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Combination radiofrequency (RF) ablation and IV liposomal heat shock protein suppression: Reduced tumor growth and increased animal endpoint survival in a small animal tumor model

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Abstract

Background—To investigate the effect of IV liposomal quercetin (a known down-regulator of heat shock proteins) alone and with liposomal doxorubicin on tumor growth and end-point survival when combined with radiofrequency (RF) tumor ablation in a rat tumor model.

Methods—Solitary subcutaneous R3230 mammary adenocarcinoma tumors (1.3–1.5 cm) were implanted in 48 female Fischer rats. Initially, 32 tumors (n=8, each group) were randomized into four experimental groups: (a) conventional monopolar RF alone (70°C for 5 min), (b) IV liposomal quercetin alone (1 mg/kg), (c) IV liposomal quercetin followed 24hr later with RF, and (d) no treatment. Next, 16 additional tumors were randomized into two groups (n=8, each) that received a combined RF and liposomal doxorubicin (15 min post-RF, 8 mg/kg) either with or without liposomal quercetin. Kaplan-Meier survival analysis was performed using a tumor diameter of 3.0 cm as the defined survival endpoint.

Results—Differences in endpoint survival and tumor doubling time among the groups were highly significant ($P < 0.001$). Endpoint survivals were 12.5 ± 2.2 days for the control group, 16.6 ± 2.9 days for tumors treated with RF alone, 15.5 ± 2.1 days for tumors treated with liposomal

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quercetin alone, and 22.0 ± 3.9 days with combined RF and quercetin. Additionally, combination quercetin/RF/doxorubicin therapy resulted in the longest survival (48.3 ± 20.4 days), followed by RF/doxorubicin (29.9 ± 3.8 days).

Conclusions—IV liposomal quercetin in combination with RF ablation reduces tumor growth rates and improves animal endpoint survival. Further increases in endpoint survival can be seen by adding an additional anti-tumor adjuvant agent liposomal doxorubicin. This suggests that targeting several post-ablation processes with multi-drug nanotherapies can increase overall ablation efficacy.

Keywords

Radiofrequency; Tumor ablation; Heat shock protein; Liposomal Quercetin; Liposomal Doxorubicin

Introduction

Minimally-invasive, image-guided radiofrequency (RF) ablation is increasingly used to treat focal tumors in a range of organs, and has been incorporated into treatment paradigms for focal tumors of the liver, lung, bone, and kidney [1]. However, additional work is required to overcome difficulties in achieving a complete ablative margin for larger tumors, where residual, untreated tumor leads to local tumor progression [2-5]. Most often, tumor cells survive ablation because of the biophysical limitations of the procedure, such as poor thermal conductivity coupled with perfusion-mediated tissue cooling, that prevent uniform heating of the entire tumor volume to a temperature sufficient for inducing coagulation necrosis (50 – 60°C), especially in the peripheral area of ablation [5-7]. Therefore, strategies that can increase the extent of RF tumor destruction are desired.

One such successful strategy has been to target residual viable cells with adjuvant chemotherapy or radiation to improve completeness of RF ablation [7, 8]. The rationale of this combined approach is to increase tumor destruction occurring within the sizable peripheral zone of sub-lethal, temperatures (i.e., largely reversible cell damage induced by mildly elevating tissue temperatures to 41 – 45°C) surrounding the heat-induced coagulation [7]. Indeed, several studies have reported increased tumor destruction with RF ablation and adjuvant IV liposome-encapsulated chemotherapeutics, such as doxorubicin and/or paclitaxel in part due to RF inducing a potentially controlled increase intratumoral nanodrug accumulation [9-11]. Several additional mechanisms accounting for the increased tumor destruction of combination therapy have been identified, including amplification of cell stress and apoptosis by the nanodrugs most notably in the rim of periablational tissue exposed to sublethal hyperthermia [12]. Interestingly, in these experiments a rim of increased heat-shock protein (HSP) production was also observed immediately peripheral to the zone of apoptosis in a concentric ring of still-viable tumor surrounding the ablation zone. As these HSPs have known cellular protective effects against apoptosis [13-15], the presence of a rim of increased HSP expression beyond the ablation zone with co-localization to persistently viable tumor suggests that protective HSP effects may be inhibiting additional gains in tumor destruction at the ablation margin.

