the parenchyma.¹²⁰ Studies with labeled liposome-encapsulated bleomycin have shown that encapsulated substances are retained where there is liposomal decay, therefore, distribution of the encapsulated substance depends entirely on the distribution of the liposomes.¹²¹ Selective toxicity of the reticuloendothelial system (RES) organs like the liver and

spleen occurs due to rapid clearance by opsonization in the plasma and uptake by the resident macrophages of those organs. RES clearance has shown improvement with administration of a loading dose consisting of empty liposomes to overwhelm the MPS/RES system before introducing loaded liposomes.¹²² Further development of the liposome model led to the creation of “stealth” liposomes where PEG is added to the surface. Two or three water molecules bind to the surface of the liposome for every PEG unit. This creates a hydrophilic shield that helps protect from enzymatic digestion and immune recognition.^{123,124} It also increases the size of the liposome to decrease renal clearance.^{125,126} With these new PEG polymers incorporated onto the surface, there is no need for a MPS blockage prior to administration.

Lastly, the third problem with the classical liposome model is ensuring intracellular delivery of the drug cargo across the cell membrane. Drugs that are hydrophobic weak bases (such as doxorubicin or vincristine) can enter cells as free drug via passive diffusion down their concentration gradient in the uncharged form, while small hydrophilic drugs can undergo active transport into the cells. Delivery of the liposome near the targeted tissue and release of the drug in its free form at a therapeutic concentration will result in activity with these drugs.¹²⁷ Some recently developed drugs cannot cross the cell membrane due to their large, hydrophilic character and instead need integration of the entire liposome into the host cell. Endocytosis of liposomes can be performed if the liposome contains fusogenic lipids or membrane active peptides to promote fusion to the host cell and delivery of the payload to the host cell’s cytoplasm.¹²⁸ This method is challenging and not commonly used. Instead, receptor-mediated endocytosis of ligand-targeted liposomes (LTLs) into the endosomal-lysosomal compartment is more commonly adapted. Ligand-targeted liposomes trigger endocytosis via binding with a host cell receptor. Antibodies initially were used to initiate host cell binding, but antibody-bound liposomes

experienced faster clearance until the development of PEGylated liposomes with antibodies conjugated to the distal end of PEG.^{129,130}

Liposome targeting can be divided into passive and active types. Passive targeting exploits the structural features of tumor tissue and accumulates in the diseased tissue via the permeation and retention effect. The permeation and retention effect refers to tumor vasculature endothelium having increased permeability due to deformed tight junctions causing retention of macromolecules with lack of clearance from lymphatics. This causes accumulation of liposomes in tumor tissue.¹³¹

Active targeting selects for cell binding and receptor-mediated endocytosis. Ligand-targeted liposomes are one example of active targeting, though often there is no obvious difference between ligand-targeted liposomes and passive targeting liposomes due to non-internalization, premature leakage of contents, poor antigen density, binding site barriers, or poor penetrability.¹²⁷ Specific receptors that have been the subject of receptor-mediated targeting include: folate receptors expressed by many types of cancer,¹³² transferrin receptors expressed by many malignant cancers,¹³³ CD-44 receptor expressed during inflammation and commonly during tumor growth,¹³⁴ etc.

Liposome development has progressed from targeted liposomes to triggerable liposomes. A new generation of “triggerable” liposomes include redox-sensitive, temperature-sensitive, enzyme-sensitive, phospholipase pro-drug activation, pH-sensitive, and ultrasound-responsive.¹³⁵ Liposomal structure is influenced by surface charge. Electron-transfer reactions can change the charge and hydrophilicity of the liposome by altering the molecular structure causing disassembly and drug release. These redox reactions can be initiated in the presence of tripeptide glutathione (TGE), a substance that is in higher concentrations in tumor cells than in normal physiological conditions.¹³⁶ Liposomes can also be designed to be susceptible to certain enzymes

that may be found in higher concentrations in diseased tissue, for example, proteases.

