

RESEARCH PLAN

Introduction: Response to prior review of 1 R01 EB 000484-01A1

This is a revised NIH R01 proposal for studies to improve minimally invasive thermal ablation by combining radiofrequency with liposomal doxorubicin. In this revision, we have responded to the concerns raised by the reviewers. We thank the reviewers for their suggestions and based upon these insights the proposal is substantially improved. We now respond to the issues raised in the summary statement with emphasis on the improvements in the project. Changes and additions in text have been marked with a bar in the right margin.

The focus of our laboratory has been to rapidly translate basic scientific advances in the field of high-temperature, image-guided tumor ablation into clinical practice. In our experience, this has been accomplished most expeditiously by balancing mechanistic approaches with empirical optimization studies. Although our former revised proposal was more geared to rapid clinical implementation, based upon the advice of the reviewers we have elected to perform “more studies to better understand the basic mechanisms driving the RF-Doxil synergy and to characterize the normal tissue damage”. As outlined below, we cite new preliminary data from our ongoing studies and the experiments outlined in new Aim 2 as an expression of our commitment to improved understanding of the underlying mechanisms of the combined RF-liposomal doxorubicin paradigm. This work was alluded to in the introduction to our previous revised proposal, where we had proposed clinical study first, followed by a second grant proposal on mechanism. After careful consideration, we realize that further pre-clinical study combining optimization and mechanistic study is warranted at this time. We wish to stress the importance of our empirical study that serendipitously identified the unanticipated enhanced treatment effect of empty liposomes, which as the reviewers point out also merits further mechanistic study. We also reiterate our long-standing commitment to patient safety in our new Aim 3 using animal models to better address the clinical concerns that the reviewers raised.

Our preliminary results demonstrate improved tumor destruction when combining RF ablation with liposomal doxorubicin in multiple tumor types and tissues. Prior to translation into the clinic, further optimization of the parameters known to influence this interaction and better characterization of local and systemic toxicity are warranted. These form the basis of Specific Aims 1 and 3, respectively. Our ***underlying hypothesis*** is that increased tumor destruction is due to interaction between the liposomal doxorubicin and sub-lethal hyperthermic temperatures that do not directly induce coagulation ($\approx 45 - 50^{\circ}\text{C}$). Characterization of thermal dosimetry, as outlined in Specific Aim 1, Experiment 3 is important to assess this hypothesis, and may potentially shed additional light on the underlying mechanisms of this process. Preliminary data outlined in Section C and prior work outlined in Section B suggest that multiple interactions account for increased tumor destruction. These include the effects of the increased intratumoral doxorubicin (Section C.5) and the effects of the liposome carrier itself (Section C.6). Experiment 2 of Aim 1 is designed to provide additional data in regards to our hypothesis that increased tumor destruction is due in part to increased delivery of the drug in the liposomal form to RF ablated tissue. Specific Aim 2 is specifically directed to testing mechanistic hypotheses as to why empty liposomes increase tumor destruction including enhanced lipid peroxidation. The experiments of Specific Aims 1 and 2, when analyzed in combination, are designed to determine the relative contribution of the doxorubicin and liposome components to the overall efficacy of the system. Thus, as requested by the reviewers, at the conclusion of the proposed project, we should be able to explain why ‘liposome encapsulation of doxorubicin is essential for the observed synergy’ between RF and Doxil. This information will be used to determine the optimal agent to be used for future study in clinical practice. Thus, the proposed experiments represent the necessary next steps in the development of RF tumor ablation, as they will permit the design of a scientifically robust clinical trial that takes into account all the improvements in technique learned from the proposed studies.

1. Improved study of hyperthermia and thermal-based parameters.

Additional attention is now given to the concept of hyperthermic dosing and the clinical need for understanding thermal dosimetry (see Section B). We now propose the formal calculation of thermal dosimetry for both the liposomal RF-mediated increase in tumor destruction and RF coagulation zones. We will express thermal dosimetry in terms of equivalent minutes at 43°C , the standard measure for hyperthermia, which will enable us to determine the utility of this parameter for high-temperature ablation. Additional thermal dosing models for high-temperature processes (i.e., RF induced coagulation) will be calculated, as appropriate.

We also further clarify the difference between RF ablation parameters (i.e., temperatures at the tip of the electrode and duration of RF application) and “thermal dosimetry parameters” (the duration of heating within the tumor). As a reviewer points out, these two are not one and the same, as RF heating is applied using a point source which necessitates thermal conduction throughout the heated zone. Specifically, the hyperthermic volume (i.e., the volume reaching sub-lethal temperatures/thermal doses at which there is liposomal doxorubicin mediated toxicity) and the ultimate coagulation volume of ablated tissue following combined RF and IV liposomal doxorubicin [versus RF alone or RF combined with IV liposomal control] will be characterized as a function of thermal dose delivered both inside and outside of the RF ablated tissue, not based solely on thermal dose at the electrode tip (Experiment 3).

Along these lines, we also agree that given the importance of interaction of the drug at hyperthermic temperatures, our thermal mapping should measure temperatures beyond the ablation volume. As such, we now propose the placement of multiple thermal sensors at 5-mm intervals from the tip of the electrode outward to the edge of the tumor and into surrounding tissues, to 5 cm. We specifically note that our goal is to capture thermal data for all points at which temperature is elevated. In addition, we have improved our proposed thermal monitoring by placing a thermal sensor perpendicular to the midpoint of the electrode, which will measure a continuous temperature profile throughout the ablation volume at defined time intervals. This will enable obtaining a more refined thermal map of heating throughout the entire tumor, including hyperthermic temperatures and will enable a stronger test of the relationship between thermal dose delivered to a defined region of tissue and drug accumulation in that sample (Experiment 3).

In order to strengthen our plan for elucidation of the role of hyperthermia, we have enlisted the help of Dr. Mark Dewhirst, a well-known expert in hyperthermia to serve as a consultant for this project. Dr. Dewhirst has a program project grant in hyperthermia and extensive experience studying liposomal doxorubicin and hyperthermia. He has assisted us with this amended proposal and will continue to provide consultation throughout the project (see letter).

2. Formulation of Specific Aims.

As requested, the prior first three Specific Aims are now presented as one stronger, cohesive focused aim. We agree that former Aim 2, “Increasing Intratumoral Liposomal Doxorubicin Accumulation Using RF Ablation,” and former Aim 3, “Characterizing Thermal Parameters that Increase Liposomal Uptake,” can be reorganized as sub-aims of Specific Aim 1, “Improving Tumor Destruction Using Combined RF Ablation and IV Liposomal Doxorubicin.”

As noted by one reviewer, Specific Aim 4, a clinical trial, was proposed in the 01A1 application in response to the study section critique of the original 01 application. Nevertheless, we agree with the second set of reviewers that the better approach will be to defer the clinical study until we can better characterize the response of normal tissue to combined RF and liposomal doxorubicin (below #4) and better understand the basic mechanisms that drive the RF-liposomal doxorubicin synergy, including the role of the liposome (below #3). Hence, we have eliminated prior Specific Aim 4.

3. Characterization of the effects of RF ablation combined with empty liposomes alone.

We concur with the reviewers that a substantial portion of the potential synergy between RF and liposomal doxorubicin may be attributable to the role of “empty” liposomes. As suggested by the reviewers, in addition to studying the potential mechanisms of hyperthermia and increased doxorubicin accumulation, we now propose a set of experiments as new Specific Aim 2 to test hypotheses about the role of liposomes as they interact with RF ablation and hyperthermia. In the proposed studies we will also compare outcomes between three test conditions – *RF alone*, *RF plus liposomes containing Doxil*, and *RF plus liposomes containing saline* – and we will attempt to explain differences between the latter two conditions, as well as differences from *RF alone*.

First, we will study RF combined with saline-filled liposomes, including the determination of the parameters and thermal dosimetry at which interaction between RF and empty liposomes occur (Experiment 4). This is important as it will enable us to gain an appreciation towards the underlying mechanism based upon prior published studies in hyperthermia. For our initial experiments, we will use liposomes without doxorubicin at the same doses and RF parameters as the Simplex Optimization from Specific Aim 1, where doxorubicin is to be used. This will enable us to use the doxorubicin uptake of the first set of experiments to determine actual intratumoral liposome doses, and permit direct comparison between the efficacy of RF and liposomal doxorubicin vs empty liposomes alone. Experiments will also be performed, as suggested, to determine the degree of dose dependence for the liposomes.

The reviewers have also suggested additional studies to define the underlying mechanisms behind the interaction between the liposomes and RF ablation. We now provide new data from our ongoing

mechanistic studies suggesting that this enhanced response in the aftermath of thermal ablation could be due to increased oxidative stress caused by increases in lipid peroxidation due to the unsaturated fatty acids in the liposomes. We now show increases in endpoints indicative of lipid peroxidation in ablated tumors as supporting evidence for this line of investigation [Section C.6]. Accordingly, we now propose a set of experiments to determine the potential role of this underlying mechanism in increasing tumor destruction. These include the administration of antioxidants such as vitamin C and/or vitamin E as “inhibitors” of lipid peroxidation chain reactions to determine if a reduced response is observed as well as thiol based enhancers and inhibitors of lipid peroxidation cytotoxicity (Experiment 5a). We will also combine RF ablation with lipids of greater oxidative potential (GLA, arachidonic acid) to determine whether this mechanism can be further potentiated. We will further attempt to define this phenomenon by correlating tumor destruction with measurements of endpoints indicative of lipid peroxidation (Experiment 5b). Other, less likely explanations for the increased activity of empty liposomes such as toxicity of highly concentrated intratumoral PEG accumulation are mentioned under Future Studies in Section D, Aim 2 and will be tested, as appropriate.

In order to strengthen our plan for elucidation of the role of lipid peroxidation in these processes, we have enlisted the help of Dr. Douglas R. Spitz, a well-known independent investigator in this field who co-directs the Antioxidant Enzyme Core Laboratory at the Free Radical and Radiation Biology Program in the Department of Radiation Oncology at the University of Iowa, to serve as a consultant for this project. He has assisted us with this amended proposal and will continue to provide advice throughout the project (see letter).

4. Characterization of normal tissue damage

In response to queries and concerns of two reviewers, we have clarified the rationale behind the current clinical practice of destroying an ablative margin of apparently normal tissue surrounding the ablated tumor [Section B.3 and Experiment 7 rationale]. Further literature is now cited confirming that, from the perspective of those performing RF ablation, destruction of 5 – 10 mm of apparently normal tissue surrounding the tumor is viewed in many circumstances as a desired end point of therapy. We also provide new preliminary data showing that the RF combined with liposomal doxorubicin treatment can extend tissue destruction several millimeters in normal tissues in the small-animal model [Section C.2g]. We therefore fully agree that characterization of normal tissue damage (i.e., determination of the thermal doses at which cytotoxicity occurs in normal tissues), using clinically relevant large-size animal models, is warranted to make this procedure safer for eventual use in people. Thus, we now propose (new Aim 3, Experiment 6) to determine the thermal dosimetry at which the desired extent of liposomal doxorubicin mediated tissue destruction occurs in normal liver and kidney parenchyma, the two organs for which RF ablation is most commonly performed.

5. Quantification of potential treatment complications.

Given the concern about potential local or systemic toxicity, we now also propose a one-month survival study in which animals receiving the optimal liposomal doxorubicin RF doses will be monitored for hemorrhage within the treatment site and for systemic toxicity (new Aim 3, Experiment 8). This will include monitoring of hematologic, renal and hepatic blood tests, as well as CT and pathologic examination of ablated tissues.

6. Approach for optimizing combination RF ablation/liposomal doxorubicin therapy

Based on the recommendations of one reviewer, we have changed our approach of Simplex optimization from large Koshul design (i.e., constructing the initial Simplex with wide mapping) to a small-step design where changes are made about the likely set of parameters to achieve maximum effect. This approach will enable us to determine a set of clinical treatment parameters having maximal effect. It also reduces the number of iterations required for the Simplex optimization (Exp. 1) to 15 – 25 parameters (from 25 – 30).

We have also eliminated large scale mapping of RF parameters in favor of a more focused approach of determining thermal dose (see point 1, above). We agree (based upon new Monte Carlo simulations), that if this approach is taken the number of proposed additional parameters for defining thermal dosimetry (Experiment 3) can be decreased from 32 to ~16 parameters.

7. Animal Usage and choice of subcutaneous animal model for initial studies

Based upon the streamlining outlined in point 6, we have reduced the number of animals in Specific Aim 1 from the previously proposed 130 to 78 dogs. This enables us to reduce the timeline of Aim 1 from three to two years. On the other hand, use of a kidney (or liver) model throughout would increase animal

utilization 5-fold and costs by \$200K each year. Our proposed approach using subcutaneous tumors for initial studies is based upon our ability to readily implant multiple tumors that can be used in a straightforward efficient manner. This high yield and ease of handling is justified for initial optimization. Then, optimized RF and drug parameters can be used in the renal tumor model for the proposed survival / complication study. Although our more efficient approach to defining the interaction between liposomal doxorubicin and RF ablation has enabled a reduction in the number of animals used for Aim 1, the number of animals has increased overall to account for the additional experimental and control groups that have been added in response to the recommendations of the reviewers for the new Aims 2 & 3.

A. SPECIFIC AIMS :

Minimally-invasive, image-guided radiofrequency (RF) tumor ablation (i.e., coagulating tumor using short duration heating [< 15 min] by directly applying temperatures $> 50^{\circ}\text{C}$ via needle electrodes) is being used to treat focal liver, renal, breast, bone, and lung tumors. Potential benefits of this thermal therapy include reduced morbidity and mortality compared to standard surgical resection and ability to treat non-surgical patients. However, clinicians have been unable to achieve complete ablation in many cases, particularly at tumor margins and adjacent to blood vessels, presenting substantial barriers toward clinical efficacy.

Our preliminary animal studies taking advantage of complementary interactions between RF ablation and IV-administered liposomal (lipid encapsulated) doxorubicin to produce more complete tumor destruction, demonstrated that combination therapy markedly increases tumor destruction and animal survival compared to RF alone, or RF combined with unencapsulated doxorubicin. RF ablation also increased intratumoral accumulation of liposomal doxorubicin seven fold compared to controls, particularly in a peripheral region immediately adjacent to the zone of RF-induced coagulation. Additionally, in our randomized, pilot clinical study on 10 patients with primary and metastatic liver tumors, combined RF ablation and IV liposomal doxorubicin (albeit, non-optimized) increased the volume of tumor destruction 25 – 30%, improved ablation of tumor margins, and eliminated residual tumor vascularity compared to RF alone. Thus, this combined treatment paradigm has the unique potential both to potentiate preferential delivery of cytotoxic agents in liposomal vehicles, and to maximize the completeness of ablation of a treated tumor, in humans.

Several issues require further study before translation of this paradigm to clinical practice. First, our data indicate that gains in both drug uptake and tumor destruction can be further increased through the systematic optimization of the relevant treatment parameters. Second, the mechanisms driving this interaction particularly the hyperthermic dosimetry of increased tissue destruction and the causes for increased tumor destruction seen when empty liposomes are given without doxorubicin need to be better understood, as this knowledge could lead to developing clinical strategies having even greater efficacy. Last, given data showing that combined therapy can extend into surrounding tissues, local and systemic toxicities need to be better defined. Our immediate goals are therefore to address these current concerns prior to initiation of larger clinical trials.

Specific Aim 1. *Improve tumor destruction* using combined RF ablation and IV liposomal doxorubicin

- a) Optimize RF ablation parameters and liposomal doxorubicin pharmacokinetics (timing and dose) that effect tumor coagulation using simplex optimization in canine venereal sarcoma (CVS). This will define maximum gains in tumor destruction that can be achieved in an appropriately scaled large animal model.
- b) Quantify the amount of doxorubicin in RF ablated CVS tumors using spectrophotometry.
- c) Characterize the relationship between the directly measured intratumoral thermal dosimetry with increased doxorubicin accumulation, RF induced coagulation, and liposomal doxorubicin mediated increased tumor destruction. This will elucidate the role of hyperthermia in the combined interactions, the threshold thermal dosage, and the thermal conditions under which combined therapy is most effective.

Specific Aim 2: *Test specific hypotheses about the possible role of the liposome carrier components*

- a) Compare the thermal dosimetry at which increased tumor destruction occurs with RF and empty liposomes to that of RF and liposomal doxorubicin. This will define the relative contributions of the lipids and of the doxorubicin to the mechanisms of cell-killing by the treatment paradigm combined with RF hyperthermia.
- b) Test the possibility that increased tumor destruction can be ascribed to *enhanced oxidative stress and lipid peroxidation*. We will compare endpoints indicative of oxidative stress and lipid peroxidation with tumor destruction following administration of liposomal lipids in the presence and absence of inhibitors and enhancers of lipid peroxidation to try and establish a causal link between these phenomena.
- c) Combine RF ablation with liposomes containing fatty acids having greater oxidative potential (GLA, arachidonic acid) to determine whether manipulation of the substrate upon which lipid peroxidation occurs can further potentiate the antitumor effect of thermal ablation.