Based upon this observation, most recently, RF ablation has been combined with adjuvant IV liposome-encapsulated quercetin, a flavanoid with known suppressive effects on the synthesis of heat shock proteins in a variety of cell lines [16]. Indeed, the rationale for this combined paradigm was to use image-guided RF to control the release of a nanodrug that is particularly active against its unwanted effects at the periphery of the thermal ablation within a given tumor target. In one study, RF ablation sufficiently increased liposomal quercetin concentrations in the tumor to increase apoptosis, reduce HSP expression,

preferentially in the periablational rim, and increased overall tumor destruction compared to RF alone at 24 hr. [17]. Even greater gains observed for triple therapy combining RF ablation with both adjuvant liposomal doxorubicin and liposomal quercetin [17]. Yet, short-term mechanistic studies alone are often insufficient to predict the actual effect of different liposomal compounds on the critical endpoint outcome of survival [11]. Accordingly, the purpose of this study is to investigate the effect of IV liposomal quercetin and/or liposomal doxorubicin when combined with RF tumor ablation on tumor growth and end-point survival in a rat tumor model as a necessary step in evaluating the potential utility of this regimen prior to consideration of clinical trials.

Materials and Methods

Experimental Overview

Approval of the Institutional Animal Care and Use Committee was obtained prior to the start of this study. The study was performed in two phases to systematically investigate the potential synergistic effects of RF ablation with adjuvant liposomal quercetin alone and then in combination with liposomal doxorubicin. A total of 48 tumors in 48 animals were used. In sections below, references to “quercetin” and “doxorubicin” imply liposomal encapsulation as no free drug (either quercetin or doxorubicin) was administered in this study.

Phase I. Effect of liposomal quercetin and RF ablation on tumor growth rates and animal endpoint survival—To investigate the endpoint survival and tumor growth for animals from different treatment strategies, 32 tumors/animals were randomized to receive one of four therapeutic regimens, including standardized RF ablation (conventional, monopolar 1 cm tip; applied for 5 min; with the tip temperature titrated to $70\pm 1^{\circ}\text{C}$) and/or IV liposomal quercetin (0.5ml/0.29mg). These groups included (n=8, each group): (a) RF alone, (b) IV liposomal quercetin alone, (c) IV liposomal quercetin followed 24hr later with RF, and (d) no treatment. The survival endpoint was a maximum tumor diameter of 30 mm or survival of 90 days, whichever was reached first. Secondary endpoints were the rate of tumor growth and local control (i.e., no visible tumor on the chest wall).

Phase II. Effect of adding liposomal doxorubicin to RF/liposomal quercetin on end point survival—This experiment was performed to determine whether the liposomal doxorubicin would affect the survival results of combination treatment with RF and liposomal quercetin. Sixteen additional tumors were randomized into two groups (n=8, each group) that received either triple therapy with combined RF, liposomal quercetin (24 hrs pre-RF, 0.5ml/0.29mg), and liposomal doxorubicin (15 min post-RF, 8 mg/kg) or RF with liposomal doxorubicin alone.

Animal Model

For all experiments and procedures, anesthesia was induced with intraperitoneal injection of a mixture of ketamine (50 mg/kg, Ketaject; Phoenix Pharmaceutical, St. Joseph, MO) and xylazine (5 mg/kg, Bayer, Shawnee Mission, KS). Animals were sacrificed with an overdose (0.2 mL/kg) of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL).

Experiments were performed with a well-characterized established R3230 mammary adenocarcinoma cell line used in multiple prior studies [10, 18, 19]. In total, 48 female Fisher rats ($120\pm 20\text{g}$; 13–14 weeks old, Taconic Farms, Germantown, NY) with R3230 tumors were used in this study. Fresh tumor was harvested from a live carrier and homogenized in a tissue grinder (Model 23; Kontes Glass, Vineland, NJ) and suspended in Roswell Park Memorial Institution (RPMI) 1640 medium (Biomedicals, Aurora, IL). One tumor was implanted into each animal by slowly injecting 0.3–0.4 mL of tumor suspension

