Pharm Res (2014) 31:3120–3126
DOI 10.1007/s11095-014-1404-5

RESEARCH PAPER

Hyperthermia Sensitization and Proton Beam Triggered Liposomal Drug Release for Targeted Tumor Therapy

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Received: 9 January 2014 / Accepted: 28 April 2014 / Published online: 23 May 2014
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ABSTRACT

Purpose The objectives of this study were to: 1) determine if mild hyperthermia (40–42°C) can sensitize tumor cells for more effective proton beam radiotherapy (PBRT); 2) characterize the survival fraction of cells exposed to PBRT; and 3) characterize release of the drug doxorubicin (Dox) from low temperature sensitive liposomes (LTSLs) without exposure to mild hyperthermia in combination with PBRT.

Methods Dox was actively loaded in LTSLs. A549 monolayer cells were incubated with 100–200 nM of Dox-LTSL (±mild hyperthermia). Cell irradiation (0–6 Gy) was performed by placing the cell culture plates inside a solid water phantom and using a clinical proton treatment beam with energy of 150 MeV. End points were survival fraction, radiation-mediated Dox release, and reactive oxygen species (ROS) production.

Results Hyperthermia effectively sensitized cells for PBRT and lowered the cell survival fraction (SF) by an average of 9.5%. The combination of 100 nM Dox-LTSL and PBRT (1–6 Gy) achieved additive to synergistic response at various dose combinations. At higher radiation doses (>3 Gy), the SF in the Dox and Dox-LTSL groups was similar (~20%), even in the absence of hyperthermia. In addition, 30% of the Dox was released from LTSLs and a 1.3–1.6 fold increase in ROS level occurred compared to LTSL alone therapy.

Conclusions The combination of LTSLs and PBRT achieves additive to synergistic effect at various dose combinations *in vitro*. Concurrent PBRT and Dox-LTSL treatment significantly improved the cytotoxic outcomes of the treatment compared to PBRT and Dox chemotherapy without LTSLs. We hypothesize that PBRT may induce drug release from LTSL in the absence of hyperthermia.

KEY WORDS Hyperthermia sensitization · Nanoparticle · Radiation guided drug delivery

INTRODUCTION

In patients with locally advanced cancer, concurrent chemo and photon (X-ray) radiation therapies are commonly used for treatment (1, 2). Despite heavy reliance on this approach in clinical practice, failure rates remain high due to systemic toxicity of chemotherapeutic agents and damage caused to

Electronic supplementary material The online version of this article (doi:10.1007/s11095-014-1404-5) contains supplementary material, which is available to authorized users.

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normal cells near the tumor by X-ray radiation (3). Corrective approaches that rely on reducing dosage and frequency of treatment often cause chemo- or radio-resistance (4). Increasingly, proton beam radiotherapy (PBRT) is being used as an alternative to X-ray based photon radiation to deliver a precise, predefined dose of radiation to a tumor in the patient with minimum exposure to nearby healthy tissues (5, 6). This is achieved using the spread out Bragg peak (SOBP) technique that provides more uniform dose coverage of the tumor with almost zero exit doses. Like photon therapy, proton beams can be used in conjunction with chemotherapeutics such as carboplatin and paclitaxel. These combinations have been shown to reduce side effects of treatment, especially in non-small cell lung cancer (2). However, symptoms such as dermatitis, esophagitis, pneumonitis, and pulmonary/pleural fistula still may occur and affect long-term patient survival. Thus, there is a critical need to better localize and target chemo-radio therapy at the treatment site.

The objective of this study was to combine PBRT with doxorubicin (Dox) encapsulated liposomes *in vitro* with the overall goal of *in vivo* dose (Radiation/Dox) reduction (Fig. 1). The idea for this combination regimen stems from clinical reports of reduced systemic toxicity of chemotherapeutic agents, such as Dox, upon encapsulation into stealth (PEGylated) liposomes. These liposomes can permeate the pores in blood vessels and accumulate selectively in the extravascular spaces of tumors *via* a mechanism known as enhanced permeability and retention; once inside the tumor, they slowly release drugs to achieve high intratumor concentrations (7, 8). Despite improved side-effect profiles, stealth liposomes have antitumor efficacies that are only equivalent to those of conventional treatments. The reason for the limited efficacy of stealth liposomes is unknown, but the intratumoral drug release rates may be too low. To achieve faster release rates, low temperature sensitive liposomes (LTSLs) have been developed. LTSLs can deliver Dox when triggered by temperature elevations greater than the melting temperature of the lipid formulation (~41–42°C) (9–12). The mild hyperthermia (~42°C) required for LTSL drug release can also enhance killing of radio-resistant hypoxic cancer cells. Thus, the combination of mild hyperthermia-triggered drug release from LTSLs with PBRT can potentially improve the efficacy of concurrent treatment (13, 14). In addition, radiation-triggered Dox release from LTSLs in the absence of hyperthermia can significantly broaden its clinical application (Fig. 1) for combined LTSL-PBRT treatment. Herein, we present findings obtained from an *in vitro* cell culture model. The results support the premise that this radiation- and hyperthermia-guided drug delivery approach should be tested in *in vivo* preclinical and clinical studies to improve the spatial targeting of therapies to tumors.