Specific Aim 3. *Characterize potential local and systemic treatment toxicity*

- a) Determine the thermal dosimetry at which liposomal doxorubicin tissue destruction occurs in normal canine liver and kidney parenchyma. A peripheral rim of these *difficult to treat* tissues must be ablated to ensure adequate clinical destruction of hepatic and renal tumors.
- b) Perform a one-month survival study in CVS renal tumors receiving the optimal liposomal preparation and RF ablation parameters. Animals will be monitored for local complications such as hemorrhage at the treatment site and systemic toxicity including monitoring of hematologic, renal, and hepatic blood tests.

B. BACKGROUND AND SIGNIFICANCE:

1. Overview of RF thermal ablation therapy as a treatment modality for focal malignancies:

Traditionally, local tumor removal has required major surgery. Over the last decade, improvements in imaging technologies have enabled the development of minimally-invasive thermal tumor ablation that uses imaging guidance for the accurate percutaneous placement of needle-like applicators [1, 2]. Tumor destruction for these methods relies primarily upon subjecting the entire tumor volume to cytotoxic temperatures that induce tumor coagulation and necrosis from energy sources such as: radiofrequency (RF) [3 – 6], microwave [7 – 9], ultrasound [10, 11], and laser [12, 13]. Potential benefits of image-guided ablation of focal neoplasms compared to conventional surgical options include: 1) the ability to ablate and/or palliate tumors in non-surgical candidates; 2) substantially reduced morbidity, lower costs, and improved quality of life [1 – 6]; and 3) performance of these procedures on an outpatient basis [1 - 3].

Historically, the greatest attention has been given to the clinical potential of image-guided ablation procedures for the treatment of colorectal metastases to the liver and to primary liver tumors due to the significant morbidity and mortality of standard surgical resection, combined with the large number of patients who cannot tolerate such radical surgery. Indeed, *over 20,000 RF ablation procedures for treating focal liver tumors have been performed over the last five years* [14 – 24]. This fact alone (i.e., rapid adoption without complete validation and comparative outcome studies attests to both the scope of the clinical need and the less than ideal treatment options for these patients. More recently, the important clinical potential of these techniques has been reported for the treatment of neoplasms in other sites, including the kidney (> 1,000 cases) [25 - 30], breast [11, 31], bone [32 - 34], lung [35], and retroperitoneum [36] (see also Appendix A).

Clinical studies using RF ablation for liver and kidney tumors report that local tumor control can be achieved in 80 – 90% of cases where the tumors measure less than 2.5 cm in diameter [14 - 30]. However, current thermal ablation techniques are proving less satisfactory for the treatment of larger tumors. Specifically, a recent study of RF ablation of hepatocellular carcinoma reports local recurrence in 65% of tumors greater than 3.5 cm in diameter, and in 75% of tumors larger than 5 cm [20]. For the percutaneous RF ablative treatment of colorectal metastases, local recurrences of 35 - 89% have been reported for tumors greater than 2.5 cm [14, 17, 21, 22]. Additionally, with further long-term follow-up of ablation therapy, there has been an increased incidence in detection of progressive local tumor growth for all tumor types and sizes despite initial indications of adequate therapy [3 – 6, 14]. This suggests that there are residual patches of untreated disease in a substantial, but unknown number of cases, a result that falls far short of our goal of completely eradicating all tumor treated by RF ablation. Therefore, **strategies that can increase the uniformity and completeness of RF tumor destruction, even for small lesions, are needed.**

From a practical perspective, the volume of ablation from a single (or few) treatment session must be optimized to encompass the entire target. Based upon classic surgical resection dogma [37, 38], for many tumors this includes the ablation of a 0.5 - 1 cm margin of normal tissue surrounding the tumor [4, 39]. However, this cannot be reliably achieved for tumors > 2.5 cm in diameter with current technology using a single application of RF due to technologic and bio-physical limitations that retard uniform heating of the entire tumor volume to temperatures sufficient for inducing coagulation [1, 40]. This necessitates multiple treatments to adequately treat the tumor. However, clinical strategies based upon multiple applications of RF have met with variable success, and are otherwise unattractive given that they can be extremely labor and time intensive, expensive, and prolong the duration of therapy [17, 39 - 41]. Thus, **further increases in thermal ablation efficiency are necessary to benefit greater numbers of patients and to treat larger tumors.**

2. Overcoming the limitations of RF tumor ablation:

We have devoted substantial effort in modifying the RF system and the biologic environment to increase the extent of coagulation that can be produced, and thus improve the clinical utility of RF ablation [42 - 51]. Our program has employed a three-pronged strategy for translating basic ideas for improving thermal ablation therapy into clinical practice. This triad encompasses: 1) animal studies in which selected ablation parameters can be systematically modulated; 2) mathematical modeling and optimization strategies that both refine and predict new approaches; and 3) rapid translation of experimental gains into clinical studies. Over the course of past investigations, we have devised several electrode modifications and adjuvant techniques that have increased tissue coagulation in animal experiments [42 - 51], and that as a result of their utility, have been rapidly adopted into clinical practice [3 – 6]. We have also used RF

as a model for understanding the underlying mechanisms and physical principles of thermal ablation therapy [8, 52]. We have further directly translated this experimental work into clinical practice [4, 43, 49], with others applying these advances more globally to different percutaneous thermal ablation therapies such as laser and microwave [8, 53].

Our initial efforts at increasing RF ablation efficacy were based upon maximizing tissue coagulation by increasing the amount of thermal energy deposited during ablation (see Appendix B). This has been accomplished largely by increasing generator output with modified RF application (i.e., pulsed RF and other algorithms [1, 44, 50]) or electrode modification (i.e., electrode arrays [17, 18, 54], or internally cooled electrodes [42, 43]) to deposit greater amounts of heat within the tissue. Such strategies have met with some success as under ideal conditions coagulation diameters of 3.5 – 5 cm can be obtained from a single RF application. While these advances represent a significant step toward the goal of inducing large volume ablation, by themselves they do not overcome a key limitation – the inability to reproducibly induce complete and uniform tumor destruction of sufficient volume to effectively treat many clinical tumors [1].

As a next step, we turned our attention to modulating biophysical aspects of the thermal ablation process to achieve more effective spatial tissue heating and greater tissue coagulation for a given RF application. Based upon predictions of the Bio-heat equation of Pennes [55], two key factors, modification of tissue electrical conductivity to enhance energy deposition by injection of NaCl solutions [46 – 48, 56], and reduction of tissue blood flow to reduce heat loss using intra-arterial chemoembolic techniques [49, 57, 58], IV pharmacologic modulation [50], and direct injection of ethanol [59] are the focus of our separate NCI-funded project (*R01CA87992-01A1; Tumor Ablation using Radiofrequency*). Indeed, substantial gains are being achieved in extending RF-induced coagulation to 5 – 7 cm in diameter (see Appendices B & C) [46 - 47]. Nevertheless, despite these advances, we and others have demonstrated that in many cases small residual foci of viable cells are scattered throughout some tumors [46]. Thus, the ability to achieve complete and uniform eradication of all malignant cells remains a key barrier to clinical success [1 - 6].

3. Combining RF ablation and hyperthermia with adjuvant chemotherapy:

We therefore sought to improve upon our results by **combining RF thermal ablation with chemotherapeutic adjuvants**, with the goal of achieving increased responses from the combined regimen. We applied the concept that tumor death could be enhanced when combining RF thermal therapy with adjuvant chemosensitizers based upon the rationale of the known synergistic anti-neoplastic effects of hyperthermia (i.e., largely reversible cell damage induced by mildly elevating tissue temperatures to 41 – 45° C) and chemotherapeutic agents, such as doxorubicin [60-65]. The goal of this approach is to increase tumor destruction occurring within a sizable zone of elevated, but sub-lethal, temperatures adjacent to regions of heat induced coagulation, including at the periphery of the treatment zone [1, 42]. Based upon this concept, we have generated significant preliminary data in animal models and in patients (see section C; Appendices D - I) that demonstrate complementary interaction between RF ablation and chemotherapy [66 - 71].

Increasing tumor destruction by adding chemotherapy to RF ablation can potentially overcome many of the current limitations posed by performing RF ablation alone. First, the addition of a systemic or intratumorally injected agent could potentially increase the diameter of tumor destruction by enabling chemotherapy to interact with the large zone of sub-lethal heating at the periphery of the ablation focus [1, 72]. Currently, thermal ablation strategies only take advantage of temperatures that are sufficient by themselves to induce coagulation necrosis (> 50°C). Yet, based upon the exponential decrease in RF tissue heating there is a steep thermal gradient in tissues surrounding an RF electrode [72, 73]. Hence, there is substantial flattening of the curve below 50° C, with a much larger tissue volume encompassed by the 45° C isotherm (*Figure 1*). Modeling studies demonstrate that were the threshold for cell death to be decreased by as few as 5° C (i.e., an isotherm shift suggested in Section C.2e), tumor coagulation could be increased up to 1.5 cm [51]. Thus, **lowering the temperature threshold of cell death** would increase the volume of tumor destruction.

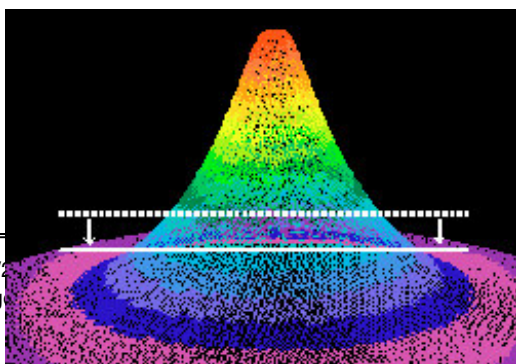


Figure 1: Schematic temperature profile surrounding a monopolar RF electrode. The dashed line represents the 50°C isotherm where coagulation of tumor is induced by RF alone. The solid white line depicts the 45°C isotherm that only undergoes RF induced necrosis in the presence of liposomal chemotherapy (see Section C.2e). This encompasses a 59%

larger spherical volume. Hence, by lowering the temperature at which cytotoxicity is induced, this specific chemotherapy has the potential to increase tumor destruction.

Improved tumor cytotoxicity is also likely to **reduce the local recurrence rate at the treatment site**. Although there is high temperature heating throughout the zone of RF ablation, heterogeneity of thermal diffusion (especially in the presence of vascularity) retards uniform and complete ablation [1 – 6]. Since local control requires complete tumor destruction, ablation may be inadequate even if large zones of ablation that encompass the entire tumor are created. By killing tumor cells at lower temperatures, this combined paradigm will not only increase necrosis volume, but may also create a more complete area of tumor destruction by **filling in untreated gaps** within the ablation zone.

Combined treatment also has the potential to achieve **equivalent tumor destruction with a concomitant reduction of the duration or course of therapy** (a process which currently takes hours to treat larger tumors, with many protocols requiring repeat sessions) [1–6]. A reduction in the time required to completely ablate a given tumor volume would permit patients with larger or greater numbers of tumors to be treated. Shorter heating time could also potentially improve their quality of life by reducing the number of patient visits and the substantial costs of prolonged procedures that require image guidance. Last, quicker therapy achieved in a single procedure session may also limit the chance of developing thermotolerance [74].

Traditionally, RF ablation strategies have only taken advantage of the coagulative effects of high temperature heating as the parameter governing tissue destruction for RF ablation has been cellular death via thermal coagulation necrosis [16, 72, 75, 76]. While, irreversible protein denaturation occurs nearly instantaneously at ~ 60°C, we and others have demonstrated that 50 - 55°C induces coagulation over 4 – 6 minutes of heating [60, 75, 77], and thus application of RF energy for ablation tends to be of short duration [1 – 6]. This differs from classic hyperthermia which is typified by long courses of uniform heating (on the order of hours) [60 - 65]. Thus, unlike lower temperature hyperthermia, the concept of thermal dose (heating temperature X time) has not traditionally played a major role for clinical RF thermal ablation. Rather, manipulation of the position of a critical isotherm (50° C) has been viewed as essential for maximizing ablation volume [1]. Given this practical, critical difference, direct translation of results at lower temperature hyperthermia must be made with caution. Indeed, there is only limited hyperthermia data for temperatures > 45 ° C [78], the range of temperature at which most, if not all, of the increased tumor destruction is observed in our system [75]. More precise and quantitative delineation, specifically of the relationship between RF ablation and classic hyperthermia dosimetry [78, 79], requires further study as outlined in this proposal.

4. Devising optimal strategies for chemotherapeutic delivery – Evolution towards Liposomal Preparations:

Judicious selection of the type of chemotherapy combined with thermal therapy is necessary to potentiate and optimize the tumoricidal effects occurring in the peripheral zone of hyperthermia created by RF heating. As will be shown, we have progressively refined our chemotherapeutic delivery for combined therapy to now include liposomal preparations [66 - 71]. Indeed, the maximum tumor destruction achieved was obtained using IV liposomal doxorubicin combined with RF ablation (see Section C.2) [67].

Although initial image-guided tumor ablation strategies sought to increase local drug delivery by direct injection [80 - 82], this approach has encountered many difficulties in clinical practice that have led to its disfavor. Many have reported difficulty in achieving uniform diffusion of percutaneously injected drugs such as ethanol and doxorubicin over larger tumor volumes [83] due to high interstitial pressures that result in poor diffusion of chemical agents throughout the tumor [84]. Intravascular direct catheter injection of highly concentrated chemotherapy has also been tried, but has had limited clinical adoption due to the substantially invasive nature of surgical or angiographic device implantation [85-87]. Hence, the use of a straightforward IV chemotherapeutic delivery route would be potentially clinically beneficial - were sufficient intratumoral drug delivery and/or accumulation possible without inducing systemic toxicity.

Recent advances in delivery of chemotherapeutic agents include the development of liposomal carriers for compounds such as doxorubicin [64, 88 - 91]. Liposome particles are completely biocompatible, cause very little toxic or antigenic reaction and are biologically inert. Water-soluble drugs can be trapped in the inner aqueous compartment, whereas lipophilic compounds may be incorporated into the liposomal lipid membrane. Incorporation into liposomes protects the drug from the destructive environment *in vivo*.

Liposomes are capable of delivering their drug load inside the cell and even inside different cell compartments [89]. The primary drawback of initial liposomal preparations, (i.e., their elimination from the systemic circulation by the reticulo-endothelial system (RES) due to rapid opsonization [92]) has now been substantially overcome by surface-modification using flexible hydrophilic polymers such as polyethylene glycol [87 – 95] to prevent plasma protein absorption on liposome surfaces and subsequent recognition and uptake of liposomes by the RES. Hence, touted benefits for the use of these “stealth” liposomal carriers include reduced systemic phagocytosis and a resultant prolonged circulation time, selective agent delivery through the leaky tumor endothelium (an enhanced permeability and retention effect), as well as reduced toxicity profiles [96 - 99]. As a result, this formulation is widely accepted for clinical practice [100 - 103].

Prior investigations have demonstrated enhanced antitumoral activity of liposomal doxorubicin in tumors infiltrating the liver and spleen [94], where it has been hypothesized that liposomes that are taken up by the RES, with free doxorubicin subsequently released to the tumors. However, the activity against extrahepatic (subcutaneous) tumors has been reported to be inferior to that of free doxorubicin at milligram equivalent doses [96, 97]. Thus, although liposomal doxorubicin is gaining increased clinical attention, **methods for improved tumor drug uptake are still needed.** Along these lines, investigators have shown that conventional hyperthermia at up to 45° C for 30 – 60 min can increase intratumoral doxorubicin accumulation for various temperature sensitive and thermally stable liposome preparations [88, 104-105], and that this increased uptake can be associated with decreased tumor growth [106 – 108]. As outlined in Section C, we too have seen complementary intratumoral doxorubicin accumulation, and more importantly greater tumor destruction when combining high temperature, short course RF ablation with liposomal doxorubicin [66, 69]. Thus, the focus of our proposal is a promising approach of combining RF ablation with liposomal doxorubicin preparations.

5. Potential mechanisms for the complementary interaction between RF ablation and liposomal doxorubicin:

The processes governing the increase in tumor destruction for combined RF therapies are complex and likely multi-factorial, as there are three therapies involved, i.e., RF, hyperthermia, and chemotherapy. Section C.2 clearly shows that IV liposomal doxorubicin increases the efficacy of RF ablation, but RF heating also produces low temperature hyperthermia, which acts in at least two ways to ultimately increase the cytotoxicity of the liposomal doxorubicin. This includes increasing intratumoral liposomal drug accumulation (see Section C.5) and improved cytotoxicity from the liposome preparation itself (see Section C.6).