into the mammary fat pad of each animal via an 18-gauge needle. Animals were monitored every 2–3 days to measure tumor growth. 1.3–1.5 cm solid non-necrotic tumors (determined on ultrasonography by an absence of more than 3 separate cystic spaces >0.5 mm in diameter within the entire tumor) were used for this study, randomized to different treatment arms. Tumors were measured in a longitudinal and transverse diameter with a mechanical caliper (W.Y., B.T.). Accuracy of the final measurement was verified by the senior author, who was blinded to treatment group) every 2–3 days until they reached 30 mm in largest diameter, at which point the animals were sacrificed. This surrogate for endpoint survival was selected because of requirements of the institutional animal committee, which mandated sacrifice at this point on the basis of animal size and tumor burden as dictated by the U.S. Department of Agriculture Animal Welfare Act (31–32). Any animal with tumors weighing more than 10% of its body weight, corresponding to a tumor diameter of 30 mm in this model, was considered moribund and underwent mandatory sacrifice. For all measurements, anesthesia was induced, as described previously, to permit accurate measurements. The largest diameter measured at every time point was recorded and plotted.

RF Application

Conventional monopolar RFA was applied by using a 500-kHz RFA generator (model 3E; Radionics, Burlington, Mass). To complete the RF circuit, the animal was placed on a standardized metallic grounding pad (Radionics). Contact was ensured by shaving the animal's back and by liberally applying electrolytic contact gel. Initially, the 1-cm tip of a 21-gauge electrically insulated electrode (SMK electrode; Radionics) was placed at the center of the tumor. RF was applied for 5 minutes with the generator output titrated to maintain a designated tip temperature ($70 \pm 2^\circ\text{C}$, mean 90.1 ± 21.6 mA, range 48–156 mA). This standardized method of RFA application has been demonstrated previously to provide reproducible coagulation volumes with use of this conventional RFA system [18, 19].

Preparation and administration of adjuvant intravenous liposomal agents

Quercetin-loaded liposomes were prepared such that the lipid composition in these liposomes was identical to Doxil, in a manner similar to prior reports [12]. Briefly, 0.29 mg of quercetin (1mg/mL solution in methanol) was added to hydrogenated soy phosphatidylcholine, cholesterol and polyethyleneglycol phosphatidylethanolamine (PEG2000-PE) (57.25:37.57:5.18 mol%, respectively) solutions in chloroform, and a lipid film was formed in a round bottom flask by solvent removal on a rotary evaporator. The lipid film was then rehydrated with 1 mL of phosphate buffered saline, pH 7.4 and the preparation was probe-sonicated with a Sonic Dismembrator (Model 100, Fisher Scientific, Pittsburgh, PA) at a power output of 7 watts for 30 min. To remove any titanium particles which may have been shed from the tip of the probe during sonication, the sample was centrifuged for 10 min at 2000 rpm. Liposomes were loaded with 5 mol% quercetin (not to saturation), and the liposomal loading efficiency of quercetin was 100% (as noted above, 0.29 mg of quercetin was loaded in each administered dose). The liposomal size was $115 \text{ nm} \pm 43 \text{ nm}$. The zeta-potential was $-34.7 \pm 5.5 \text{ mV}$. The liposomal formulation was tested and stable for 1 week at 4°C in phosphate buffered saline (PBS) at a pH of 7.4 and for 24 hrs at 37°C in PBS with 10% fetal bovine serum. When prepared for the experiment, all formulations were administered within 24 hrs of preparation. Finally, the polydispersity coefficient was 0.315. For Phase II, a commercially-available preparation of liposomal doxorubicin (Doxil; ALZA Pharmaceuticals, Palo Alto, CA) was used.

Liposomal quercetin (0.5 ml) was injected slowly (for 30 sec via a 27-gauge needle) into the tail vein 24 hr pre-RF ablation in the combined therapy group; in order to provide maximal drug uptake and potential HSP suppression prior to the thermal ablation. Liposomal doxorubicin (1 mg; of a 2 mg/ml preparation) was injected separately with the same

approach (0.5 ml IV per animal), but at a later time point, 15 min post-RF. This timing of IV liposomal doxorubicin administration was selected based upon prior studies demonstrating maximum tumor coagulation and intratumoral drug accumulation with this paradigm [10, 19].