MATERIALS AND METHODS

Synthesis of Dox-LTSLs

Dox-LTSLs were prepared by hydration of a phospholipid film followed by extrusion through a polycarbonate membrane filter (15). A mixture of three phospholipids [1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine, 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine, and N-(carbonyl-methoxypolyethyleneglycol-2,000)-1, 2-distearoyl-sn-glycero-3-phosphoethanolamine] was dissolved in a minimum volume of chloroform at a molar ratio of 85.3:9.7:5.0. The organic solvent was evaporated to dryness in a rotary evaporator and the resulting thin film of phospholipids was hydrated using 300 mM citrate buffer at pH 4.0. The hydrated lipids were extruded five times through two stacked polycarbonate filters

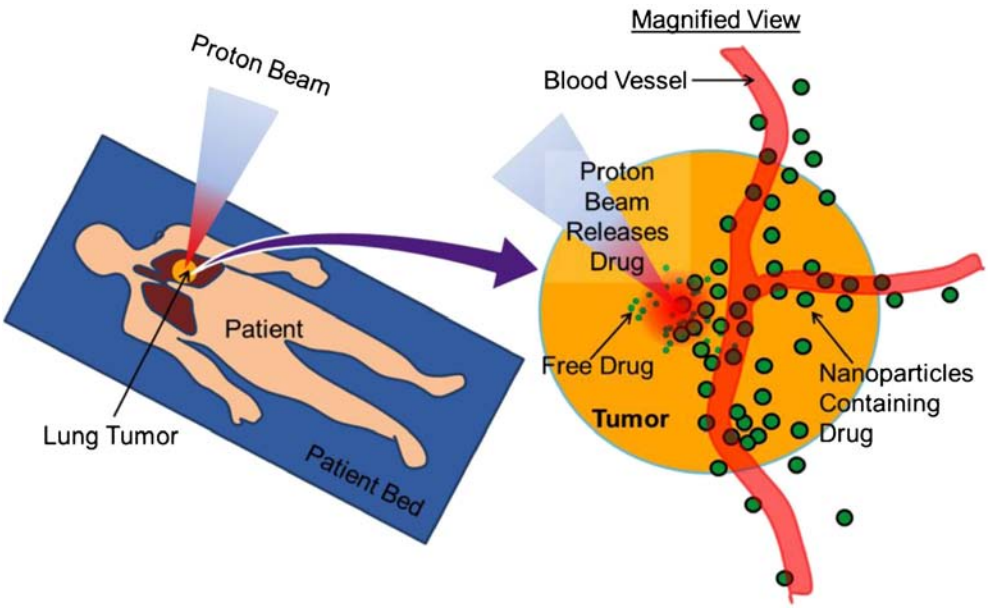

Fig. 1 Proposed Radiation guided drug delivery paradigm. PBRT triggered loco-regional drug delivery.

of 100 nm pore size to yield bland liposomes (LTSLs). Encapsulation of Dox into LTSLs (2 mg of Dox per 100 mg of lipids) was carried out actively using a pH gradient method described by Mayer *et al.* (16). Unencapsulated Dox in the LTSL solution was removed using a PD-10 size exclusion column (GE Healthcare Life Sciences, Buckinghamshire, UK). Prior to *in vitro* studies, LTSLs were characterized for size using a dynamic light scattering instrument (Zetapals, Brookhaven Instruments Corporation), and Dox release as a function of temperature (25, 37–42°C) was assessed using fluorescence (SpectraMax M2^e, Molecular Devices, Sunnyvale, CA).

Monolayer Cell Culture

All studies were conducted using A549 lung cancer cells maintained in RPMI-1640 medium (Mediatech Inc., USA) supplemented with 10% fetal bovine serum and 1% antimicrobials (10,000 IU/mL penicillin and 100,000 μg/mL streptomycin) in a humidified incubator with 5% CO_2 at 37°C. Prior to irradiation, the cells were gently scraped and seeded onto 6-well plates at six different seeding levels (50, 75, 100, 150, 200, and 300 cells/well) for 12 h under 5% CO_2 at 37°C.