Increased intratumoral liposomal drug accumulation: Hyperthermia, and hence thermal ablation, has been proposed to increase vascular permeability likely as a result of endothelial injury [88, 109 - 111], which results in improved intratumoral delivery of cytotoxic agents. Under normal conditions, the intratumoral delivery of systemic chemotherapeutics is limited [112]. Although tumor microvasculature is known to be “hyperpermeable”, most tumors have regions of hypopermeable microvessels, where the transvascular channels are limited in number and or size, limiting the extravasation of larger (> 100 nm) anti-neoplastic agents such as liposome vectors [113, 114]. This in turn results in less than optimum therapeutic efficacy. However, Yuan et al. [111], and Kong et al. [114] have demonstrated that low temperature hyperthermia increases the vascular endothelial pore size, which allows for greater deposition of the liposome containing doxorubicin. Thus, non-coagulative hyperthermia in regions adjacent to the thermally mediated RF induced coagulation may behave as other permeabilizing agents, opening inter-endothelial gaps, thus augmenting microvascular permeability (and transvascular transport) to drug. Alternatively, low temperature hyperthermia (42° C) doubles maximum blood flow which could increase drug delivery [110]. Although higher temperatures (> 45° C) cause vascular stasis, we have recently demonstrated reversibility of this process with increased blood flow during reperfusion in this zone [115].

Our preliminary data using RF ablation [66 – 71] confirm prior hyperthermia data that demonstrate dramatically enhanced local drug accumulation for liposome delivery vectors [104, 105, 116] (see Section C.5). Augmentation of liposomal doxorubicin delivery and increasing its accumulation in tumors by combining it with RF ablation could potentially account for the increased efficacy of the drug. Thus, the use of short courses of RF to concentrate liposomally delivered drugs could potentially expand the clinical use of this and other chemotherapeutic agents that have previously lacked efficacy due to an inability to achieve sufficient intratumoral drug concentrations. Additionally, selective intratumoral deposition of high drug concentrations could potentially allow an overall reduction of drug dosage, thereby reducing the potential for systemic toxicity, while maintaining delivery of high doses to the tumor target. Thus, **liposomal delivery into RF ablated tumors has the unique potential to act as a focal targeting**

mechanism to guide the deposition of liposome encapsulated agents.

Although our preliminary data establish increased tumoral accumulation of doxorubicin when liposomal doxorubicin is combined with RF, this mechanism alone is unlikely to be the only reason for improved tumor destruction. Section C.1 shows that direct intratumoral injection of doxorubicin also potentiates RF-induced coagulation [66]. This result supports the contention that the improved treatment effect is at least in part due to other, non-vascular mechanisms. For many tumor cell lines, doxorubicin is actively pumped out of cells by the multi-drug resistance membrane protein, which can be transiently damaged by heat [117, 118]. Hence, transient hyperthermic damage to cellular homeostatic mechanisms that permit increased cellular or nuclear membrane permeability to doxorubicin and increased intracellular accumulation could be partly responsible [119, 120]. Alternatively, RF has been shown to transiently reduce blood flow at 45 – 50° C [115], and cellular uptake of doxorubicin is known to increase under hypoxic conditions [121]. Finally, liposomal carriers have been shown to circumvent vesicular drug transport [122, 123].

Improved cytotoxicity with empty liposomes: Section C.6a demonstrates that RF combined with empty liposomes increases tumor destruction greater than RF or empty liposomes alone. Thus, improved tumor destruction from combined RF ablation and IV liposomal treatment is not due solely to increased drug delivery. Prior studies at low temperature hyperthermia (41 - 43° C) demonstrate increased cytotoxicity in the presence of unsaturated lipids [124 - 127], which has been ascribed to hyperthermia inducing enhanced oxidative stress and increased lipid peroxidation that could be amplified in the presence of highly oxidizable lipids. Also, the diffusible cytotoxic byproducts that form during lipid peroxidation (lipid hydroperoxides and lipid aldehydes) can also potentially enhance cell killing at the margins during thermal ablation therapy, and these species would also be expected to increase in cells treated with liposomal preparations containing GLA and arachidonic acid [128, 129]. As will be shown, new preliminary data suggest that similar mechanisms may be playing a role in our system where we see parameters indicative of steady state levels of lipid hydroperoxides increasing following thermal ablation (Section C.6b). These novel results suggest that alteration of liposomes with lipid components that increase their susceptibility to lipid peroxidation can increase tumor destruction (Section C.6a). As such, a better understanding of the underlying mechanism for this interaction may lead to therapies with even greater efficacy and that are potentially safer, especially if equivalent efficacy can be demonstrated without the need for systemic doxorubicin administration.

7. Summary:

The long-term goal of this project is to optimize RF ablation to increase the extent and completeness of tumor destruction and thereby improve treatment efficacy in a safe and straightforward manner. Achieving this objective would effectively improve therapy in terms of local control and overall survival, thereby dramatically improving the suitability of this strategy for treating the clinical spectrum of focal tumors. We have made significant progress in our research over the past six years to overcome the limitations of the monopolar RF electrode model, as our prior studies and innovation have pushed ablation sizes to the 5 – 7 cm range [5, 52]. Additional barriers to be overcome include a reduction in the rate of local ablation failure due to inadequate ablation of margins and residual viable cells throughout the tumor volume. Preliminary data as shown in Section C strongly suggest that ***a new paradigm, combining RF ablation with liposomal chemotherapy, has the unique potential to address these limitations in a robust manner.*** In the present proposal, our objectives are to continue systematically optimizing this paradigm for rapid translation into clinical practice, while simultaneously gaining a better understanding of the underlying mechanisms that when validated, may permit further potentiation of this paradigm, particularly by the construction of appropriately tailored liposomal drugs. Accordingly, we propose optimization and safety studies, as well as experiments that both validate specific hypotheses as to underlying mechanisms for the various components of this complex system. This will include determination of the relative importance of each of the components within this paradigm. We trust that the rationale for our approach as outlined in this section and Section D, coupled with our robust preliminary data amply illustrate the need for further study as proposed.

C. PRELIMINARY RESULTS:

Extensive, additional studies supporting the proposed hypotheses outlined in Specific Aim 2 and underlying the rationale for the need to study combined RF ablation with liposomal doxorubicin in normal tissues (Aim 3) is now provided. While the reviewers have been quite positive about our preliminary data

in the prior proposal, in order to permit appropriate space to present our new findings, we summarize some of our earlier findings that are now published. All of the previously presented data are provided in the Appendix, with reference to specific figures and tables noted below.

1. Intratumoral doxorubicin injection increases RF induced tumor destruction in animals (Appendix D) [66]:

R3230 mammary adenocarcinoma (1.2 - 1.5 cm diameter, n = 46) was implanted in female Fisher rats, and treated with: 1) monopolar RF alone ($70 \pm 2^{\circ}$ C for 5 min, 250 ± 25 mA); 2) direct intratumoral doxorubicin injection (250μ l; 0.5 mg total) alone; 3) combined doxorubicin followed immediately by RF; or 4) no treatment. Tumor destruction 48 hr following the last intervention measured 6.7 ± 0.6 mm for RF alone, and doxorubicin alone produced only 2-3 mm of necrosis ($p < 0.01$). Increased coagulation was observed with combination doxorubicin and RF therapy (11.4 ± 1.1 mm; $p < 0.001$) (Appendix D, Fig. 2).

Next, the total amount of doxorubicin injected immediately prior to RF ablation was varied from 0.02 to 2.5 mg (n = 48). A logarithmic dose response curve ($r^2 = 0.97$) to a maximum 12.1 mm diameter of tumor destruction at a doxorubicin dose of 2 mg was seen (Appendix D, Fig. 5). The timing of intratumoral doxorubicin injection was also varied in relation to the RF application from 2 days prior to 2 days following RF (n = 40). Greatest tumor destruction was observed for doxorubicin administered within 30 min prior to or after RF ablation (11.5 ± 1.1 mm). Marked, progressive reduction in tumor destruction was observed at 6 to 48 hrs. following RF ablation ($p < 0.01$) (Appendix D, Fig. 6).

These results are important in that they demonstrate that **intratumoral doxorubicin injection not only increases tumor destruction in solid tumors** compared to RF ablation alone, but also show that these increases are **dependent upon drug dose and the timing** of administration in relation to RF ablation. However, the maximal increase seen was only several millimeters, and a very narrow therapeutic window was identified for optimal doxorubicin injection. Thus, we postulated that improved methods of chemotherapeutic delivery such as IV administration would further improve upon this adjuvant, combined therapy.

2. Intravenous liposomal doxorubicin further increases RF induced tumor destruction (Appendix E) [67]:

2a: Effect of combined intravenous liposomal doxorubicin and RF ablation. R3230 was implanted in 40 female Fisher rats. Tumors were randomized into five groups including: 1) RF alone ($70 \pm 2^{\circ}$ C for 5 min); 2) IV liposomal doxorubicin injection alone (1 mg Doxil; ALZA Pharmaceuticals, Palo Alto, CA); 3) IV injection of the empty liposome carrier; 4) RF ablation followed 15 – 30 minutes later by IV Doxil; 4) RF ablation followed by IV injection of the empty liposome carrier; and (5) no therapy. Animals were sacrificed 48 hours following the last intervention. Tumor destruction measured 6.7 ± 0.6 mm for tumors treated with RF alone and 11.1 ± 1.5 mm for RF and empty liposomes ($p < 0.05$ compared to RF alone). Maximal increased tumor destruction (13.1 ± 1.5 mm) was observed with combination Doxil and RF therapy ($p < 0.001$), an amount of tumor destruction that was also greater than that achieved for the intratumoral injection of a similar dose of free doxorubicin (11.4 ± 1.1 mm; $p < 0.01$) (Appendix E, Fig. 1).

2b: Evolution of pathologic changes over time. The temporal evolution of tumor destruction from combined RF and liposomal doxorubicin and RF alone were studied from 15 min to 120 hr after ablation. For tumors receiving combined therapy, a progressive increase in tumor destruction was observed to 48 hr (13.1 ± 1.5 mm) with no additional increase observed at 72 hrs. Initially, histopathology revealed only typical findings of RF induced coagulation. However, progressive evolution to frank coagulative necrosis was observed by 48 hr only in regions where increased tumor destruction was seen (Appendix E, Fig. 2). By comparison, for tumors treated with RF ablation alone, no further changes in coagulation diameter (6.6 ± 0.4 mm) or histopathologic findings were noted for 0 – 120 hrs. post-RF. These morphologic and temporal differences suggest that the ***thermal damage from combined RF and liposomal chemotherapy produces cytotoxicity via different mechanisms than that of RF alone***, possibly involving metabolic biochemistry.

2c: Effect of altering the timing interval between liposomal doxorubicin and RF ablation therapy. The timing of liposomal doxorubicin administration (1 mg Doxil) was varied in relation to the RF application from 3 days prior to 3 days following RF application (n = 48). Equivalent amounts of tumor destruction were observed at 48 hours after the last intervention when the liposomal doxorubicin was administered three days prior to 24 hours after RF application, ($p = \text{NS}$) (Appendix E, Fig. 5) [67]. However, progressive reduction in the amount of tumor destruction was observed 48 and 72 hrs following

RF ablation ($p < 0.01$).

These results are important in that they demonstrate that **IV liposomal doxorubicin not only increases the extent of RF induced tumor destruction, but also permits a greater window for optimal timing of doxorubicin administration compared to direct intratumoral injection of unencapsulated doxorubicin** where reduced necrosis was observed by 6 hours following the RF therapy [66].

2d. Effect of liposomal doxorubicin dose. The dose effect of the amount of IV liposomal doxorubicin on RF-mediated tumor destruction was assessed. Tumors ($n = 20$) were treated with increasing drug dose given 15 – 30 minutes following RF ablation [67]. Over a range of 0.063 – 2 mg of doxorubicin, the amount of IV liposomal doxorubicin injected did not significantly influence the overall diameter of tumor destruction or the histologic findings at 48 hours post-therapy. (*Appendix E, Fig. 4*)

These results differ from the dose dependent response curve observed for intratumoral injection of similar quantities of free doxorubicin in this model [66], and suggest that very little doxorubicin may be needed to potentiate enhanced tumor destruction when coupled to liposomes, a finding that would be very useful in limiting chemotoxicity in patients. Synergistic interaction between the RF heating, doxorubicin, and the additional lipid may very well reduce the amount of chemotherapeutic drug necessary to achieve cytotoxicity.

2e. Effect of RF thermal dose. RF ablation was applied for 5 min to 1.5 cm R3230 nodules ($n = 65$). RF tip temperature was varied from 30° to 80°C. Tumors received liposomal doxorubicin (1 mg Doxil) or no additional therapy. The amount of ablation at 48 hours is presented in *Figure 2*. Significantly greater tumor destruction was seen at all temperatures for RF combined with liposomal doxorubicin compared to RF alone ($p < 0.001$), with an equivalent increase in tumor destruction achieved at each tip temperature.

Additional experiments ($n = 16$) varying duration of RF application demonstrated differences in increased tumor destruction from combined therapy. RF ablation at 70° C for 1 minute (4.1 ± 0.5 mm) was smaller than that obtained for 5 min (6.1 ± 0.6 mm; $p < 0.01$). RF for 10 min at 60 °C with liposomal doxorubicin also produced 12.1 ± 1 mm of effect, but only 8.0 ± 0.5 mm alone (a 4.1 mm difference; $p < 0.05$). This difference was 3.1 mm larger than the 1 mm increase in tumor destruction from 5 min of heating ($p < 0.05$).

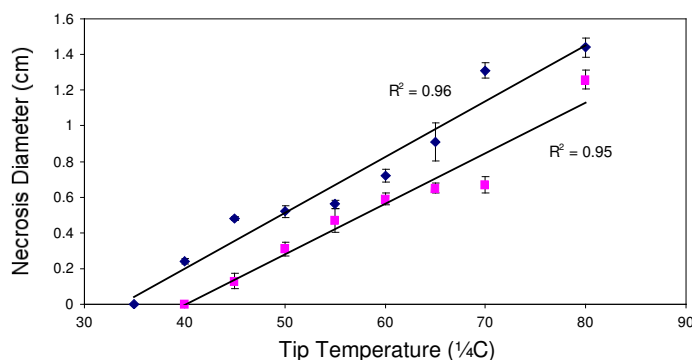


Figure 2: Correlation of tumor destruction to RF tip temperature. The figure demonstrates tumor destruction at varied tip temperatures for 5 min of RF heating combined with liposomal doxorubicin (diamonds) and RF alone (squares). Linear correlations are suggested with greater tumor destruction seen for combined therapy. Data further suggest that the threshold to induce necrosis is at least 4 - 5° C lower for RF combined with liposomal doxorubicin than for RF alone [71].

These preliminary data suggest the **thermal dependency of the interaction between RF ablation and liposomal doxorubicin**. However, the range and threshold of actual thermal dosing at which increased tumor destruction can be observed and the maximum increase in tumor destruction are as yet unknown.

2f. Increased tumor destruction in a large animal tumor model. Experiments were performed in 8 subcutaneous canine venereal sarcoma nodules (4.8 ± 0.7 cm), the proposed tumor model. RF ablation was applied to 1 cm internally-cooled electrodes (1,000 – 1,200 mA pulsed technique for 12 min). Four tumors received 1 mg/kg liposomal doxorubicin immediately prior to RF ablation. Pathologic changes at 48 hours following ablation demonstrated 1 cm of increased tumor destruction for the combined therapy group versus for RF alone (3.2 cm diameter vs. 2.2 cm; $p < 0.05$) (*Figure 3*). Volumetrically, this translated into a **212% increase in tumor destruction** when liposomal doxorubicin is added to RF ablation.

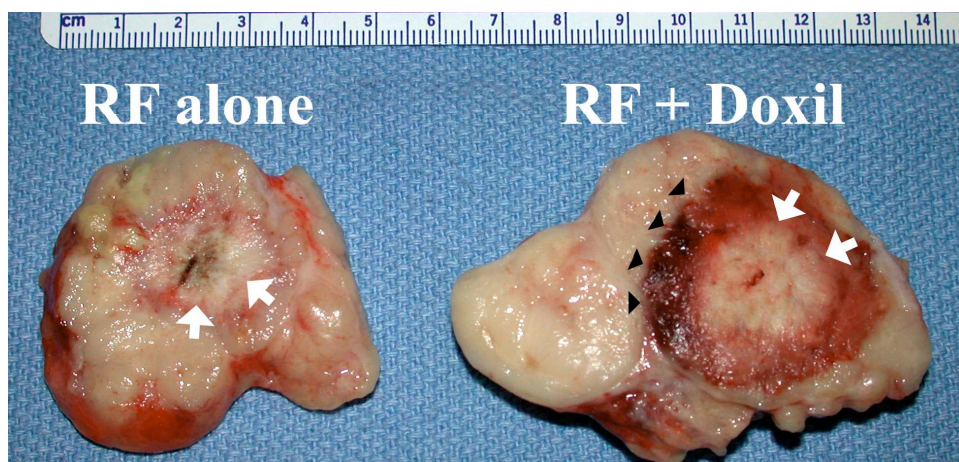


Figure 3: Effect of combined RF ablation with liposomal doxorubicin. The central white zone (arrows) that corresponds to RF-induced coagulation is slightly larger (3 mm) in the combined therapy tumor, while the peripheral red zone (black arrowheads) is dramatically increased in size (0.21 to 0.93 cm). In the combined therapy tumor this red zone of increased tumor destruction is comprised of frank coagulative necrosis.

This experiment demonstrates **clinically meaningful increases in tumor destruction for a large animal tumor model**. However, optimization of the extent of tumor destruction achievable, and the thermal conditions under which it occurs, awaits the systematic study proposed in Section D.