Statistical Analysis

SPSS 13.0 software (SPSS Inc., Chicago, IL) was used for statistical analysis in this study. All data were provided as mean plus or minus SD. The *P* value is less than 0.05 was considered as significant. On the basis of studies that demonstrated exponential tumor growth patterns, tumor growth rates and doubling times were also calculated by means of regression analysis of an exponential model. The Kaplan-Meier method and log-rank test were used for endpoint survival analysis. Given the absence of censoring of our data, one-way analysis of variance was then performed on the survival endpoints for each animal for the comparisons reported. Pairwise *t* tests ($p < 0.05$; two-tailed test) based on the least square means were subsequently performed only if the overall *P* values were significant. We then used two way analysis of variance to determine the contributions of RF ablation and intravenous injection of liposomal quercetin to endpoint survival. One-way and two way analyses of variance were also performed to determine the contribution of RF ablation and intravenous liposomal quercetin to the parameter of tumor doubling time.

Results

Endpoint Survival

The mean endpoint survival (i.e., the time from treatment for the tumor to reach 30 mm in diameter) in the control group was 12.5 ± 2.2 days. Mean endpoint survival for the groups treated with either IV liposomal quercetin alone or RF ablation alone was similar, but was significantly improved when compared with the control (no treatment) group. Endpoint survival was 16.6 ± 2.9 days for tumors treated with RF ablation alone and 15.5 ± 2.1 days for tumors treated with IV liposomal quercetin alone ($P < 0.001$ compared with control). Significantly greater endpoint survival of 22.0 ± 3.9 days was obtained for tumors treated with combined RF ablation and IV liposomal quercetin ($P < 0.001$ compared with other groups) (Table 1, Figure 1).

When liposomal doxorubicin was added to RF/quercetin (i.e., triple therapy), survival increased to 48.3 ± 20.4 days, compared to 29.9 ± 3.8 days for RF with only liposomal doxorubicin ($P < 0.01$). Pairwise comparisons indicated that tumor growth rates and mean endpoint survival with the different treatments protocols (i.e. single agent, combination of two treatments, and combination of three treatments) were all significantly different ($P < 0.005$, all comparisons). Although, animals that received combined RF/doxorubicin survived longer than did animals that received combined RF/quercetin ($P < 0.005$), triple therapy (quercetin/RF/doxorubicin) showed the greatest survival benefit. The maximum endpoint survival in this study was limited to 90 days and was obtained for one animal in the combined treatment group of triple therapies.

Tumor Growth Rate

Tumor growth rate curves were calculated as exponential functions to the formula $y = ae^{bx}$, where $a = 1.3$ – 1.5 , initial tumor size, according to study design. All curves had R^2 values ranging from 0.90 to 0.99 (Table 1). The value representing tumor growth rate (b) varied from 0.035 to 0.009 (Table 1). This corresponded to doubling times ranging from 19.7 ± 3.2 days (untreated controls) to 81.6 ± 6.8 days in animals treated with triple therapy (i.e., quercetin/RF/doxorubicin) (Figure 2). Results of analysis of variance demonstrated significant differences in tumor doubling times for the combined treatments compared over

no or single treatment (either RF, quercetin, or doxorubicin alone), and the longest tumor doubling times with triple therapy.

Local Control

Local tumor control was achieved only once, in 1 (12.5%) of the 8 animals that received triple therapy. In this particular animal, tumor size decreased progressively after treatment and completely cured until the animal was sacrificed at 90 days, when no viable tumor was identified at histopathologic examination. No local tumor control was seen in any other groups. Thus, a significant difference was seen in local control achieved in the group of rats that received the triple therapy or not ($P<0.01$ for all comparisons) (Figure 3).

Discussion

Exposure to low, non-lethal levels of hyperthermia results in reversible cellular injury and upregulation of protective cellular pathways [20]. One such pathway, mediated by the heat shock protein family and the HSP70 protein in particular, has known protective effects in the face of cellular injury from exposure to low level hyperthermia and chemotherapeutic agents [21]. Along these lines, several studies have demonstrated increased HSP70 expression in patient serum and in the ablative zone up to 5 days after RF ablation [22-24]. Similarly, elevated HSP70 levels were also detected in biopsy specimens obtained 24 hr after RF ablation of human hepatocellular carcinoma [25].