Phospholipase A2 is a commonly targeted enzyme because it is overexpressed in prostate tumors. Phospholipase can be used to trigger payload release or activate pro-drugs.^{137,138} pH-sensitive liposomes are triggered by a decrease in environmental pH. Specialized bonds or polymers are used in liposome development to create the pH sensitivity.¹³⁹

Thermosensitive liposomes transition from a stable (gel) state to an unstable (liquid) state in the tissue of interest when exposed to its phase transition temperature. Hyperthermia is often employed in the tissue of interest because it leads to liposome conformational changes and increases vascular permeability while being directly cytotoxic to tumor tissue.¹⁴⁰ This method may only have clinical utility if it is non-invasive and able to target one tissue type, minimizing collateral damage to other organs. Various modalities have been developed including microwaves, radio waves, and ultrasound.

Lipid composition can greatly affect the liposome phase transition temperature. Incorporation of small amounts of water-soluble lysolipids like monopalmitoylphosphatidylcholine (MPPC) or monostearoylphosphatidylcholine (MSPC) into dipalmitoylphosphatidylcholine (DPPC) liposomes can decrease the phase transition temperature from 42°C to 40.5°C (MPPC) or 41.3°C (MSPC).¹⁴¹ These altered DPPC liposomes also have an increase in the amount and rate of release of entrapped contents at the phase transition temperature. Other lipid supplementation can include distearoylphosphatidylcholine (DSPC) and hydrogenated soy phosphocholine (HSPC) to also increase the rate and amount of drug release.^{142–144} Mixing lipid types can create a packing incompatibility that will result in increased permeability. Polymer-modified liposomes are formulated with temperature sensitive polymers that become membrane disruptive and promote drug release when exposed to either the lower critical solution temperature or an upper critical solution temperature of the polymer.¹⁴⁵

Thermosensitive liposomes typically are made with PEG to prolong plasma circulation and with cholesterol to increase serum stability, although the latter has been shown to decrease liposome thermosensitivity, leading to low release even at moderately high (43°C) temperatures.¹⁴³

Based on this information, thermosensitive liposomes loaded with gemcitabine by pH trapping with ammonium sulfate could be an effective treatment for pancreatic ductal adenocarcinoma when paired with a targeted hyperthermia treatment such as radiofrequency hyperthermia. Theoretically, such liposomes would have a high loading efficiency, targeted delivery, and high payload delivery to tissue that is being heated.

Chapter 4 - Liposome integration into treatment for pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal malignancy that is the third leading cause of cancer death in the US. The 5-year survival rate is only 10%.¹⁴⁶ It is proposed that by 2030 PDAC will be the leading cause of cancer-related death due to advancements in lung and breast cancer treatment.¹⁴⁷ There are many risk factors for PDAC, with genetic conditions including hereditary breast and ovarian cancer syndrome, lynch syndrome, familial adenomatous polyposis, multiple melanoma syndrome, hereditary pancreatitis, cystic fibrosis, and ataxia telangiectasia.^{148–151} Modifiable risk factors include tobacco usage, obesity, and prolonged and excessive alcohol usage.^{152,153}

Late detection due to clinical silence is one of the main reasons the majority of patients are diagnosed at an advanced stage. Surgical resection can increase the 5-year survival rate to 25%, but many patients do not qualify because of dissemination or locally advanced disease. Patient management can vary depending on CT results, patient performance status, symptom burden, and co-morbidity profile.¹⁵⁴ For metastatic disease, treatment is heavily based on performance status scoring. Those who have minimal clinical symptoms will receive gemcitabine plus nab-paclitaxel or folfirinox. Patients with mild clinical symptoms will receive gemcitabine as a monotherapy.^{155,156}

Gemcitabine is a pro-drug that requires cellular uptake and intracellular phosphorylation for effectiveness. The main metabolites include gemcitabine diphosphate, triphosphate, and the terminal complex dFdCTP/DNA. Gemcitabine diphosphate inhibits ribonucleotide reductase, resulting in the depletion of the deoxyribonucleotide pool useful in DNA synthesis.¹⁵⁷ Gemcitabine triphosphate binds to DNA polymerase causing termination of the DNA helix.