2g. Increased tissue destruction when combining the RF ablation with liposomal doxorubicin in normal tissues. Monopolar RF (90° C x 5 min.) was applied to normal small animal tissues (rat muscle and rabbit liver and kidney). Ten RF applications were made in each of the tissues. For half of these, 1 mg/kg liposomal doxorubicin was administered 30 min following RF ablation. Results (see accompanying Table) show significant greater coagulation for all 3 tissues when treated with combined therapy ($p < 0.01$).

	RF/Doxil (mm)	RF alone (mm)

This experiment is important in that it demonstrates the relatively non-specific, but focal tissue destruction that can be achieved by combined therapy. While this can be of potential benefit for clinical practice, as a peripheral rim of liver and kidney need to be ablated to ensure complete clinical destruction of hepatic and renal tumors, characterization of this process, including thermal dosimetry in normal tissues is needed to permit the precise and safe use of this strategy. Significant differences between the necrosis observed in different tissues ($p < 0.05$) further points to the need for characterization of each tissue individually.

3. Combined RF with liposomal doxorubicin increases local tumor control and survival (Appendix F [70]):

Rats with solitary 1 - 1.2 cm R3230 nodules (n=56) were divided into 8 groups receiving: 1) Liposomal doxorubicin alone (1 mg dose); 2 - 4) RF ablation alone (5 minutes) at 70°, 90°, or 98° C tip temperature; 5 - 7) combined therapy (RF ablation at 70°, 90° or 98° C tip temperature followed by liposomal doxorubicin); or 8) no therapy. Tumor growth was measured to 60 days or until a defined surrogate survival endpoint of 3 cm diameter (10% tumor body weight) was reached. Kaplan-Meier curves (Appendix F, Fig. 3) demonstrate significant increases in survival for combined therapy that was related to RF thermal dose ($p < 0.01$). Mean survival of control animals was 10 days, of the 70° C RF or Doxil alone was 18 days, and combined therapy (70° C) was 28 days ($p < 0.03$). Mean survival was 32 days for animals receiving 98° C RF alone, while all 5 animals receiving liposomal doxorubicin and 98° C RF survived to the defined 60 day endpoint as local control was achieved. Tumor growth rate likewise was influenced by thermal dose ($p < 0.01$).

This study demonstrates combined therapy achieves not only increased tumor destruction at pathology, but also that **combination of RF and IV liposomal doxorubicin can impact tumor growth, local control, and survival**. More importantly, the data further suggest the **thermal dependency of the interaction between RF ablation and liposomal doxorubicin**.

4. Increased destruction of human liver tumors from RF and IV liposomal doxorubicin (Appendix G) [69]:

Ten patients with 18 intrahepatic tumors were randomized to receive either liposomal doxorubicin (20 mg Doxil) 24 hr prior to RF ablation or RF ablation alone. 14 RF treated tumors measured 4.0 ± 1.8

cm. Four small tumors (1.4 ± 0.4 cm) had chemotherapy alone. Each tumor was treated with only a single session of RF ablation with a standardized RF paradigm using internally cooled electrodes and pulsed technique [14]. Immediately following RF, no difference in the amount of tumor destruction was seen between patients receiving liposomal doxorubicin (51.8 ± 66.5 cc of induced coagulation) or the controls receiving RF ablation alone (80.2 ± 70.9 cc; $p = \text{NS}$). However, at 2 - 4 weeks, patients receiving liposomal doxorubicin had an increase in tumor destruction of 17.2 ± 15.9 cc (4.7 to 49.0 cc range). This translated to a 24% to 342% volumetric increase (median = 32%). By contrast, the ablation zone of all tumors treated with RF alone decreased in size at 2 - 4 weeks post therapy to 76 - 88% of the initial volume, a finding concordant with prior observations [14, 22]. No treatment effect was observed in the 4 tumors treated with Doxil alone. Given that **all of the tumors treated with combined RF and IV liposomal doxorubicin had an increase in treatment effect, while all tumors treated with RF alone had a decrease in the size of the coagulated focus**, the difference between therapies was highly statistically significant ($p < 0.01$).

Several unexpected, but clinically beneficial findings were also observed only in the combination therapy group. Increased diameter of treatment effect up to 15 mm was observed in multiple tumor types including primary HCC, as well as intrahepatic metastases from colorectal, neuroendocrine and breast origin. Equally as important, **more complete internal tumor destruction, particularly adjacent to intratumoral vessels was demonstrated**. Furthermore, in one case the increase in treatment effect was sufficient to encompass the entire tumor, suggesting that in some cases the use of adjuvant liposomal doxorubicin can result in a **reduction in the number of treatment sessions required** to adequately ablate a given tumor. Additionally, treatment effect extended in most cases to encompass peri-tumoral liver. This is important given that the Doxil enabled the destruction of the difficult to treat 0.5 - 1 cm "ablative margin," an area, that similar to a surgical resection margin, must be ablated to reliably eradicate the entire tumor (**Figure 4**) [6].

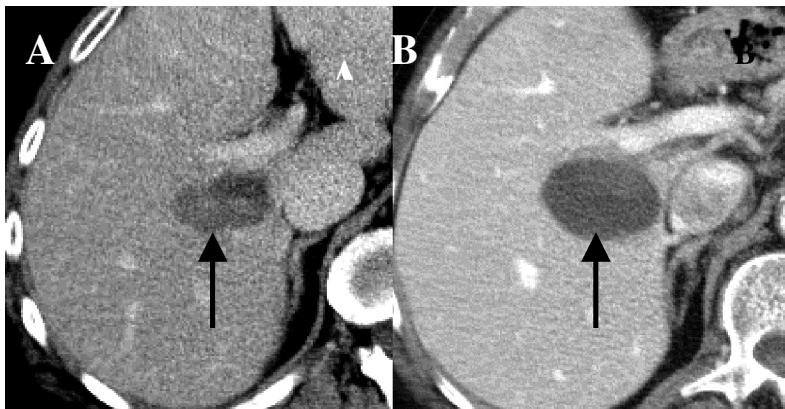


Figure 4: Improved ablation of surgical margins with combined treatment. CT scan of the liver in "A" depicts coagulation enveloping a 2 x 3 cm colorectal metastasis (arrows) in a 72 year old male. 3 weeks later CT scan "B" demonstrates marked increase in treatment effect of 17 cm³, predominantly involving a 1 cm rim of "normal" peri-tumoral tissue. The ablation of this "surgical safety margin" is necessary for ensuring complete treatment of the tumor. This patient had no evidence for local recurrence of his two metastases at 12 months.

Given the exciting **clinically meaningful results** of this study there is marked enthusiasm to embark upon multi-center trials comparing the effects of RF alone versus RF combined with liposomal doxorubicin for inducing local tumor control (i.e., complete ablation) and its impact on long term survival. Indeed, based upon this study we are getting several calls a month from clinicians asking us how to best put this strategy into immediate clinical practice. However, gaps in our knowledge preclude us from providing more than a "best guess" answer. Specifically, given that only one arbitrary dose of Doxil and RF parameters was selected, the maximum benefit achievable with this therapy is currently unknown. Yet, modeling studies (see Section B.3) combined with the preliminary data of Section C.2e suggest that even greater increases in tumor destruction and normal tissue than those seen in this study are theoretically possible. Additionally, the **ablation of normal tissue surrounding the tumor**, while desired based upon surgical resection criteria, **strongly suggests that characterization of this damage is needed to ensure precision and safety**. Together these realities make a compelling case for additional animal studies prior to commencing the design of appropriate, rational strategies for the anticipated implementation of large-scale clinical trials.

5. RF ablation increases intratumoral accumulation of liposomal doxorubicin (Appendix H) [68]:

5a. Increased intratumoral accumulation of liposomal doxorubicin. Two R3230 tumors were grown in the fat pad of each of 19 rats. One tumor of each pair was treated with RF ($70 \pm 2^\circ$ C, 5 min) while the other served as an internal control. IV liposomal doxorubicin (Doxil; 1 mg) or IV free, unencapsulated doxorubicin was given 15 min following RF. Acid alcohol extraction and fluorescent spectrophotometry

were used to quantify intratumoral doxorubicin (see Section D, Exp. 2). RF combined with liposomal doxorubicin had $5.6 \pm 2.1 \mu\text{g/g}$ of doxorubicin present in tumors, while $1.0 \pm 0.4 \mu\text{g/g}$ of doxorubicin was present in non-RF treated control tumors ($P < 0.05$). This represented **a 7.1 ± 4.9 fold increase in intratumoral doxorubicin accumulation** ($P < 0.05$). This markedly elevated doxorubicin accumulation was only observed for doxorubicin given as a liposomal preparation, as increased accumulation was not seen for animals receiving free doxorubicin either with or without RF ($0.4 \pm 0.1 \mu\text{g/g}$ vs. $0.8 \pm 0.4 \mu\text{g/g}$; $P = 0.12$).

These data are important as they demonstrate that **RF ablation augments the preferential accumulation of liposomal doxorubicin** into tumors. Because no increase is observed for unencapsulated doxorubicin, it is likely that the liposome delivery vector further enhances local drug accumulation in the RF heated tumor. It is also important to note that these data suggest the unique potential for using **RF ablation as a vehicle for targeted delivery via liposomal carriers**.

5b. The complementary distribution of liposomes in tumors treated with RF ablation. Autoradiography performed on six rat tumors demonstrates the spatial distribution of the intratumoral accumulation of liposomes in the presence or absence of RF ablation. ^3H -cholesterol labeled liposomes of equivalent to Doxil were constructed. Liposomal accumulation in a 1 – 2 mm circumferential rim of tumor immediately peripheral to the zone of RF coagulation was noted (**Figure 5**). To a lesser extent, the liposomes also diffused into the adjacent “normal” tumor tissue, as well as into the adjacent central coagulated tissue. Minimal heterogeneous accumulation of ^3H -liposome was seen in control tumors not receiving RF ablation. Densitometry confirmed a 2.6 fold increase in average liposomal accumulation for the RF ablated tumors as compared to the non-ablated pair matched controls (3.0 ± 0.7 intensity units [IU] vs. 7.8 ± 3.7 IU, $p < 0.05$). Likewise, the maximum intensity of liposomal uptake was 2.1 times greater for the RF treated tumors compared to the paired control tumors (157.7 ± 25.2 IU vs. 75.3 ± 13.7 IU; $p < 0.01$).

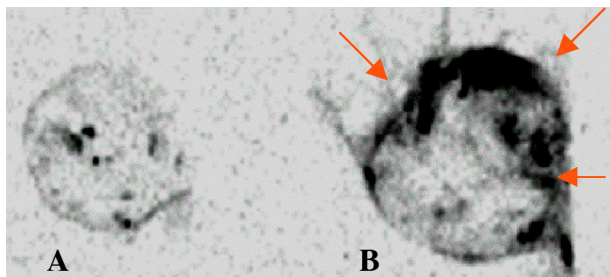


Figure 5: Autoradiography of two paired tumors from the same animal 24 hours following the IV administration of tritiated liposomes. (A) did not receive prior RF ablation, while (B) received RF ablation immediately preceding liposome injection. For the RF ablated tumor, the central zone with little uptake corresponds to the zone of RF coagulation. A peripheral rim of increased liposome uptake is seen (small red arrows) [68].

These findings help explain why liposomal doxorubicin is likely to be complementary to RF ablation. The majority of the liposomes concentrated in a zone immediately peripheral to the area coagulated by RF heating and were within the region where increased coagulation is observed [67]. Additionally, the patchy penetration of liposomes into the zone of coagulation implies infiltration of chemotherapy into the coagulated focus (possibly through residual patent vessels) which may improve the completeness of tumor destruction.

5c: Confirmation in large animal tumor model. Quantitative spectrophotometric analysis of doxorubicin accumulation (as outlined in Sections C.5a and D [Aim 2]) of various portions of the CVS tumors treated with RF ablation in Section C.2f further confirmed the preferential drug accumulation in the region immediately surrounding the zone of acute RF-induced coagulation. Specifically, 72.1 ± 28 nG/G doxorubicin were found in this (red zone) in tumors treated with combined therapy (**Figure 3**), whereas 42.9 ± 5.4 nG/G doxorubicin were seen in the center (white zone) and periphery of these tumors, as well as in control (unablated) tumors ($p < 0.05$, all comparisons). Thus, there is complementary preliminary data demonstrating this variable spatial distribution of quantitative doxorubicin uptake in our CVS model.

5d: RF-mediated doxorubicin accumulation depends on timing and drug dose (Appendix I) [71]. Doxorubicin uptake was quantitated at 0 - 120 hrs following liposomal doxorubicin administration to rats with two tumors ($n = 80$). In each rat, one tumor underwent RF ablation (70°C , 5 min) 30 min prior to chemotherapy. Parabolic curves with maximum uptake at approximately 72 hours following liposomal doxorubicin administration were observed (**Appendix I, Fig. 2**). Tumors receiving additional RF ablation had greater doxorubicin accumulation compared to tumors not receiving RF ($p < 0.01$). In subsequent experiments, the total amount of IV liposomal doxorubicin was varied in 48 rats from 0 to 45 mg/kg

(beyond an LD₅₀ of 38.3 mg/kg in mice) [125]. Results documenting the dramatic progressive increases in liposomal doxorubicin uptake into the tumor with increasing dose are presented as **Figure 6**.

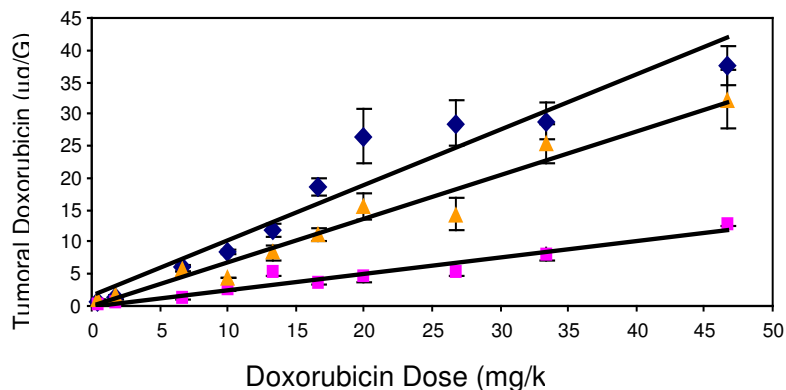


Figure 6. Effect of drug dose on accumulation. The figure demonstrates intratumoral doxorubicin concentrations ($\mu\text{g}/\text{gm}$ of tumor tissue) in paired rat breast tumors 24 h after receiving increasing doses of IV liposomal doxorubicin with (diamond plots) or without (box plots) RF. Concentrations of doxorubicin in liver tissue are presented for comparison (triangle plots). Linear but diverging correlations are suggested with predominantly greater intratumoral doxorubicin accumulation occurring in RF/liposomal doxorubicin tumors ($R^2 = 0.92$) at higher doses as compared to either liver ($R^2 = 0.96$) or tumors receiving IV liposomal doxorubicin alone ($R^2 = 0.94$) [71].

These data are important in that they show: 1) there are **non-linear, time dependent differences in the pharmacokinetics of intratumoral doxorubicin accumulation** following the administration of liposomal doxorubicin in the presence of RF; 2) **RF ablation augments the preferential accumulation of liposomal doxorubicin into tumors**; and 3) **RF enables intratumoral concentrations of doxorubicin which would otherwise require lethal doses of IV drug**.

5e. RF dose influences and increases intratumoral doxorubicin accumulation. RF ablation was applied for 5 min with tip temperature varied from 30° to 90°C to one of two paired 1.5 cm R3230 nodules ($n = 70$). Liposomal doxorubicin (1 mg) was administered IV. Intratumoral doxorubicin accumulation was assessed at 24 hours using fluorescent spectroscopy. Beyond a threshold of 50° C, progressive increases in liposomal doxorubicin accumulation were observed with increased RF ablation temperature (*Appendix I, Fig. 6*) [71]. Further delineation of this relationship awaits fulfillment of Experiment 2 of Specific Aim 1.

6. Liposomal carrier components demonstrate antitumoral effects.

6a. Effect of liposomal lipid: Here, we provide additional preliminary data demonstrating that RF combined with empty liposomes increases tumor destruction, and that this cytotoxic effect can be further potentiated by incorporating lipids with greater oxidizability, a hypothesis that will be pursued in Aim 2.

Rats with R3230 tumor nodules ($n = 30$) were divided into 6 groups receiving RF ablation combined with: 1) phosphatidyl-choline [PC] containing empty liposomes; 2) PC liposomes with doxorubicin; 3) empty liposomes comprised of 5% gamma-linolenic acid (GLA, a polyunsaturated fatty acid thought to enhance the peroxidizability of lipids); 4) 5% GLA containing liposomes plus doxorubicin; and 5), no liposomes. Results presented in the Table demonstrate that statistically significant increases in coagulation were demonstrated for both types of empty liposomes compared to RF alone ($p < 0.05$), and that greater tumor destruction was achieved with GLA containing liposomes ($p < 0.05$). In addition, even greater coagulation and tumor destruction were seen when doxorubicin was added to the liposome preparations ($p < 0.05$).