Recently, markedly increased HSP70 expression after RF ablation has been localized to the transitional zone at the ablation margin where the greatest exposure to sub-lethal hyperthermia occurs [12]. Immunohistochemical characterization in subcutaneous rat breast tumors using co-localized staining for both cleaved caspase 3 (a marker of apoptosis) and HSP70 demonstrated a rim of apoptosis at the ablative margin, immediately surrounded by a rim of HSP70 that corresponds to partially-injured, viable tumor cells [12]. Most recently, Yang et al have endeavored to exploit this observation further into a factor that can improve nanoparticulate mediated cancer therapy by attempting to target the HSP70 cellular stress pathway to increase overall tumor coagulation through combining RF ablation with liposome encapsulated quercetin, a compound with known inhibitory effects on HSP expression [17]. In their report, combination therapy resulted in reduced HSP70 expression in the ablative margin with correlative increases in both apoptosis and tumor coagulation. This correlated to an early (4hr) reduction in HSP70 expression and increased apoptosis for combination quercetin-RF compared to RF alone. However, whether gains in apoptosis and tumor destruction (with reductions in HSP70 expression) translate into correlative increases in animal endpoint survival and reduction in tumor growth has not been determined.

In this study, combining RF ablation with adjuvant liposome encapsulated quercetin resulted in reduced tumor growth (as evidence by tumor doubling time) and increased animal endpoint survival over individual treatments (either RF or liposomal quercetin alone). This correlates well with the increased tumor coagulation and immunohistochemistry staining (increased apoptosis and reduced HSP70 expression) observed in prior studies. Quercetin acts through several mechanisms, including phosphorylation of AMP-activated protein kinase to down-regulate HSP70 expression [26], interfering with translocation [27], reducing the availability of heat shock transcription factor, suppressing initiation and elongation of the HSP70 mRNA [28], and separately increasing the activity of caspase 3 [26]. This affects both of the pathways (cleaved caspase 3 and HSP70) that were interrogated by Yang et al, and likely contributes to gains observed here in animal endpoint survival. Interestingly, liposomal quercetin also provided a survival benefit over control, untreated tumors (and similar to RF alone), suggesting a separate and independent susceptibility of this tumor model to quercetin. The effects of quercetin on tumor cell growth

have been separately demonstrated in in vitro studies [16, 29], and in addition, liposomal quercetin has been shown to slow tumor growth in several separate murine xenograft tumor models [30].

A number of experimental and clinical studies have demonstrated the benefits of combining RF ablation with IV liposomal doxorubicin, including RF-induced local increases of intratumoral drug accumulation, leading to increased tumor coagulation and animal endpoint survival (as is also confirmed in our results) [7, 10, 31]. In our study, RF and liposomal doxorubicin resulted in longer animal survival times (mean 29.9 days) compared to RF and liposomal quercetin (mean 22 days), despite the similar tumor coagulation observed in prior studies (13.2 mm vs. 13.1 mm for RF with liposomal doxorubicin or liposomal quercetin, respectively) [17]. This discordance underscores the need to carefully address the most relevant endpoints, such as tumor coagulation and endpoint survival, and that delayed evaluation of tumor coagulation must be viewed as primary, and separate, outcome measure. Thus, while early evaluation of coagulation diameter can identify early temporal differences between groups, the actual time it takes for a full effect to take place may vary. Hence, the determination of a full dose response curve likely needs to be tailored for each agent, and may make comparisons between different drugs even more difficult. Similarly, differences between intratumoral drug accumulation and coagulation demonstrate that identifying the drug concentration required for a threshold effect requires continued investigation. Finally, while tumor coagulation reflects local improvements in tumor destruction, the systemic impact of adjuvant therapy may be under-represented. For example, extracellular HSP expression upregulates the immune-mediated response to tumor cells [32-35], and therefore, gains in tumor coagulation from intracellular HSP suppression does not account for systemic anti-tumor effects, and may not translate directly to improvements in animal endpoint survival.