Lastly, the terminal complex can induce apoptosis. Gemcitabine acts between the G1 and S phase of the cell cycle preventing cell proliferation.¹⁵⁸

When gemcitabine is indicated, it is given intravenously in four-week cycles. The cycles consist of three once-weekly infusions followed by a week off before undergoing another cycle.¹⁵⁴ The addition of nab-paclitaxel improves the efficacy of gemcitabine,¹⁵⁹ but exacerbates some of the side effects such as neutropenia, febrile neutropenia, fatigue, and neuropathy. Non-hematological toxicities include edema, cutaneous toxicity (rash, pruritus), fever, pulmonary toxicity, nausea, vomiting, and GI upset.¹⁶⁰ Gemcitabine's short plasma half-life results in large volumes needed to elicit an anti-tumor effect but consequently results in systemic toxicity and undesirable side effects. Clinical usage of a targeted drug delivery system, like thermosensitive liposomes, may reduce systemic side effects by initiating release of the anti-cancer agent only in the tumor vasculature.

Expanding on the concept of thermosensitive liposomes and targeted drug delivery, we investigated the effect of gemcitabine-loaded thermosensitive liposomes and heat treatment on KPC and BXPC-3 cells in culture. This study focused on characterizing the efficiency and release of encapsulated gemcitabine via triggering the phase transition temperature of the liposome with various heat modalities. We hypothesize that at 24- and 48-hours post-heat treatment the cells treated with liposomes will have lower viability than the cells that were not treated with liposomes. This study was comprised of three aims, one of which has yet to be completed. The first two aims were designed to define a liposome composition with an optional phase transition temperature. First, a liposome model was developed with defined encapsulation efficiency and percent release of gemcitabine. Next, the effects of temperature-released gemcitabine were applied in vitro on pancreatic cancer cell lines and cell viability was measured.

For the first aim, liposomes were synthesized with carboxyfluorescein to determine percent release from a given liposome formulation. Liposome synthesis was completed by another graduate student using the classical method. Carboxyfluorescein liposome synthesis involved mixing 500 μ l of an organic solvent (e.g. chloroform) with various lipids in a predetermined ratio. For example, 60:35:5 DSPC, DPPC, and PEG. For the entire synthesis procedure, it is crucial that the solution temperature is maintained approximately 5-10°C above the expected phase transition temperature to ensure that the liposomes are always in their liquid (crystalline) phase. Once mixed, the organic solvent is vacuum evaporated. Once dried, the lipid film is rehydrated with 6-carboxyfluorescein. The vial is vortexed until the solution is a milky consistency (about 10-30 seconds) and dark red or orange in color. Rehydration of the lipid layer generally results in multi-lamellar vesicles. The encapsulation efficiency of MLV is generally too small for clinical application. Freeze-thaw cycles can increase the encapsulation efficiency by decreasing the lamellarity, creating large and small unilamellar vesicles. The glass vial is passed between a water bath set to 45 °C and liquid nitrogen ten times with one minute of complete submersion, sometimes less for liquid nitrogen. To obtain homogenous unilamellar vesicles, the liposome solution is placed in a glass syringe within an extrusion system that is suspended in a hot water bath. The solution is pushed through the 100nm pore diameter polycarbonate membrane filter ten times. Following extrusion, the solution contains large unilamellar vesicles.