Liposome	Doxorubicin	Coagulation (cm)
None	-	6.8 ± 0.1
PC	-	1.05 ± 0.07
5% GLA	-	1.24 ± 0.05
PC	+	1.28 ± 0.08
5% GLA	+	1.44 ± 0.28

This study demonstrates the antitumor activity of the empty liposomes when combined with RF, and suggests that **alteration of liposome lipid components can potentiate tumor destruction** with more highly unsaturated lipids giving the greatest potentiation.

6b. Enhanced oxidative stress and/or lipid peroxidation as a potential underlying mechanism.

Twelve R3230 tumors ($n = 4$ / group) were subject to 1) RF alone, 2) IV liposomal doxorubicin [LD], 3) combined RF and liposomal doxorubicin, and 4) no therapy.

Tumors were harvested one hour following the last intervention and subject to assessment of lipid peroxidation by quantitative colorimetric assay for lipid hydroperoxides (Fe oxidation, Bioxytech LPO-560, OxisResearch [130-131]). Results are presented in the accompanying Table, expressed as a ratio compared to untreated tumor from the same animal (mean \pm SD). A statistically significant increase ($p < 0.05$) in lipid oxidation in tissues treated with RF, and

even greater lipid peroxidation when RF is combined with liposomes (85% increase was noted over baseline, $p < 0.01$).

Treatment	Hydroperoxide Ratio
RF + LD	1.85 \pm 0.1
RF alone	1.49 \pm 0.2
LD alone	1.15 \pm 0.3
Untreated tumor	1

Since lipid hydroperoxide content represents an indication of the extent to which lipid peroxidation reactions have occurred, **these results provide support for the hypothesis that enhanced oxidative stress and lipid peroxidation may be an underlying mechanism** for the enhanced tumor killing by liposomal lipids combined with RF, a hypothesis that will be rigorously explored in Specific Aim 2.

D. RESEARCH DESIGN AND METHODS

Our preliminary results demonstrate improved tumor destruction when combining RF ablation with liposomal doxorubicin in multiple tumor types and tissues. Prior to translation into the clinic, further optimization of the parameters known to influence this interaction and better characterization of local and systemic toxicity are warranted. These form the basis of Specific Aims 1 and 3, respectively. Our **underlying hypothesis** is that increased tumor destruction is due to interaction between the liposomal doxorubicin and sub-lethal hyperthermic temperatures that do not directly induce coagulation ($\approx 45 - 50^\circ\text{C}$). Characterization of thermal dosimetry, as outlined in Specific Aim 1, Experiment 3 is important to assess this hypothesis, and may potentially shed additional light on the underlying mechanisms of this process. Preliminary data outlined in Section C and prior work outlined in Section B suggest that multiple interactions account for increased tumor destruction. These include the effects of the increased intratumoral doxorubicin (Section C.5) and the effects of the liposome carrier itself (Section C.6). Experiment 2 of Specific Aim 1 is designed to provide additional data in regards to our hypothesis that increased tumor destruction is due in part to increased delivery of the drug in the liposomal form to RF ablated tissue. Specific Aim 2 is specifically directed to testing mechanistic hypotheses as to why empty liposomes increase tumor destruction including enhanced lipid peroxidation. The experiments of Specific Aims 1 and 2, when analyzed in combination, are designed to determine the relative contribution of the doxorubicin and liposome components to the overall efficacy of the system. Thus, as requested by the reviewers, at the conclusion of the proposed project, we should be able to explain why 'liposome encapsulation of doxorubicin is essential for the observed synergy' between RF and Doxil. This information will be used to determine the optimal agent to be used for future translational and clinical studies.

Specific Aim 1. *Improve tumor destruction* using combined RF ablation and IV liposomal doxorubicin

Experiment 1: Optimize RF ablation parameters and liposomal doxorubicin pharmacokinetics (timing and dose) that effect tumor coagulation using multivariate simplex optimization in canine venereal sarcoma.

Objective: To measure RF induced coagulation as well as the liposomal doxorubicin induced increase in tumor destruction in order to define maximum gains in tumor destruction that can be achieved with the combined therapy in an appropriately scaled large animal model.

Question asked: What is the maximum increase in coagulation necrosis that can be achieved over RF ablation alone in this animal model, and under what conditions can this increase be reliably achieved ?

Rationale: Sections C.2 and C.4 show clinically meaningful gains in tumor destruction via complementary interactions between RF ablation and IV liposomal doxorubicin. Yet, the full extent of potential gains is unknown at this time. Indeed, we have observed variable increases in tumor destruction by varying RF and liposomal doxorubicin parameters in animal models (see Section C.2). Optimization of parameters that alter the interaction between RF and liposomal doxorubicin are essential for achieving optimal clinical benefit. The most efficient, and straightforward approach to the systematic study and optimization of combined RF ablation and liposomal chemotherapy is through the continued use of the

proposed CVS animal tumor model. These animal studies will minimize many of the problems that may be encountered if we directly proceed to further clinical trials, including patient risk, population heterogeneity, cost, and enrollment.

Importance: This experiment is necessary to *establish the maximum extent of increased tumor destruction possible* when adding liposomal doxorubicin as an adjuvant to RF ablation, and to *determine the experimental parameters that achieve maximum effect*. This experiment will be performed first as it will permit the definition of the most important variables affecting combined RF ablation with liposomal doxorubicin. Additionally, throughout this experiment, the baseline optimized parameters about which subsequent response surface contours (including the study of RF combined with empty liposomes [Exp. 4] and other lipid preparations [Exp. 5]) will be defined.

Study Design: Simplex optimization provides a robust, efficient method for optimization, particularly when the precise form of the equations governing the process are not known [132 - 134]. Once the maximum is found, the response surface around the maximum can be determined by factorial search algorithms (either rectangular or skewed) which are grid searches around this maximum value [135 - 137]. We have previously used this approach to determine the surface response contour for RF coagulation and electrical conductivity using altered NaCl volume and concentration [45], and MR spectroscopic processes [138, 139].

Canine venereal sarcoma tumors (n = 6 per condition) will be subject to standardized RF ablation with or without adjuvant IV liposomal doxorubicin. Experimental parameters including RF thermal dose, drug dose, timing of measurement, and interval between therapies will be optimized using multi-dimensional simplex. The optimized variable will be *increase in tumor destruction* from adding liposomal doxorubicin to RF.

Experimental groups: Four parameters have been identified by our preliminary data (see Section C.2) to influence tumor destruction in this paradigm. These include the thermal dose of RF (here measured as the duration of RF application); two parameters for liposomal doxorubicin administration (dose and the interval between the drug and RF ablation); and the time interval after treatment at which coagulation is measured. We will incorporate these four variables into our multi-simplex optimization.

Control groups: Our main parameter of interest is the increase in *tumor destruction* induced by adding liposomal doxorubicin to RF ablation. However, alteration of RF parameters will also alter the amount of tumor *coagulation* produced. To eliminate this confounding variable, the absolute difference between these two processes (i.e., increased tumor destruction) will be calculated and defined as the independent variable. Thus, for each iterative data point in the simplex, two groups of tumors will be treated (RF alone and combined with liposomal doxorubicin).

Simplex characteristics: A small step, variable-size sequential simplex design will be used [134]. The first five (k + 1) points chosen to construct the initial six-dimensional hypertetrahedron will be selected near and centered upon optimal parameters generated in the preliminary data (see Section C.2). This includes RF duration of 10 min, coincidental RF ablation and doxorubicin administration, pathologic examination at 48 – 72 hr, and high doses of liposomal doxorubicin (25 – 35 mg/kg). Thus, greatest information will be obtained near the optimum enabling precise definition of the optimal conditions, with sparse information obtained from regions away from the optimum. To help visualize the multidimensional simplex, successive iterations will be predicted using the assistance of a simplex computer software package (Multisimplex 2.1, Grabitech Solutions AB, Timra, Sweden). Out of bounds predictions will be substituted with defined boundary conditions. Successive iterations will be performed until convergence, which will be defined as achieving three iterations producing changes ≤ 0.3 cm (≤ 1 std. dev).

The maximum ranges for the four variables to be imputed into our simplex model are based upon data generated in preliminary studies, constrained to the boundary conditions of clinically useful parameters. For 1) RF duration a range of 1 – 30 minutes has been selected, as the upper boundary is at the upper limit of clinically accepted RF heating times, and is well beyond that which is presently clinically desired (ideally < 15 min). For 2), the timing of drug administration will initially range from 1 day prior to 1 day beyond RF ablation, based upon data of Section C.2c suggesting that optimal coagulation occurs when the two therapies are administered in proximity, coupled with the known 24 hr half life circulation time of the liposomal doxorubicin [98]. For 3), the dose of doxorubicin will range from 0 – 38.3 mg/kg (LD₅₀) [128, 140]. For 4), the interval at which the tumor coagulation will be measured will range from 0 – 5 days based upon data of Section C.2b showing greatest variability over this range, with peak tumor destruction expected between 48 and 72 hr. To ensure robustness of the simplex, we will perform reference experiments every 10 iterations to ensure stability of the system, as well as re-evaluate suspiciously good

data points at $(k + 1)$ iterations [141].

Sample size: Monte Carlo simulations have determined that between 15 and 25 data points are necessary to converge a small-step multivariate simplex optimization of 4 parameters [133]. Each component of the data point (i.e., RF with liposomal doxorubicin and RF alone) will be run in triplicate to ensure a conventionally accepted standard error $\leq 10\%$ [142]. Power calculations determine that this number of replicates could detect a standard error of 1.5 mm which falls well within the bounds of the 1.0 – 2.0 mm 10% margin of error for the 10 - 20 mm anticipated increase in coagulation in this model. Thus, a total of six tumors will be required for each data point. Therefore ~120 tumors will be used ($20 \times 6 = 120$). Given that we can treat multiple ($n = 3$) tumors in each animal, this will require 40 animals.

While the experimental design of performing all ablations for a given Simplex iteration in a single animal will reduce the animal to animal variation, it does not take into account animal to animal variability. An additional three dogs will be used to confirm the degree of variability in tumor destruction from animal to animal at optimal RF settings. Thus, a total of 43 animals are proposed.

Animal model: Experiments will be performed using a canine venereal sarcoma model. This model has been selected for two reasons: 1) the tumor can be grown to a size of clinical relevance (4 – 6 cm), and 2) it has been previously used to represent tumor growth for the study of minimally-invasive thermal ablation [47, 143 - 145]. Most importantly, we have demonstrated complementary effects between RF ablation and liposomal doxorubicin in this model (see Sections C.2f and C.5c).

To facilitate tumor growth, moderate immunosuppression will be administered in the form of oral cyclosporin A at 10 mg/kg bid for 3 days prior to injection to 14 days post-implantation, followed by 10 mg/kg qd. Female dogs (20 – 50 kg) will be inoculated subcutaneously with 1×10^7 tumor cells under direct ultrasonographic guidance and will be monitored every 7 - 10 days to determine tumor growth. Fresh tumor will be initially harvested from a live carrier, and prepared in our standardized manner as previously described [47]. To maximize the benefits from each animal and increase the usable tumor yield of this model to $> 90\%$, each animal will be injected with multiple tumors. This approach can be justified given that this untreated tumor burden has not been shown to alter intratumoral doxorubicin accumulation or tumor destruction in this model. Each animal will have four injections underneath a nipple in each of the four quadrants of the abdomen, and four injections on the back. Solid, non-necrotic tumors measuring 4 - 6 cm in diameter (10 – 18 weeks approximate incubation) will be used. This range in tumor size is necessary given expected variability in tumor growth. For this model, tumor yield is anticipated at 3 – 4 tumors per animal.

RF application: RF will be applied using a 500 kHz RF generator capable of an output 2,000 mA (200 Watts) (Radionics, Model CC-1). This generator has been selected because it is the most powerful that is currently available for clinical practice and is capable of monitoring relevant ablation parameters. Four standardized grounding pad electrodes will be placed on the animal's thighs and back. One of the most commonly used electrodes in clinical practice, a 3 cm, internally-cooled electrode will be used to apply RF energy [42]. Pulsed high current RF energy will be applied for 12 minutes according to a well-validated algorithm that has been shown to maximize energy deposition and tissue coagulation [44]. This standardized method of RF application provides reproducible coagulation (i.e., a std. deviation ≤ 0.3 cm) [42]. Total energy deposition is expected to vary with local impedance and the extent of baseline RF coagulation is expected to vary among the treatment groups, ranging from 3.1 – 4 cm. RF parameters including: tip temperature, tissue impedance, and applied current will be recorded throughout the procedure.

Liposomal doxorubicin: A commercially available liposomal doxorubicin preparation (Doxil; ALZA pharmaceuticals, Mountain View, CA) will be slowly administered into a large extremity vein over 30 seconds via an 18 gauge angiocath. The phosphotidyl choline – cholesterol liposome in this preparation is approximately 100 nm in diameter and is stearylly stabilized with polyethylene glycol to increase circulation time [146]. Doxorubicin concentration is 2 mg/mL.

Pathologic studies: Animals will be sacrificed at defined endpoints. Tumors will be excised and sectioned at 3-mm intervals through the entire region of ablation. The extent of visible coagulation at gross pathology will be measured with calipers by consensus of at least two observers who will be blinded to the treatment group at the time of measurement. Histopathologic studies will include hemotoxylin-eosin staining, as well as staining for mitochondrial enzyme activity by incubating thin representative tissue sections for 30 minutes in 2% 2,3,5-triphenyl tetrazolium chloride (TTC). This latter test determines irreversible cellular injury during early stages of RF induced necrosis [66, 67]. The effect of the RF treatment on intratumoral vascularity will also be assessed by IV administration of 50 ml of the perfusion

agent, 2% Evans' Blue in representative animals 15 min prior to sacrifice [46, 66].

Data analysis: Convergence of the simplex results will define optimal parameters for increasing tumor destruction by combining RF ablation with liposomal chemotherapy. Partial differential equation analysis of the data generated in the iterations of simplex optimization will also be performed to determine the degree and form of interaction between the variables and their influence on tumor destruction [132].

Anticipated Results: We anticipate that increased tumor destruction from the combined paradigm can be optimized to a definable predictable maximum at specified RF and liposomal doxorubicin parameters. We further predict that the results will be predominantly influenced by RF thermal dosimetry, as preliminary data suggest a wide range of efficacious liposomal doxorubicin dosing. Although preliminary data suggest that a wide range of doxorubicin doses do not largely influence tumor destruction in other animal models [67], we will test this by including doxorubicin dose in our simplex model. We will subsequently analyze the data to determine whether this variable influences the results, and if so, perform systematic univariate analysis to better delineate its role on coagulation. Likewise, if simplex identifies other dependent variables that have not been previously optimized, these variables will be systematically studied to determine their true influence.

Pitfalls: It is conceivable that our initial estimates of the maximal gain will not occur within our initially selected defined range of parameters. However, multivariate simplex modeling will be able to readily identify the need for further study along one of the continuous variables, as it will suggest that the simplex model has not converged and point to the most efficient solution. It is also possible that we have underestimated the number of iterations necessary to achieve convergence. If this occurs we will increase the number of points studied until convergence is achieved. It is also theoretically possible that we will not achieve convergence due to multiple maxima or an oscillatory collapse about a false maxima. To minimize this possibility, we will test our system every 10 iterations along the lines outlined by Spendley et al. [141] for these events, and in the unlikely event that these occur, we will transpose our simplex as proposed by these investigators.

One potential pitfall is that higher thermal doses from RF ablation alone could completely ablate enough of the tumors so that optimization will not be possible. We find this to be unlikely given our prior work in this model where we have been unable to produce tumor destruction larger than 3.5 cm in diameter using RF alone [47]. However, if we are fortunate enough to identify a set of parameters that reproducibly enable the destruction of 5 – 6 cm tumors, we will immediately commence the studies with empty liposomes (Experiment 4) to determine whether an equivalent effect can be obtained without the need for doxorubicin.

Experiment 2. Quantify the amount of doxorubicin in RF ablated CVS tumors using spectrophotometry.

Objective: Determine the influence of RF thermal dosing and parameters of drug administration on the spatial distribution and local concentration of intratumoral doxorubicin accumulation.

Question asked: In what ways do liposomal doxorubicin dosing and RF thermal parameters influence intratumoral liposomal drug *accumulation* and *distribution* within an RF ablated tumor ?

Hypothesis: Increased tumor destruction is due in part to increased delivery of the drug in the liposomal form to RF ablated tissue.

Rationale: Section C.5 presents data demonstrating seven fold greater doxorubicin accumulation when IV liposomal doxorubicin is given following RF therapy compared to non-ablated tumors [68]. Increased tumor destruction for this combination is likely due in part to increased tumor drug concentrations. However, this increase in doxorubicin accumulation occurs only under some, but not all, thermal conditions (Section C.5d). Further quantitative studies will enable us to better define the anticipated intratumoral drug concentrations at optimal RF parameters, and in conjunction with Exp. 3 will enable us to demonstrate how this uptake is influenced by tumor heating. Thus, this experiment will also assess the unique potential of RF ablation to achieve a main aim of chemotherapy which is often thwarted by high interstitial tumor pressures - to preferentially drive as much chemotherapy into the tumors and away from normal tissues [84].