Immunohistochemical characterization in small animal tumors treated with combination RF and liposomal doxorubicin has also shown increased HSP70 expression in the periablational zone with compared to RF alone, and has formed the basis for recent studies in which Yang et al reported that introducing liposomal quercetin into the treatment paradigm could result in HSP70 suppression with triple therapy, resulting in the largest tumor coagulation compared to single or dual therapy regimens (RF with or without a single adjuvant liposomal chemotherapy agent). Our results support these findings, with significantly longer animal endpoint survival compared to other treatment arms. Potential mechanisms of synergy for triple therapy include improved therapeutic index and efficacy (quercetin may reverse doxorubicin cell resistance in hypoxic conditions [36]) and targeting separate pathways of cellular stress (quercetin predominantly through anti-HSP effects, while adjuvant doxorubicin has been shown to both increase apoptosis and HSP expression [12]). Interestingly, Yang et al reported only partial HSP70 suppression with the addition of liposomal quercetin to the RF and liposomal doxorubicin, possibly reflecting limitations with intratumoral spatial delivery or temporal effects of HSP70 suppression. This suggests that refinement of the treatment paradigm (for example, by increasing liposome loading of quercetin or using a multi-dosing approach) could result in even greater gains in animal endpoint survival. Finally, in a separate study, Yang et al have also previously combined RF and liposomal doxorubicin with adjuvant liposomal paclitaxel, an agent with predominant pro-apoptotic effects but with some secondary anti-HSP activity [11]. In the same rat breast tumor model, combination RF/doxorubicin/paclitaxel therapy resulted in a mean of 56.8 days of survival, compared to the maximum of 48.3 days observed here with combination RF/doxorubicin/quercetin. This suggests both that multiple drug regimens have the potential to increase overall tumor destruction and that continued investigation into the optimal selection of drug(s) is required.

Clearly, HSP70 has known cellular protective effects in the setting of low-level hyperthermic exposure that one may wish to eliminate when attempting to treat a tumor. Fortunately, as these results and several other studies demonstrate, HSP70 is locally increased when cells are exposed to sublethal hyperthermia immediately surrounding the RF ablation zone, and this focal effect can be selectively suppressed with targeted local delivery of inhibitors/antagonists including quercetin [17]. Yet, a word of caution and a call for additional study is warranted prior to rapid clinical adoption. For example, the HSP70 family of proteins has other extensive roles in protein folding and transport, molecular chaperone activities, and immune-related antigen presentation [37, 38], such that downregulation of HSP activity may have unintended and even unwanted side effects on tumor growth. Given the ubiquitous nature of HSP70, it is also theoretically possible that systemic HSP70 suppression from systemic administration of specific antagonists could have unrelated secondary effects on non-target tissues. However, in our current paradigm in which a single dosing regimen is used with nanoparticle encapsulated platforms, the non-target systemic exposure is likely minimal. Finally, quercetin itself has separate non-HSP-based effects (for example, anti-oxidative, anti-hypoxic, and anti-angiogenic effects [39-42]) such that manipulation of these pathways may also alter the cellular microenvironment and tissue response at least in part through non-HSP mechanisms. The degree to which these potential secondary effects from targeted and localized HSP70 suppression occur, and their ultimate clinical significance, is currently unclear, but will hopefully be more fully elucidated in the near future.

There are several additional limitations of this study worth mentioning. Although this well-characterized R3230 tumor model to allow comparison to other studies of RF ablation and liposomal chemotherapy performed in this model, careful interpretation and application of the results is required. For example, the differences identified between quercetin and doxorubicin-based regimens likely reflect in part underlying tumor susceptibility to each of these agents for this particular model, and so further study will be required in other models to determine which tumor types will respond to RF combined with either or both liposomal agents. We have also selected a commercially available pegylated stealth liposomal doxorubicin agent and prepared similarly constructed quercetin-containing liposomes. Prior studies have demonstrated good success with these preparations, such as increased tumor destruction and intratumoral drug accumulation while reducing tumor growth rates [7]. As it has been previously shown that the empty liposome itself contributes to increases in observed tumor destruction, the continued use of this delivery vehicle allows a standardized comparison of the effects of different chemotherapy agents. However, additional characterization and optimization of the delivery vehicle and timing of the agent administration in future studies may improve in vivo drug delivery. For example, administering liposome-encapsulated quercetin at a different time point or dosage (or multiple doses) may potentially result in even greater HSP70 suppression, and therefore, further gains in tumor destruction and animal endpoint survival. Similarly, we also acknowledge that other approaches, such as the incorporation of thermosensitive liposomal preparations into our combination therapy algorithm may yield different, and perhaps improved, results[43, 44]. However, given the timing (4-12 hrs) of anti-HSP and pro-apoptosis effects that have been previously observed, it is far from clear that an additional early burst of drug release (0-1 hr), both spatially and temporally, will definitely increase tumor destruction compared to a slower, more prolonged drug release. In particular, the paradigm of tissue heating (both timing and variances in temperature ranges in the treatment zone) vary significantly between focal high-temperature RF tumor ablation and the low-temperature conventional hyperthermia that has been combined with thermosensitive liposomes in existing literature – such that, it is likely too early to assume that thermosensitive liposomal preparations will definitely be “better” than non-temperature

sensitive agents. Clearly, further investigation into identifying the delivery vehicle characteristics and administration paradigm is still required.