To remove unencapsulated contents, the liposome solution is passed through size-exclusion chromatography filtration using Sphadex G-10 medium. A sample from the liposome fraction is subjected to dynamic light scattering with a Zetasizer (Malvern Panalytical, Westborough, MA) to determine size and distribution. Liposome stability is determined by heating liposomes to 37°C, 39°C, 41°C, 43°C, 45°C, 47°C, or 49°C in water baths for 5, 10, and

15 minutes at each temperature. Fluorescence was measured following incubation before total release was induced with Triton X and percent release was calculated (Figure 4.1).

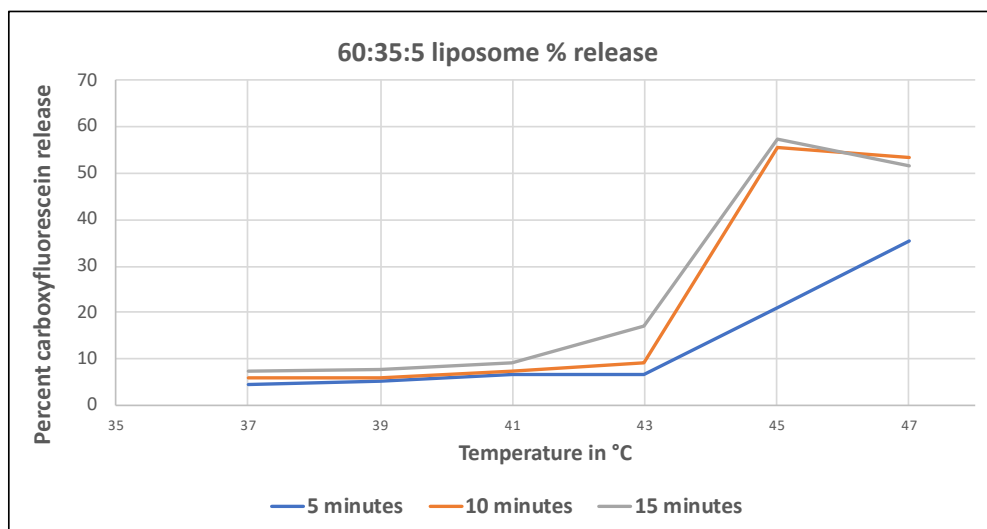


Figure 4.1 Percent release of carboxyfluorescein at various temperatures from liposomes made with a 60:35:5 DSPC, DPPC, and PEG ratio submerged at each temperature for either 5 minutes, 10 minutes, or 15 minutes.

The second aim was to analyze the ability of thermosensitive liposomes to deliver a payload to pancreatic cancer cells under hyperthermic conditions. Liposomes were synthesized similar to the previous study with lipid film hydration, freeze-thaw cycles, and extrusion. The lipid film was rehydrated with phosphate-buffered saline (PBS) and 250 mM of ammonium sulfate instead of 6-carboxyfluorescein. Once large unilamellar liposomes are made, the excess ammonium sulfate will be removed with ultra-centrifugation at 14000 rpm, 4°C for 1 hour. The supernatant was removed, and the liposomes were remotely loaded via suspension in 1 mM gemcitabine and incubation at room temperature for 3 hours. Gemcitabine is a weak base and will diffuse into the liposome along its concentration gradient in an un-ionized form. The ammonium sulfate within the liposome dissociates to ammonia and a proton. The ammonia diffuses out and the proton remains within the liposome. When gemcitabine diffuses into the

liposome it will become ionized and will not be able to diffuse out of the liposome, trapping it. Non-encapsulated gemcitabine is removed via ultracentrifugation. Liposome size distribution, again, was measured via dynamic light scattering and liposomes were incubated at the same temperatures and times as the last study. Following treatment, liposomes were removed with centrifugation and gemcitabine release within the supernatant was characterized by absorbance (269 nm). The remaining liposomes were disrupted with Triton X to obtain 100% release.