Sections C.5b&c also demonstrate that the spatial distribution of liposomal drug accumulation is not uniform throughout an RF ablated tumor [68]. However, it is currently unknown in what ways RF and thermal dose parameters alter the distribution of the liposome accumulation, and the relationship between liposomal distribution and increased tumor destruction is also currently insufficiently defined. Yet, this

has important implications for *defining both how and where tumoricidal interaction occurs*, as well as for *defining how RF can be used to increase the deposition of liposomally delivered substrates*. Here, fluorescent spectrophotometric assessment of doxorubicin uptake will be used to determine the actual concentration of drug delivered to various portions of the RF ablated tumor.

Study design: CVS tissue obtained from Experiment 1 will be analyzed for doxorubicin content. Intratumoral accumulation of doxorubicin will be quantitated using fluorescent spectrophotometry as described below. Representative tissue samples will be collected from specified portions of the tumor including: the central zone of RF coagulation, the peripheral zone of increased tumor destruction, the 1 cm zone of tissue peripheral and adjacent to ablative changes, and apparently normal, unablated distant tissue on the same tumor. The amount of doxorubicin accumulating in each of these zones will be compared for tumors treated with RF alone, and RF combined with liposomal doxorubicin. All of the thermal parameters and dosing strategies tested in prior experiments will be interrogated. The relationship between the RF parameters of thermal dose (i.e., duration of heating), liposomal doxorubicin dose, timing and measurement interval with intratumoral doxorubicin accumulation will be mathematically modeled.

Quantitation of intratumoral doxorubicin uptake: The fluorescent properties of doxorubicin will be used to quantify the amount of intratumoral doxorubicin. Selected representative samples of tumor (0.25 – 1 g) will be collected from the defined regions of the tumor. These samples will be weighed and homogenized in acid alcohol (0.3N HCl, 50% EtOH) for 24 hours at 5° C to ensure complete extraction of all bound and free doxorubicin. For each tumor, three representative sections will be obtained from each of the four defined regions to minimize sampling error. As a control, an unablated tumor from each dog will be subject to similar doxorubicin extraction. Extracted doxorubicin will be quantified by fluorescent spectroscopy at an excitation wavelength of 470 nm and by measuring the intensity of emission at 590 nm [99]. Doxorubicin accumulation will be measured in terms of $\mu\text{g}/\text{G}$ tissue, based upon standardized concentration curves constructed for each experimental assay.

Sample Size: The anticipated difference in intratumoral doxorubicin uptake relative to the standard error of measurement is greater than the anticipated difference in increased tumor destruction relative to its standard error. Thus, Monte Carlo simulations suggest that the data points generated in Experiments 1 & 3 will likely produce sufficient data to establish the relationship between intratumoral doxorubicin accumulation and RF and liposomal doxorubicin parameters.

Data analysis: We will first define the relationship between intratumoral doxorubicin accumulation and the dose of IV liposomal doxorubicin for the specified regions of individual ablated tumors. Subsequently, we will define the relationship between intratumoral doxorubicin accumulation and the RF thermal dose, as well as the dose of IV liposomal doxorubicin, timing and measurement interval. The ratio of accumulation to thermal dose and other parameters will be plotted and subject to linear and non-linear regression analysis to determine the best fit for the response curve using Chi-square functions [147].

As a secondary analysis, we will also compare increased doxorubicin accumulation between the RF and paired, control non-RF tumors for each set of liposomal doxorubicin parameters. One-way Analysis of Variance will be performed. Pair-wise T-tests ($\alpha = .05$; two-tailed test) based upon the Least Square Means will be subsequently performed, if and only if the overall p-values are significant. Dunnett's T-test and Tukey's test will be used in the analyses of variance comparing groups to correct for multiple comparisons.

Anticipated results: We expect a linear relationship between the amount of liposomal doxorubicin administered and intratumoral doxorubicin accumulation. We predict that increased drug accumulation will correlate with tumor destruction to a maximum. It is also possible that we will not observe a one to one correlation between intratumoral doxorubicin accumulation and IV liposomal doxorubicin administration, particularly at higher doses. Defining such saturation kinetics for either tumor destruction or drug accumulation is important for clinical studies, as they would help define appropriate dosimetry of potentially toxic drugs. Even if we do not demonstrate that the increase in coagulation is completely dependent upon increased accumulation, the data generated will be important for other systems where dose dependence of doxorubicin (or other therapies) may play a greater role.

We anticipate demonstration of increased drug accumulation with a well-defined spatial distribution that conforms with the zone of increased tumor destruction over RF-induced coagulation alone. Together, these data would provide further justification for pursuing expanded study of the unique potential of using RF ablation as a method for inducing preferential delivery of many types of liposomally encapsulated agents to tumors and other tissues. Alternatively, we will find that a larger area of increased liposomal distribution will be observed at higher thermal doses. Lack of correlation would suggest that other

mechanisms play a greater role mediating increased treatment effect.

Potential pitfalls: It is conceivable that we will find a non-linear correlation between administered liposomal doxorubicin and intratumoral doxorubicin accumulation. As such, further data points may be necessary to better define the relationship mathematically and/or define parameters for maximum doxorubicin accumulation. If subsequent study (see Aim 2) continues to bear out the importance of doxorubicin as a key mediator of this interaction, we will perform simplex optimization using the metric of “increased intratumoral doxorubicin accumulation.” If additional points are required to define the relationship between liposomal doxorubicin accumulation and the drug and RF parameters, Box-Wilson and skewed factorial grid search techniques outlined in Experiment 3 will be performed as well.

Experiment 3. Characterize thermal parameters that increase liposomal uptake and tumor destruction

Goal: Characterize the relationship between the directly measured intratumoral thermal dosimetry associated with increased doxorubicin accumulation, RF induced coagulation and liposomal doxorubicin mediated increased tumor destruction. This will elucidate the role of hyperthermia in the combined interactions, threshold thermal dosage, and the thermal conditions under which combined therapy is most effective thereby permitting the prediction of clinical outcomes when using combined therapy.

Objectives: To a) define the relationship between thermal dosimetry and the ultimate volume of tumor destruction; b) elucidate the threshold and optimal range of thermal dosimetry at which combined interaction works, and c) determine the influence of thermal dose on the spatial distribution of intratumoral doxorubicin to define the range and extent of control attainable for local drug concentration and distribution.

Questions asked: a) At what local intratumoral thermal dosimetry (tissue temperatures and heating duration) is increased liposomal uptake and increased tumor destruction observed ?

b) Can the threshold for either RF induced coagulation or the increased zone of liposomally mediated tumor destruction be related to classic parameters of hyperthermic dose?

Rationale: Section C.2e demonstrates that both parameters of thermal dose (i.e., the temperature of RF ablation as well as duration) influence the amount of tumor destruction when RF is combined with liposomal chemotherapy. Although clinically acceptable thermal doses for RF heating alone (i.e., 4 – 6 minutes of heating at 50 - 55° C) have been reported [75], data of Section C.2e and prior hyperthermia studies [88, 104, 105] suggest that this likely to be altered several degrees lower for a given heating duration by the presence of liposomal doxorubicin. Given that combined liposomal doxorubicin and RF can be used to create large, but currently unpredictable volumes of tumor destruction, it is important to determine the thermal dosimetry prior to the initiation of clinical studies. *Such quantitation is of critical importance if we are to match the initial tumor size to an appropriate volume of tissue destruction in a predictable fashion when treating patients.* However, the formal relationship between these parameters, specifically the determination of threshold thermal doses, has not been well defined. Thus, systematic study and definition of the relationship between RF thermal dose and tumor destruction is required and proposed here.

The definition of accurate thermal intratumoral thermal dosimetry might also allow us to *gain better mechanistic insight* into combined RF ablation with liposomal doxorubicin by enabling better comparison and translation of the hyperthermia literature. Specifically, validation of hyperthermia thermal dosimetry (as to whether or not it extends to the temperatures of RF ablation [$> 50^{\circ}$ C]) is important as it will determine the applicability of the well-defined mechanisms of lower temperature hyperthermia to RF thermal therapy [61-63, 78]. This would include inferences into how doxorubicin functions in our system [148], and how endothelial leakiness increases intratumoral liposome accumulation [113 - 115]. The definition of thermal dosimetry may also facilitate translation of our results when combining liposomal doxorubicin with other high temperature thermal ablation technologies such as ultrasound, microwave, and laser.

Definition and characterization of the threshold parameters that induce increased tumor destruction for combined therapy is also necessary for *designing optimal strategies of RF application*. Currently, additional RF treatments are applied to regions of the tumor that are heated $< 50^{\circ}$ C, as detected by MR imaging or remote thermometry [11, 12, 149, 150]. Thus, knowledge of the actual thermal dose responses and threshold temperature(s) at which tumor destruction can be anticipated from the combined therapy would be an essential and indispensable clinical tool. First, it could potentially be used to decrease the

number of RF applications necessary to treat a given size tumor (which entail increased patient risk and substantial time and cost penalties to perform). Second, it could prevent over-treatment of tumor margins (an important *clinical safety* issue when tumors abut structures, such as the colon, gallbladder or bile ducts) [151].

Experimental study design: Experiments will be performed concurrently during RF ablation of the canine venereal sarcoma tumors treated in Experiment 1. The actual threshold thermal dose to induce tumor destruction in tumors ablated with RF alone or with combination therapy will be determined by comparing the extent of tumor destruction with thermal mapping of temperatures throughout the tumor. The relationship between thermal dose and duration of RF heating to achieve optimal and or threshold tumor destruction will be calculated and compared between RF alone and RF combined with liposomal doxorubicin. Measurements of liposomal doxorubicin uptake will be correlated with tissue samples obtained at the exact location of defined thermistors. The relationship between RF thermal doses and doxorubicin accumulation will be determined by comparing measured temperatures with intratumoral doxorubicin by quantitatively assessing additional tissue samples that were immediately adjacent to thermistors during RF heating using fluorescent spectrophotometry as outlined in Experiment 2.

To ensure appropriate interrogation of thermal dosimetry, Box-Wilson and skewed factorial grid search techniques [136, 152] will identify any additional parameters of RF duration and liposomal doxorubicin dosimetry required, based upon an overlay of initial data obtained in Experiment 1. The grid will be optimized to cover three key regions of interest 1) peak thermal dose (likely covered by the simplex of Experiment 1); 2) regions of greatest variability of response to liposomal doxorubicin and RF dose; and 3) threshold doses to observe increased tumor destruction. Based upon initial data, RF heating will be varied from 1 to 30 min, and liposomal doxorubicin dose from 0 – 38.3 mg/kg (LD₅₀) [128]. Multiple ablations in several animals will be performed at optimal RF and drug parameters to assess animal to animal variability.

Temperature measurements: Direct measurement of tissue temperatures will be performed over other strategies, as this method is most commonly used in clinical practice and is therefore most directly translatable [5, 14]. Multiple thermal sensors will be placed at 5-mm intervals from the tip of the electrode to the edge of the tumor and into surrounding tissues, to at least 5 cm (i.e., beyond the ablation volume), to enable the capture of thermal data for all points at which temperature is elevated [46, 47]. Tissue temperatures over the dynamic range of tissue heating will be monitored during RF application using laser-Doppler thermometry, as these probes are not perturbed by RF interference. Correct positioning of temperature sensors will be assured using a stabilizing device and ultrasound visualization and guidance.

In addition, a thermal sensor will be placed perpendicular to the midpoint of the electrode to measure a continuous temperature profile throughout the ablation volume at defined intervals to obtain a thermal map of heating throughout the entire tumor, including hyperthermic temperatures.

Sample size: Monte Carlo simulations of the Box-Wilson factorial design strategy suggests that in addition to the parameter combinations concentrated about the peak (Experiment 1), an additional 10 parameters will be sufficient to adequately model the thermal dose threshold in terms of temperature and duration. Conservatively, an additional 6 points will be required to determine the mathematical form of the response to liposomal doxorubicin dose to a given thermal dose. Thus, a total of 16 additional parameters will be studied (for a total of 41). As justified in Experiment 1, each parameter is comprised of two components (i.e., RF + IV liposomal doxorubicin, and RF alone). These will be run in triplicate to reduce standard error to $\leq 10\%$, for a total of six tumors for each combination of parameters. Thus, a total of 96 tumors will be required ($16 \times 6 = 96$). Conservatively, these data can be obtained from 35 dogs.

Data analysis: The ultimate coagulation diameter of ablated tissue following combined RF and IV liposomal doxorubicin [versus RF alone or RF combined with IV liposomal control (Experiment 4)] will be characterized as a function of thermal dose delivered both inside and outside of the RF ablated tissue. Additionally, both the white zone of RF -induced coagulation and the zone of liposomal doxorubicin mediated increase in tumor destruction (i.e., the zone of presumed hyperthermic temperatures) will be analyzed separately and correlated with temperatures and thermal dose dosimetry. The thermal dose at given distances from the electrode will be calculated as duration X directly measured temperature and defined for discrete distances from the electrode. Thermal dosimetry will be further calculated in terms of equivalent minutes at 43° C, the standard measure for hyperthermia [78], to enable determination of the utility of this parameter for high-temperature ablation. Multivariate multiple regression analysis will be performed to correlate thermal dose with intratumoral liposomal doxorubicin uptake, and RF current applied. This will permit the calculation of the threshold thermal doses for increased doxorubicin uptake

and the definition of the relationship between RF dosing and ultimate tumor destruction. We will further define the relationship between the local thermal dose and the spatial distribution and regional intensity of intratumoral doxorubicin using linear and non-linear mathematical modeling.

Anticipated results: We predict a relationship between thermal dose and the spatial distribution and regional intensity of intratumoral doxorubicin, with greatest accumulation at defined isotherms near the threshold for RF induced coagulation. We further anticipate defining a threshold thermal dose associated with increased tumor destruction, and that a relationship between thermal dose to increased tumor destruction in the RF ablation system can be mathematically modeled. We also predict that liposomal doxorubicin enables tumor destruction at lower temperatures than RF ablation alone, in the range of 45 – 50° C. We also anticipate defining the threshold thermal dosage at which liposomal doxorubicin mediates an increase in tumor destruction and that the size of this zone correlates linearly with the predicted 43° C equivalent minute model. Confirmation of correlation of induced tumor destruction to the 43° C equivalent minute model is important as thermal dosimetry in this range has been less well studied. Regardless, we anticipate defining an appropriate temperature baseline to determine dosimetry time equivalent doses for RF-induced coagulation and liposomal doxorubicin mediated increase in tumor destruction.

Specific Aim 2: Test specific hypotheses about the possible role of the liposome carrier components

Experiment 4: Optimize parameters for RF ablation with empty liposomes alone, and compare the thermal dosimetry at which increased tumor destruction occurs with RF and empty liposomes to that of RF and liposomal doxorubicin. This will define the relative contributions of the lipid to the improved treatment effect of the combined treatment paradigm. By serving as an appropriate control for comparative studies, this experiment will also help elucidate the role of doxorubicin in this interaction.

Questions asked: a) To what extent is the increase of tumor destruction from combined RF ablation with liposomal preparations due to synergy between RF heating and the carrier components itself?

b) To what extent is the increase in tumor destruction from combined RF ablation and liposomal doxorubicin due to an increase in doxorubicin accumulation within the tumor?

Rationale: Preliminary studies (Section C.6) document that at least some of the antitumoral effect of liposomal doxorubicin is due to the liposome interacting with RF heating. The study of RF combined with empty liposomes, including the determination of the parameters and thermal dosimetry at which interaction between RF and empty liposomes occur, is important as it will enable us to gain an appreciation of the underlying mechanism based upon prior published studies in hyperthermia [61 - 63, 78]. It may also enable us to devise safer, less toxic, and more efficacious therapies for use with RF ablation.

Study Design: We will study RF combined with saline-filled liposomes, including the determination of the parameters and thermal dosimetry at which interaction between RF and empty liposomes occur. Animal models and set up will be performed as outlined for Experiment 1, and thermometry will be performed as outlined for Experiment 3. For our initial experiments, we will use liposomes of similar composition to those used in Aim 1, but without doxorubicin at the same doses and RF parameters as the Simplex Optimization from Specific Aim 1. This will enable us to use the doxorubicin uptake of the first set of experiments to determine actual intratumoral liposome doses, and permit direct comparison between the efficacy of RF and liposomal doxorubicin vs empty liposomes alone. Experiments will also be performed to determine the degree of dose dependence for the liposomes. Assessment of intratumoral doxorubicin uptake and parameters of thermal dosimetry will be assessed as outlined for Experiments 2 and 3, respectively.

Preparation of liposomes: Liposomes exactly repeating the lipid composition and size of Doxil will be prepared. A lipid mixture of 6.38 mg of N-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (MPEG-DSPE), 19.16 mg of fully hydrogenated soy phosphatidylcholine (HSPC), and 6.38 mg of cholesterol will be prepared using solutions of individual lipids in organic solvents. The lipid film obtained will be dispersed in 2 ml of 10 mM HEPES, 140 mM NaCl, pH 7.4 (HBS). Liposomes will be formed by extrusion of the crude lipid dispersion through appropriately sized polycarbonate filters at 50° C. The size of liposomes obtained will be measured by dynamic light scattering using Coulter® N4 Plus Submicron Particle Sizer to ensure that the liposomes measure 100 ± 20 nm. Incorporation of 5% GLA and 1- 5% arachidonic acid will be added for Experiment 5b.