In conclusion, combining RF ablation with liposome-encapsulated quercetin to modulate heat shock reaction can reduce tumor growth and increased animal endpoint survival. Thus, our studies further supports the paradigm of using image-guided ablation to selectively control delivery of a nanodrug that is particularly active against its unwanted effects at the periphery of the thermal ablation zone. Even further gains can be achieved by combining RF ablation with multiple adjuvant agents that target separate underlying cellular stress pathways. Continued investigation into single and multi-drug combinations as adjuvants to ablation therapy, including optimization of drug delivery vehicles and dosing regimens, may yield continued gains in tumor destruction.

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Figure 1. Kaplan-Meier analysis of animal endpoint survival following treatment with RF and/or intravenous liposomal quercetin (Qu) and/or liposomal doxorubicin (Doxil)

Animal endpoint survival was defined as tumor diameter greater than 30 mm, or survival to 90 days, whichever came first (the graph was truncated at 60 days, as the final one animal had complete local control/tumor regression, and survived beyond 90 days). Mean survival for animals that received RF and single agent therapy (quercetin-RF or RF-doxorubicin) was greater than RF alone (16.6 ± 2.9 days), quercetin alone (15.5 ± 2.1 days), or the control group (12.5 ± 2.2 days; $P < 0.05$, all comparisons). Greatest endpoint survival was observed with triple therapy, quercetin-RF-doxorubicin (mean endpoint survival of 48.3 ± 20.4 days, $p < 0.005$ for all comparisons).

Figure 2. Tumor growth following treatment with RF ablation and/or intravenous liposomal quercetin (Qu) and/or liposomal doxorubicin (Doxil)

The mean tumor doubling time for animals that received RF ablation and liposomal quercetin was greater than RF alone (24.3 ± 1.9 days, $p < 0.05$), liposomal quercetin alone (21.3 ± 2.8 days, $p < 0.05$) or the control group (19.7 ± 3.2 days; $P < 0.005$). Triple therapy (quercetin-RF-doxorubicin) showed the greatest survival benefit, with a mean tumor doubling time of 81.6 ± 6.8 days. This was significantly greater than quercetin-RF (29.2 ± 1.4 days; $p < 0.001$) or RF-doxorubicin (39.8 ± 2.0 days, $p < 0.001$).

Figure 3. Gross tumor local control and regression in animals treated with combined RF ablation and IV liposomal quercetin and doxorubicin (A-D), and tumor had no treatment (E-F) (A) The initial tumor on day 0 (prior to any treatment) measures 14×13 mm. (B) At day 14, there were signs of tumor necrosis. (C) At day 35, tumor size decreased to 10×11 mm with necrosis. (D) At day 90, no evidence of tumor can be seen, a fact verified by autopsy. (E) The control, untreated tumor on day 0 (prior to any treatment) measures 13×12 mm. (F) The tumor quickly grew up and increased to 3 cm in size (and was sacrificed) at day 14.

Table 1

Effect of RF Ablation and/or adjuvant liposomal chemotherapy (quercetin and/or doxorubicin) on endpoint survival and tumor doubling time

Treatment	No. of Tumors	Mean Endpoint Survival (d)	Median Endpoint Survival (d)	Exponential Growth Rate*	Tumor Doubling Time (d)	R ²
Control	8	12.5±2.2	11.5	0.035	19.7±3.2	0.90
RF alone	8	16.6±2.9	17	0.028	24.3±1.9	0.97
Quercetin alone	8	15.5±2.1	15.5	0.032	21.3±2.8	0.94
Quercetin-RF	8	22.0±3.9	22.5	0.024	29.2±1.4	0.99
RF-doxorubicin	8	29.9±3.8	31	0.017	39.8±2.0	0.98
Quercetin-RF-Doxorubicin	8	48.3±20.4	45	0.009	81.6±6.8	0.91

Note.—Monopolar RF was applied for 5 minutes by using a 1-cm tip electrode, with the temperature titrated to 70°C. Endpoint survival is defined as 30 mm in diameter.

* Refers to *b* in the formula in Results, Tumor Growth Rates.