The third aim of this project was to treat two pancreatic adenocarcinoma cell lines, BxPC-3 (human PDAC cell line) and KPC (mouse PDAC cell line), with gemcitabine-loaded thermosensitive liposomes at various temperatures and evaluate cell viability. My contribution to this project involved cell culture and setup and processing of the plates. The KPC cell line was grown in culture medium consisting of 89% Dulbecco's Modified Eagle Medium (DMEM) high glucose, 1% penicillin-streptomycin, and 10% fetal bovine serum. The BxPC-3 cell line was grown in culture medium consisting of 89% RPMI + pyruvate, 1% penicillin-streptomycin, and 10% fetal bovine serum. Cells were cultured to 80% confluency and split in a 1:2 density reduction. To subculture, phosphate buffered saline (PBS) was utilized to rinse the plate and trypsin-EDTA was utilized for lifting adhered cells off of the plate surface. The KPC cell line is faster growing and was seeded at a density of 50,000 cells/cm² per well. The BxPC-3 cell line is slower growing and was seeded at a density of 80,000 cells per well. The cell lines were seeded in triplicates with a control group for each cell line and treatment group that did not receive liposomes. The KPC and BxPC-3 control and treatment wells were duplicated on each plate to provide 24 and 48-hour treatment groups (Figure 4.2).

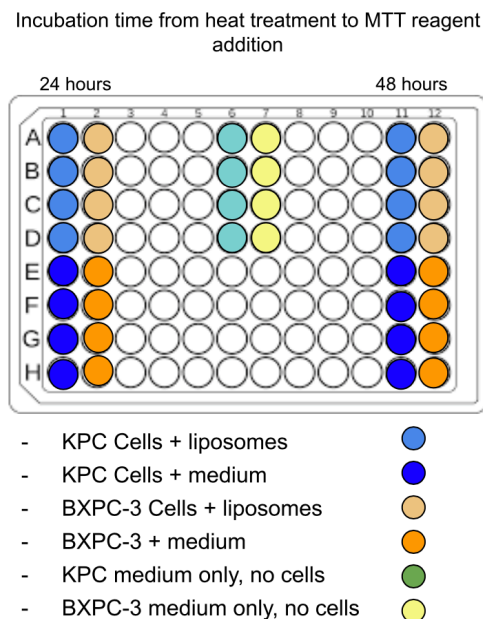


Figure 4.2 Example of plate layout on 96-well plate.

Gemcitabine-loaded liposomes synthesized by another graduate had a composition of 60:35:5 DSPC, DPPC, PEG, and a determined phase transition temperature of approximately 44°C. Each cell line was subjected to rapid heating in a water bath reaching temperatures of 37°C, 39°C, 41°C, 43°C and 49°C, maintaining each temperature for 10, 20, 30, and 60 minutes. Each temperature treatment group had 4 plates, one for each time allotment for heat treatment. The internal temperature of the wells was monitored in real-time by fiber-optic thermocouples placed on an empty 96-well plate that underwent the same heat treatments as the experimental plates (Figure 4.3). The plates were initially subjected to rapid heating in a 70°C water bath until they reached 2 degrees below the treatment temperature to mimic the rapid heating of microwave hyperthermia. Once the plates reached 2 degrees below target temperature, they were transferred to a water bath at the target experimental temperature where they inoculated for the predetermined duration (10, 20, 30, and 60 minutes). Two hours post heat-treatment, the liposome and media mixture was removed and fresh media was distributed. The plates were

cultured for either 24 or 48 hours before MTT reagent (thiazolyl blue) was added. If the cells in the well were viable, they would contain NADPH oxidoreductase enzymes that will reduce MTT to formazan crystals. Four hours after application of the reagent the crystals were dissolved with an MTT solubilizing solution (10% SDS in 0.01M HCL) and were read via spectrophotometry 550 nM. The crystals formed by the NDAPH oxidoreductase enzyme are purple in color. The darker the purple solution following crystal dissolution, the higher quantity of metabolically active cells.