Sample Size: Monte Carlo simulations suggest that 8 parameters about the peak parameters determined in Experiment 1, where RF alone and RF and liposomal doxorubicin were administered, will be sufficient to adequately determine thermal dosimetry and maximal tumor destruction (assuming translation of result with liposomal doxorubicin to empty liposomes alone; see Potential Pitfalls). Once the optimum RF parameters are established, a univariate determination of the effect of liposome dose will be performed through the maximum. This will include a control of no liposome. Monte Carlo simulations further demonstrate that this will require an additional 6 data points, and that together these 14 parameters will likely be sufficient to determine the relationship between thermal dosimetry and improved tumor destruction from empty liposomes. Given that RF controls will already be available for 8-parameter combinations, 24 tumors (8 dogs) will be required for the optimization phase, and an additional 36 tumors (6 x 6 = 36; 12 dogs) will be required for the dose-dependency study. Thus, at least 20 dogs will be studied.

Data Analysis: Convergence of the simplex results will define optimal parameters for increasing tumor destruction by combining RF ablation with empty liposomes. Results of this experiment will also be correlated with the results of Experiment 1 using linear and non-linear regression analysis to determine the extent that increased tumor destruction is due to empty liposomes. Subtraction masking techniques will be used to determine and mathematically model the role of increased focal intratumoral doxorubicin accumulation in the combined paradigm [152]. Thermal dosimetry will be calculated as outlined for

Experiment 3 and compared with the results of that experiment. Thus, this experiment will also serve as a necessary control to the prior experiments.

Anticipated results: We anticipate demonstrating increased tumoricidal efficacy when combining RF with empty liposomes compared to RF alone, but demonstrating that liposomes with doxorubicin have even greater efficacy. This will establish the importance of adding chemotherapy to optimize tumor destruction. Alternatively, we may find equivalent or near equivalent tumoricidal efficacy administering empty liposomes compared to liposomal doxorubicin. This finding would also be of importance as it would lead us to administer a potentially less toxic preparation for clinical trials.

As for Experiments 2 and 3, we anticipate demonstrating a linear relationship between the amount of liposome administered and induced tumor destruction for a given thermal dose. We predict an increase in tumor destruction compared to RF alone, but less than that with liposomal doxorubicin. (This is important, as it will enable us to determine the utility of adding the more toxic doxorubicin). It is also possible, but less likely that there will be no correlation between the liposome dosage and the extent of tumor destruction. This could potentially be due to saturation effects. As explained in Experiment 2, this too would be important, as it would define a minimal concentration of liposome necessary to achieve optimal results. Indeed, if lower liposome doses are found to be equally efficacious, we will attempt to construct liposomes containing even greater concentrated doxorubicin with the lower lipid dose.

Potential Pitfalls: If we do not observe concordance between the optimal settings for RF combined with liposomal doxorubicin with that of empty liposomes, we will proceed to determine the optimal parameters for empty liposomes combined with RF ablation using simplex optimization, as outlined for Experiment 1.

Future studies: If any empty liposome preparation (Experiment 4 or 5) demonstrates equal or greater efficacy than liposomes containing doxorubicin, we will continue with safety studies as outlined for Specific Aim 3 as a necessary step for translating the use of these compounds to clinical practice.

Experiment 5: Determine whether increased tumor destruction seen with liposomal treatment during thermal ablation can be attributed to *enhanced oxidative stress and lipid peroxidation*.

Hypothesis: Increased tumor destruction observed when adding liposomes to RF ablation is due to enhanced oxidative stress and lipid peroxidation (presumably via free radical mediated cytotoxicity).

Overall plan: Two experiments (5a & 5b) are proposed to assess this hypothesis. In 5a, we will attempt to diminish or enhance tumor destruction by inhibiting or enhancing oxidative stress and lipid peroxidation via pharmacological manipulations that increase or decrease cellular antioxidants (vitamins E and C as well as thiols). In 5b, we will attempt to potentiate tumor destruction by providing lipid substrates that enhance lipid peroxidation via incorporation of polyunsaturated fatty acids (GLA and arachidonate) into the liposome vesicles. In both experiments, parameters indicative of lipid peroxidation and oxidative stress will be monitored in tissues undergoing RF ablation to determine if the desired effects on these parameters have been achieved and if they correlate with increases and decreases in tumor killing.

Experiment 5a: Compare endpoints indicative of oxidative stress and lipid peroxidation with tumor destruction following administration of liposomal lipids in the presence and absence of inhibitors of lipid peroxidation to try and establish a causal link between these phenomena.

Rationale: Lipid peroxidation chain reactions occur in three phases: initiation, propagation, and termination reactions. Initiation reactions usually involve an oxidant-(such as hydroxyl radical)-mediated abstraction of a hydrogen atom from a polyunsaturated fatty acids (or cholesterol) to form $-R^{\bullet}$. Once formed, the lipid radical ($-R^{\bullet}$) can react with O_2 to form $-ROO^{\bullet}$, which can then propagate the chain reaction by abstracting a hydrogen atom from a neighboring polyunsaturated fatty acid to form another $-R^{\bullet}$ and $ROOH$, which is a lipid (or cholesterol) hydroperoxide. These lipid hydroperoxides ($ROOH$ s) are cytotoxic as well as highly reactive with transition metal ions (like Fe and Cu) and capable of further propagating lipid peroxidation chain reactions as well as decomposing to form lipid aldehydes [i.e., 4-hydroxy-2-nonenal (4HNE)], which are also toxic. The lipid hydroperoxides and lipid aldehydes formed during lipid peroxidation chain reactions are detoxified enzymatically via glutathione (GSH) dependent reactions involving glutathione peroxidases and glutathione transferases [153 - 155] and by small molecule antioxidants [156, 157]. These antioxidants, such as vitamin E (α -tocopherol), can donate a hydrogen atom to $-R^{\bullet}$ to form $-RH$ and terminate lipid peroxidation chain reactions. The tocopheroxyl

radical formed in the previous reaction can then be reduced by a reductant such as vitamin C to regenerate α -tocopherol which is then available to scavenge another lipid radical. In this way vitamins E and C work in concert to limit lipid peroxidation chain reactions and protect against oxidative stress [156, 157].

We have selected the CVS model for these experiments, over small animal models, to be consistent with the remainder of the proposal. This decision is further beneficial in that the larger animal model will permit us adequate sampling and differentiation between the regions of RF-induced coagulation, the liposome-mediated increase in coagulation, and untreated tumor. The clinically relevant tumor size is particularly important for our experiments in 5b, where we will attempt to potentiate the interaction with fatty acids having greater lipid oxidation potential. Indeed, although we have already shown (see section C.6a) that one such polyunsaturated fatty acid, GLA, increases tumor destruction in a small animal model, it is important to define the maximum gains in tumor destruction that can be achieved using these agents in appropriately scaled large animal models prior to translation into clinical practice.

Study Design: We will assess both enhancement and inhibition of lipid peroxidation. Buthionine sulfoximine (BSO), a clinically available inhibitor of GSH synthesis, has been used as strategy for enhancing cell killing during lipid peroxidation reactions in intact animals [157]. BSO (4 mmol/kg) will be injected SQ 4 days prior to thermal ablation to deplete glutathione and enhance the cytotoxicity of byproducts of lipid peroxidation. Depletion of GSH in tumor tissue will be verified using a spectrophotometric recycling assay available in Dr. Spitz's lab [158]. In separate animals, a combination of α -tocopherol (100 mg/kg in vegetable oil) and vitamin C (100 mg/kg in saline) will be administered SQ daily for 4 days prior to thermal ablation \pm liposomes because this protocol has been shown to inhibit lipid peroxidation and oxidative stress in animals [157 - 160]. The levels of vitamin E in tumor tissue will be confirmed using HPLC, as described previously [157]. In all cases, the animals will be treated and evaluated histologically to determine the extent of injury and the tissue specimens carefully harvested in the presence of metal chelators (1 mM DETAPAC) and antioxidants (50 μ M BHT) (to inhibit artifactual oxidation of the sample *ex vivo*) and shipped immediately to Dr. Spitz's lab for analysis [161]. Increases in lipid peroxidation will be confirmed using Western blotting of tumor tissue homogenate with antibodies that specifically recognize 4HNE-modified proteins that are commercially available (Alpha Diagnostics, San Antonio, TX) [162] as well as using the thiobarbituric acid reactive substance (TBARS) assay utilized in Dr. Spitz's lab with malondialdehyde as the standard [163]. In addition, a well accepted endpoint indicative of oxidative stress is the ratio of GSH to glutathione disulfide (GSSG) [164]. This will also be monitored in tumor tissue using standard spectrophotometric assays available in Dr. Spitz's lab. The diameter of liposome mediated increased tumor destruction will serve as the metric of tumor injury for all these experiments. The extent of tumor destruction will again be correlated with parameters indicative of lipid peroxidation and oxidative stress in an attempt to define a potential causal relationship.

Study groups: Studies will be performed using optimal RF and liposome parameters established from Experiment 4. Initially, a total of 12 groups will be studied (6 with RF ablation and 6 without RF ablation to serve as control). These include combination of a) empty liposomes with the Vitamin C / Vitamin E inhibitor, or b) enhancer of oxidative stress and lipid peroxidation (BSO), c) empty liposomes alone, d) the inhibitor or e) enhancer alone, and f) normal tumor. Next, three groups will then be injected with liposomal doxorubicin – a) alone and in the presence of b) the inhibitor or c) the enhancer - both with and without RF (for a total of 6 additional groups) and then evaluated to help determine the role of doxorubicin in the observed reactions. Endpoints of lipid peroxidation will be measured in different parts of the treated tumor, as outlined in Exp. 2 to enable more precise analysis of the spatial distribution of the reaction.

Sample Size: Given that the standard deviation for coagulation in this model is typically \pm 0.6 cm [47], power calculations predict that treatment of five tumors in each group will render an 80% capability of detecting an 8 mm difference between paired groups at a corrected significance level of 0.05. Thus, with 5 tumors (2 dogs) required for each of the 18 (12 + 6) groups, a total of 36 dogs are needed.

Data Analysis: We will compare endpoints indicative of oxidative stress and lipid peroxidation with tumor destruction following administration of liposomal lipids in the presence and absence of inhibitors or enhancers of lipid peroxidation to try and establish a causal link between these phenomena. Dunnett's T-test and Tukey's test will be used following the analyses of variance comparing groups to correct for multiple comparisons. We will further compare endpoints of lipid peroxidation with spatial distribution of doxorubicin (Experiment 2). Mathematical modeling of the dose response curve will also be performed.

Anticipated Results: It is expected that thermal ablation will result in increases in 4HNE-modified proteins, TBARS, and the ratio of GSSG/GSH, which will indicate that oxidative stress and lipid

peroxidation is being induced. It is also anticipated that treatment with liposomes and thermal ablation will enhance cytotoxicity to the tumor tissue as well as result in increases in endpoints indicative of oxidative stress and lipid peroxidation, relative to thermal ablation alone. Furthermore, **depletion of glutathione** using BSO prior to thermal ablation \pm liposomes **should further enhance tumor toxicity** as well as endpoints of lipid peroxidation and oxidative stress. It is expected that pretreatment with vitamins E and C will result in protection against thermal ablation-induced injury in the tumors and parameters indicative of lipid peroxidation and oxidative stress will be blunted.

The proposed hypothesis predicts reduced, but not completely eliminated increases in tumor destruction from liposomal *doxorubicin* combined with the inhibitor, as the effect of doxorubicin and hyperthermia is not expected to be blocked if doxorubicin is playing a major role in this interaction. Yet, it is possible that we will observe complete elimination of the enhanced liposomal doxorubicin mediated effect in the presence of antioxidants because doxorubicin is a quinone capable of redox cycling to produce oxidative stress as well as being a DNA intercalating agent [165]. This possibility will be further assessed by comparing measured lipid peroxidation to the amount of tumor destruction observed. It is also possible, but less likely, that we will not see an effect on the amount of tumor destruction in the presence of the selected inhibitors and enhancers of lipid peroxidation and oxidative stress. Potential Pitfalls describes how we plan to address this.

We anticipate demonstrating a linear relationship between the amount of liposome and the amount of lipid peroxidation observed. It is also possible that there will be progressively increasing enhanced oxidative stress without corresponding increased tumor destruction. This would warrant further study in other tissues (including normal tissue, as outlined in Specific Aim 3) and other tumors where increased lipid peroxidation has been shown to lead to greater cytotoxicity [124 - 126]. It is also possible that no correlation will be found between lipid peroxidation and tumor cytotoxicity. This will lead us to explore other mechanisms as outlined in Potential Pitfalls. We further anticipate greatest measurement of endpoints of lipid peroxidation to correlate with the spatial distribution of doxorubicin measured in Experiment 2. If this does not occur, we will perform experiments to determine whether this discrepancy is due to non-linear dose dependency of lipid peroxidation or intratumoral dissociation between the liposome carrier and the doxorubicin.

We anticipate that thermal dosimetry for optimal effect will be for empty liposomes alone and will be similar to that for liposomal doxorubicin. If different thermal doses are required, it would point to the fact that the enhanced tumoricidal effect of doxorubicin is not due solely to oxidative stress mechanisms.

Potential Pitfalls: Subcutaneous injection of the initially selected inhibitors and enhancers of oxidative stress and lipid peroxidation may not provide sufficient intratumoral concentration to elicit the desired effects on lipid peroxidation. Thus, it is possible that a single negative result may not exclude lipid peroxidation as a responsible mechanism. If this occurs, we will perform ultrasound-guided intratumoral injection of these agents. Additional experiments using other free radical inhibitors/scavengers such as adenoviral vectors that enhance superoxide dismutase, glutathione peroxidase, or catalase activity (available in Dr. Spitz's lab) will also be studied if we cannot block the effect of the empty liposomes in our initial experiments [166].

Future Study: If the hypothesized results are obtained, then this mechanism of injury will be further confirmed by pre-treating the animals with N-acetylcysteine (SQ 100 mg/kg) [157] for 4 days prior to thermal ablation to determine if this thiol antioxidant, which is capable of contributing to the detoxification of lipid peroxidation byproducts, can inhibit the toxicity seen during thermal ablation. The endpoints indicative of oxidative stress, lipid peroxidation, and thiol antioxidant content (including HPLC analysis of NAC) will be determined using standard techniques in Dr. Spitz's lab [167, 168].

Experiment 5b: This experiment combines RF ablation with liposomes containing fatty acids having greater oxidative potential by virtue of containing more unsaturated double bonds which provide the substrate upon which lipid peroxidation chain reactions are propagated (GLA, arachidonic acid [128 - 129]), to determine whether manipulation of the substrate upon which lipid peroxidation occurs can further potentiate the antitumor effect of thermal ablation.

Rationale: Alterations in polyunsaturated fatty acid content have been shown previously to enhance lipid oxidation as well as oxygen toxicity in isolated lipids and cultured cells [129, 160, 169]. Thus, the proposed experiment is the logical extension of these studies into the field of thermal ablation \pm liposomes to test our hypothesis regarding oxidative stress and lipid peroxidation as the mechanism of enhanced

injury.

Study Design: Initially, we will utilize liposomes enriched with GLA and arachidonic acid in the presence and absence of RF in the CVS model, as outlined for prior experiments. The fatty acids will be obtained in their pure form from Cayman Chemical Company, converted to the sodium salt and incorporated into the liposome preparations as described [129, 160]. The liposomes will then be subjected to fatty acid analysis as previously described to verify the incorporation of the desired fatty acid. This will also be done with the tissues from animals treated with the liposomes [129, 160]. Once the incorporation of the desired fatty acids has been confirmed the tumors will be subjected to thermal ablation and the parameters indicative of oxidative stress and lipid peroxidation will be determined as in Experiment 5a and correlated with changes in the parameters indicative of tumor destruction. These results will then be compared to animals treated with normal (phosphatidyl choline) liposomes. Dose-dependency studies at optimal RF ablation parameters will then be performed using the agent(s) showing the greatest treatment effects. The correlation between extent of tumor destruction and endpoints indicative of oxidative stress and lipid peroxidation will then be assessed.

Sample Size: As justified above in Experiment 5a, the 6 arms of the initial experiment will require 30 tumors, which can be obtained from 12 dogs. As justified in Experiment 4, eight additional points for the dose-dependency study will require another 16 animals. Thus, a total of 28 dogs are required.

Data analysis: Data analysis will be performed as outlined for Experiment 5a. Thermal dosimetry for the different liposome preparations will be calculated.