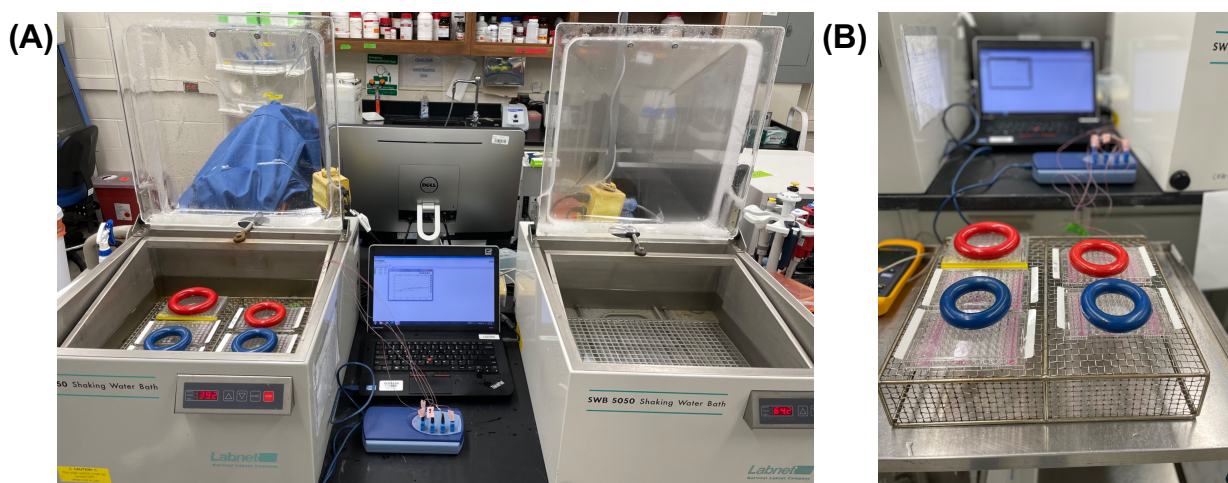


Figure 4.3 Example of heat treatment set up.

An analysis of variance (ANOVA) test and a Tukey's Honest Significant Difference post-hoc test were performed to compare the treatment groups (+/- liposomes at each temperature) within each heating time category (10, 20, 30, or 60 minutes). For both the KPC and BXPC-3 cell lines, there is no significant difference between the group treated with liposomes and the group without liposomes at 41°C and 43°C for all time points. This can suggest that the heat begins killing cells at around 41°C negating the effects of the liposomes. The 20- and 60-minute time categories were significantly different only between the group treated with liposomes and

the group without liposomes at 39°C for both cell lines. The 10- and 30-minute time categories were significantly different between the two treatments groups at 37°C and 39°C for the KPC cell line, and only 39°C for the BxPC-3 cell line (Figure 4.4). Overall, these weak but preliminary data demonstrate that temperature-triggered release of gemcitabine has a negative impact on cell viability as shown by the significantly lower viability of the treatment groups as compared to control groups at 24 hours post-heat treatment of both cell lines.