Anticipated results: We anticipate greater tumor destruction in the presence of lipids having greater polyunsaturated fatty acid contents relative to standard liposomes as well as seeing increases in parameters indicative of lipid peroxidation and oxidative stress. Positive results would support our hypothesis, and suggest that we could potentially *increase clinical efficacy* by changing the lipid composition of the liposomes. We further expect linear correlations between the dose of lipid administered, endpoints of lipid peroxidation, oxidative stress, and tumor destruction. Confirmation (or refutation of this assertion) will be important in ascertaining the role of enhanced lipid peroxidation as an underlying mechanism for this effect. We predict that the different lipid preparations will have similar thermal dosimetry for activation. If variance is found, we will further characterize these differences along the lines of previous Experiments. Negative results from both Exp. 5a and 5b would lead us to consider alternative hypotheses to the enhanced tumor cell killing seen in the presence of liposomes (see Future Studies).

Future studies: Support for the enhanced lipid peroxidation hypothesis will lead to further parametric evaluation of the lipid preparation showing greatest antitumoral effects (as performed for Doxil – see Section C.2) and a potential *search for lipids with clinically beneficial properties*, such as maximal efficacy with greatest safety profile. This likely would include combinations of agents from Experiments 5a & 5b.

Negative results for Experiment 5 would lead us to test alternative explanations for RF liposome synergy. This would include, for example, further characterization of polyethylene glycol [PEG] toxicity at anticipated intratumoral doses, based upon the rationale of our unpublished data demonstrating that the direct injection of highly concentrated PEG leads to tumor coagulation. This possibility is considered less likely given that PEG-coated liposomes have been shown to be safe for human use at normal doses [100-103], and that is why we selected to test lipid peroxidation first in the present proposal. Yet, increased accumulation of liposomes in RF ablated tumor could theoretically lead to high concentrations of PEG that are sufficient to induce cytotoxicity. As in Experiment 5b, a two-phase approach will be undertaken. In the first phase, three formulations will be studied (PEG alone, liposomes containing PEG, and liposomes without PEG) with and without RF ablation. Dose-dependency of the effects of PEG will be studied, if PEG is shown to have an antitumoral effect at intratumoral concentrations achievable by IV delivery on a liposome carrier. Support for a positive role of PEG in mediating enhanced tumor destruction will lead to a testing of several hypotheses regarding its mechanistic function including induction of metabolic acidosis [170], cell fusion [171], osmotic load imbalances [172], and parametric studies as outlined in Section C.2.

Specific Aim 3. Characterize potential local and systemic treatment toxicity

Experiment 6: Determine the thermal dosimetry at which liposomal doxorubicin tissue destruction occurs in normal canine liver and kidney parenchyma. A peripheral rim of these *difficult to treat* tissues must be ablated to ensure adequate clinical destruction of hepatic and renal tumors.

Objectives: a) Establish the extent of variability between tumor and normal tissue for the interaction between RF ablation and liposomal doxorubicin. b) Define thermal dosimetry of treatment effect in normal tissues

Question asked: To what extent does doxorubicin accumulation and tumor coagulation change in different tissue types when RF ablation is combined with liposomal doxorubicin?"

Rationale: Based upon the classical surgical resection paradigm [37, 38], destruction of an "ablative margin" of apparently normal tissue surrounding focal tumors is a desired endpoint in most cases, as it eliminates residual microscopic foci of tumor and improves local tumor control [1, 39]. Yet, control of this process is mandated. Indeed, both under-treatment and over-treatment of the surrounding tissue are to be avoided [1, 151]. Nevertheless, tailoring of this zone to the desired dimensions will require knowledge of thermal dosimetry for the RF ablation liposomal doxorubicin interaction.

Clinical data of Section C.4 suggest that combined RF ablation with liposomal doxorubicin can destroy and increase coagulation of normal liver tissue, particularly in this peripheral peritumoral rim. However, the parameters that influence tumor destruction in normal tissues have yet to be characterized. Data of section C.2g also demonstrate that the extent of induced coagulation from combining RF ablation with liposomal doxorubicin varies with tissue type. This may be due to variable responses to heating due to differences in blood flow and tissue composition [1 – 6], as well as tissue specific thresholds for cytotoxicity [72, 73, 78, 173]. Variable clinical response to liposomal preparations has also been seen [100-103]. Regardless, given the enhanced treatment effect (and therefore the relatively non-specific nature of this phenomenon) in small animals, the scope of response for clinically relevant RF electrodes and parameters is required. Here we determine the thermal dosimetry at which liposomal doxorubicin mediated tissue destruction occurs in normal liver and kidney parenchyma, the two organs for which RF ablation is most commonly performed.

Importance: *Establishing the extent of variability* in response that can be expected when the combined treatment paradigm is translated into clinical practice, including *characterization of normal tissue damage* using clinically relevant large-size models, is warranted to make this procedure safer.

Study design: Simplex optimization will be repeated in normal canine liver and kidney for determination of the metric "maximum increase in coagulation" using the apparatus, parameters and approach outlined for Experiment 1. Correlation of tissue ablation to tissue doxorubicin accumulation (using techniques of Exp. 2) and endpoints of lipid peroxidation (techniques of Exp. 5) will also be performed. Results will be compared among the three tissue models (including the CVS tumor) to determine the potential variability of response.

Animal Models: The use of canine liver and kidney as a human tissue surrogate is widely accepted and has been used by our laboratory to characterize the effects of RF energy deposition [42-50, 173]. It will also improve utilization of some animals in which tumor implantation is unsuccessful.

Simplex design and sample size: In an attempt to maximize our efficiency, and based upon our prediction of similar responses between the models, a small-step simplex design will be used [133]. As in Experiment 4, we will center the selection of our initial simplex data points at small increments from the defined maxima of Experiment 1. Monte Carlo simulation suggests that this would reduce the number of data points required for simplex convergence. Hence, only 8 – 10 iterations are anticipated. Thus, 60 data points will be required for liver and for kidney. Given that we can perform 3 trials in each liver and one per each kidney, a total of 20 dogs would be required for liver and 30 for kidney. Yet, it is likely that we will be able to perform ablations in two organs on many of these dogs reducing the requested number of dogs to 40.

Data analysis: The maximum increase in coagulation, the ratio of increased intratumoral doxorubicin accumulation, endpoints of lipid peroxidation, and thermal dosimetry will be determined as previously outlined. Direct comparison of the simplex and surface response contours will allow us to determine the magnitude of variance between these models. If differences are noted, further comparisons will be performed to identify the parameters responsible for variance among the tissue types. The equations representing the best fit correlation for the different models will be compared and differences in the residual error for each curve will be assessed for statistical significance based on their chi-squared ratios, corrected for the number of parameters in each model.

Anticipated results and interpretation: We anticipate demonstrating statistically significant correlations between the liposomal dose, tissue doxorubicin uptake, endpoints of lipid peroxidation, and the amount of

RF induced coagulation necrosis. We expect that the overall relationship between thermal dosimetry and increased coagulation in normal tissues will conform to that observed in tumors. We may also find a non-linear correlation, suggesting that new mathematical models will need to be constructed.

Potential pitfalls: We may determine that one-to-one correlation, particularly between normal liver and tumor, does not exist. If this occurs, we will continue with relevant parametric studies of the interaction between RF ablation and liposomal doxorubicin along the lines performed for tumor models [Section C.2].

Experiment 7: Survival study in renal CVS tumors using optimal liposomal and RF ablation parameters.

Rationale: Although ablation of tissue immediately surrounding the tumor is often a desired endpoint, systemic toxicity should ideally be minimized. Thus, while we and others have demonstrated that RF ablation alone has very limited morbidity (< 5%) [1 – 6, 26, 151], RF heat can theoretically alter liposome components, which in turn could potentially result in unexpected systemic toxicity. Indeed, if mechanisms such as enhanced oxidative stress and lipid peroxidation are invoked, it is possible that the heating of liposomes could engender systemic downstream effects. Section C.2g already shows that RF combined with liposomal doxorubicin has effects on locally heated tissue. However, the extent of possible local and systemic effects is unknown. Here we propose a one-month survival study in which animals receiving combined therapy are monitored for systemic toxicity, including monitoring of hematologic, renal, and hepatic blood tests as well as for treatment site complications such as hemorrhage.

Study design: We will begin our safety studies using liposomal doxorubicin, as this agent has the greatest potential risk of toxicity from the additional adriamycin, but is nevertheless, most likely to be used in the clinic given its FDA approval. A renal tumor model is proposed over a liver model due to poor yield of focal liver tumors in this model [145, 173, 174 & personal experience]; the fact that we have been successful using the proposed kidney model [173]; and that RF ablation is currently receiving much attention as a viable strategy for treating renal malignancy [25 - 30].

CVS tumors will be grown to 3 - 5 cm as determined by CT scanning. Percutaneous RF ablation will be performed using sterile technique and ultrasound guidance. RF ablation and liposomal doxorubicin dosing will be performed using optimal parameters as determined by Exp. 1 and 6. Following the procedure, animals will be monitored daily for signs of distress to the defined sacrifice endpoint of 30 days. Blood tests, including CBC, renal function tests (creatinine and BUN), liver function tests (ALT, AST, alkaline-phosphatase, bilirubin) and electrolytes will be obtained at baseline (prior to the procedure), immediately following the procedure, 48-72 hours post-ablation, at one week, and at 30 days. Multiphaseic, contrast enhanced, helical CT scan will also be performed at 48 - 72 hours to determine the presence or absence of local or intratumoral hemorrhage [69]. Pathology will be obtained at day 30 for histopathologic analysis.

Study Size: Initially, 8 animals will be studied. Power calculations determine that this will render at least an 80% likelihood of detecting mean changes of 1 std. deviation from baseline at 0.05% significance for the blood tests to be studied. For example, for creatinine the sample size would enable us to detect a change of 0.2 mg/dl at this level of confidence. This endpoint has been accepted by the FDA for our prior animal safety studies. Given an approximate 75% yield on the renal tumors (lower yield than the subcutaneous model) and a need for 8 animals, we are proposing 10 animals.

Pathologic Studies: The tumor and margin of normal renal tissue subject to RF ablation will be resected *in toto* and subject to rigorous histopathologic analysis (see Experiment 1). TTC staining will be also performed to determine the extent of induced tumor necrosis.

Data Analysis: The results from the specified blood tests will be compared to baseline values and subject to ANOVA. The percentage of animals with complications, such as hemorrhage, will be recorded. A secondary endpoint will be to determine the percentage of tumors with complete ablation and the extent of tumor destruction achieved in this model (in terms of volume and coagulation diameter). This will be compared to a result in the subcutaneous model to determine the extent of variability between different tumor environments to be expected in clinic.

Anticipated Results: We anticipate that no long-term sequelae will be identified, either at pathology or from the blood tests. Nor do we expect clinically relevant changes to be identified at CT. It is possible that we will see mild reversible biochemical abnormalities within the first week [45]. Regardless if any abnormalities are identified, we will perform further animal study in terms of larger numbers and longer follow up to quantify these issues in an attempt to identify underlying causes. This information will also be used to identify which parameters ought to be followed in clinical practice.

Potential Pitfalls: It is possible that our initial protocol for monitoring of the blood parameters may be insufficient to characterize the extent of biochemical abnormalities. If abnormalities are seen, particularly at one week, we will increase the frequency of serologic monitoring. Likewise, if abnormalities are identified at the initial follow-up CT scan, we will continue to follow these complications by CT at appropriate intervals, extending the length and scope of the survival study accordingly.

TIME LINE

Based upon prior experience working with this animal model and the proposed percentage effort of the principal investigator, a five year period of study will be required to achieve all of the objectives outlined for the 7 animal experiments. We can manage a colony of approximately 12 dogs with utilization of approximately 4 dogs each month (40 - 50 year). It is further important to stress that the rate limiting step in performing many of the experiments will be the iterative nature of the simplex methodology for which each iteration will require 2 to 3 weeks to complete.

<u>Specific Aim</u>	<u>Description</u>	<u>Animals</u>
<u>Year</u>		
Aim 1 Exp. 1 & 2	Optimization of combined treatment	43 dogs 1
Exp. 3	Definition of thermal dosimetry for Doxil	35 dogs 1 - 2
Aim 2 Exp. 4	Optimization of empty liposomes	20 dogs 2 - 3
Exp. 5a	Study lipid peroxidation inhibition	36 dogs 3
Exp. 5b	Study lipid peroxidation potentiation	28 dogs 4
Aim 3 Exp. 6	Effect on normal tissues	40 dogs 5
Exp. 7	Survival safety study	10 dogs 5

E. HUMAN STUDIES: None**F. VERTEBRATE ANIMALS:**

1. The total number of animals to be used on an experiment by experiment basis is provided in the following table. Mongrel female dogs will be used in the proposed work.

<u>Experiment</u>	<u>Dogs</u>
1	43
2	--
3	35
4	20
5a	36
5b	28
6	40
7	10
Totals:	212

2. The use of vertebrate animals for this study is required as computer models and other *in vitro* systems do not adequately approximate tumor physiology and the changes which will be observed with the proposed minimally-invasive interventions. The species of animals (dogs) has been selected given that this models is of sufficient size to permit meaningful translation of our work to tumors diameters of clinical relevance (i.e., 3.0 – 6.0 cm). **Larger tumor models (than rat and rabbit) were specifically requested by several reviewers of the initial proposal.** The proposed canine venereal sarcoma model has been used as a model for large volume thermal tumor ablation [50] (and NIH R01-CA87992-01A1), and will therefore be used in a similar fashion for this proposal. The number of animals proposed is necessary to allow for adequate statistical evaluation of study variables (please see sample size justifications in Section D).
3. Beth Israel Deaconess Medical Center and the Harvard Institute of Medicine maintain fully accredited AALAC animal care facilities. All animal surgery will be performed in special designated animal operatory suite under the supervision of a licensed veterinarian. All animals inoculated with tumors will be monitored on a daily basis by veterinarian staff.
4. All radiofrequency experiments and adjuvant manipulations will be performed in fully-anesthetized, animals. Anesthesia will include isoflurane inhalent anesthesia (2 – 4% in oxygen carrier). Dogs will be allowed to recover from tumor inoculation and RF ablation. Post-procedure pain medication (Buprenex 0.03 – 0.3 mg/Kg) will be administered, prn). Veterinarian staff will monitor these animals on a daily basis. Any animal showing signs of discomfort or weakening will be euthanized immediately. Thus far, we have not needed to sacrifice any of our tumor carriers. However, two of greater than 50 animals have required early sacrifice for infection or wound.
5. All animals will be sacrificed with phenobarbital overdose (Somlethal 0.2 mL/kg), consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

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H. CONSORTIUM/CONTRACTURAL AGREEMENTS:

CONSORTIUM - There is one subcontract with Northeastern University, Department of Pharmacology. The facilities for pharmaceutical preparation at this institution are required to ensure the synthesis of appropriately purified, high-quality preparations of liposomes for experiments of Specific Aim 2. Total costs to Northeastern University will be \$ 32,610 in year 2 and \$ 49,523 in years 3 – 4. Financial and administrative costs for clinical studies at that institution are 57%. Full details of the budget for this institution can be found in preceding paragraphs.

I. CONSULTANTS:

Mark W. Dewhirst, DVM, Ph.D. Consultant, is the Gustavo S. Montana Professor of Radiation Oncology and Adjunct Professor of Biomedical Engineering and Pathology at Duke University, Durham, NC. Dr. Dewhirst has a program project grant in hyperthermia and extensive experience studying liposomal doxorubicin and hyperthermia. He will assist by providing expertise in hyperthermia, and analysis and calculation of thermal dosimetry.

Douglas R. Spitz, M.D., Consultant, is well-known independent investigator in the field of lipid peroxidation and oxidative stress. He co-directs the Antioxidant Enzyme Core Laboratory at the Free Radical and Radiation Biology Program in the Department of Radiation Oncology at the University of Iowa. He will assist us in performing all assays related to lipid peroxidation and oxidative stress, and provide guidance in selecting appropriate lipid substrates for further study.

Elkan F. Halpern, Ph.D., Consultant, is Director of Statistics, MGH Decision Analysis and Technology Assessment Group, Massachusetts General Hospital. Dr. Halpern has served as our statistician over the last eight years and has therefore gained significant understanding of RF ablation. For this study, Dr. Halpern has assisted with the experimental design by performing sample size and power calculations. He will consult on our planned analysis of the data using linear and higher order regression models as well as multivariate multiple regression analyses.

Sabina Signoretti, M.D., Consultant, is a Pathologist at Dana Farber Cancer Center, Harvard Medical School, Boston, MA. Dr. Signoretti will be responsible for the pathologic determination of RF induced coagulation and interpretation of immunohistochemistry. She has gained considerable expertise in this endeavor as she functions as our pathologist for our other RF ablation studies including NIH grant *R01CA87992-01A1; Tumor Ablation using Radiofrequency* and our Project 3 “*Combined RF ablation with antivasular / antiangiogenesis therapy*” of Renal Cancer SPORE NIH grant 1 P50 CA101942-01.

Eric R. Cosman, Ph.D., Consultant, is Professor of Physics, Emeritus at the Massachusetts Institute of Technology. He will provide additional physics support. Dr. Cosman is a specialist in RF generation in medicine, nuclear structure and reactions, isobaric analog and shape-isomeric nuclear states, and stereotactic, (image-guidance) methods. He has collaborated with our team over the past nine years, and has jointly authored several original scientific manuscripts and patents with team members.