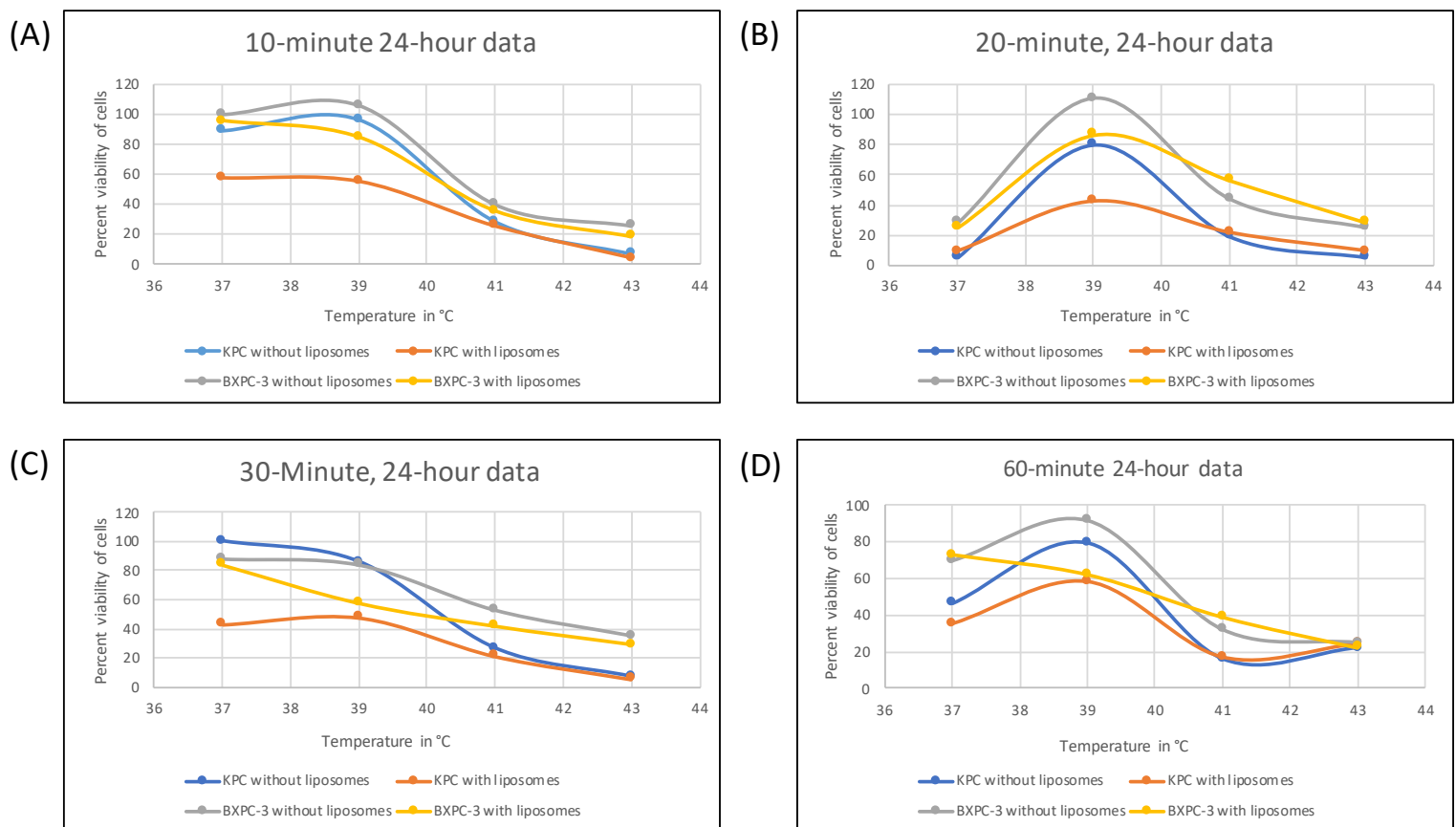


Figure 4.4 Percent viability of KPC and BxPC-3 cells following heat treatment for various amounts of time at increasing temperatures.

These data suggest that gemcitabine-loaded thermosensitive liposomes can release their payload when heated close to their phase transition temperature. The cytotoxic effects of the heat-triggered gemcitabine release can first be seen at 39°C as compared to non-heated samples at 37°C at every time point in the BxPC-3 model and perhaps the 20-minute time point in the KPC model. While these data are preliminary and weak, they demonstrate proof of concept. The high toxicity of the liposomes in the KPC model at 37°C may suggest that these initial studies were performed with too high a concentration of gemcitabine leading to excessive passive release and uptake without heat treatment. In addition, the difference in phase transition temperature of the carboxyfluorescein liposomes and the maximum cellular toxicity may indicate that the heat treatment is too cytotoxic and needs to be reduced. These preliminary data are being used to inform continuing work on this project.

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