LTSL-PBRT Treatment and Cell Survival Assay

Monolayer cells in six-well plates were treated with LTSLs suspended in culture medium containing 100 to 200 nM Dox. Immediately after treatment, cells were incubated in humidified incubators (Eppendorf Inc. New York, NY) in 5% CO_2 atmosphere at 37°C and transported for proton irradiation. To compare response with (40–42°C) and without (37°C) mild hyperthermia, one set of LTSL treated cells was heated to 42°C for 30 min in the humidified incubator. Cell irradiation was performed by placing the six-well plates inside a solid water phantom to mimic the tissue environment present inside a patient's body. A radiation dose of 0, 1, 2, 3, or 6 Gy was delivered using a clinical proton treatment beam with energy of 150 MeV, a 25 cm diameter lateral irradiation field size, and a spread out Bragg peak of 10 cm. Following irradiation, the cells were placed in the incubator, and after 6 h the culture medium was carefully removed and the plates were rinsed with phosphate buffered saline (PBS). The rinsed plates were maintained for 10–12 days in the 5% CO_2 incubator at 37°C in fresh culture medium until countable colonies formed. The fraction of cells that survived under each drug and/or radiation dose delivered was determined using a clonogenic assay (17). For this assay, the culture medium was removed, the plates were rinsed twice with PBS, and the cells were incubated with a mixture of 6.0% glutaraldehyde and 0.5% crystal violet for 1 h. Afterwards, the glutaraldehyde crystal violet mixture was removed carefully and the plates were rinsed three times with tap water in a large bucket (washing the plates under the running tap can affect the colonies). The plates were allowed to air dry at room temperature. Colonies in each well of the six-well plates were counted using at least three replicates for each treatment. Plating efficiency and cell survival fraction (SF) were calculated as described previously (17).

HPLC Quantification of PBRT-Triggered Dox Release from LTSLs

To quantify drug release from LTSLs, 2 mL of culture medium containing LTSLs with 30 μM Dox (this concentration was chosen based on the instrument's detection limit) were preheated to 37°C and irradiated to an absorbed dose of 3 Gy. This radiation dose was selected based on a prior cell survival assay that indicated significant reduction in cell survival in combination of Dox. To rule out the possibility that mild hyperthermia triggered the drug release, a infrared camera was utilized to obtain temperature maps during irradiation (the positioning of the culture plates and the infrared camera with respect to the PBRT patient bench is shown in Fig. 4(a-b)). An ETIP 7320 P-Series infrared camera (Infrared Cameras Inc., Beaumont, TX) with a micro-bolometer 320×240 UFPA VOX sensor and a 25 mm lens was used for thermography measurement. The infrared sensor had a spectral response of 8–10 mm, a thermal sensitivity of 0.027°C at 25°C, and an accuracy of ±2°C or ±2%. The dynamic range of the temperature measurement controlled by a laptop computer using USB protocol was 16 bits. The laptop computer was placed outside the PRBT room and controlled the infrared camera near the culture plate *via* an approximately 11 m long active USB extension cable. At the default screen resolution of 320×240 pixels, the maximum acquisition rate was 23 frames per second. The frames were continuously stored in the dynamic memory during the exposure, and transferred to hard-drive after the acquisition was completed. For infrared thermometry, culture plates were kept under a 11 cm depth of solid water phantom, and readings were taken from the transparent side walls while the irradiations were performed. After irradiation and thermometry, 50 μL of Daunorubicin (DNR, 2.5 μg/mL) were added to the samples as an internal control, and the mixtures were centrifuged at 150,000 g (Beckman Coulter, Brea, CA) for 1 h. The supernatant was collected and HPLC analysis was performed as described previously (18).

Measurement of Reactive Oxygen Species (ROS) Production

Radiation-induced oxidative stress in A549 cells following LTSL (200 nM) and PBRT treatment was assessed by measuring ROS production (n=8–12, 3 replicates). For the ROS assay, cells were seeded at ~20,000 cells/well in a 96-well plate, allowed to attach for 24 h, and then loaded with 75 μL of chloromethyl-2,7-dichlorodihydro-fluorescein diacetate (CM-H2DCFDA, 5 μM, Invitrogen) for 30 min in a 5%

CO_2 incubator at 37°C. Prior to irradiation, extracellular CM-H2DCFDA was removed by gentle rinsing with Dulbecco's phosphate buffered saline. Next, a serum free medium containing 1% penstrep was added to each well, irradiation was performed, and normalized fluorescence intensity from each well was recorded after 15 h using a spectrophotometer (excitation/emission: 494/525 nm; SpectraMax M2e, Molecular Devices, Sunnyvale, CA).

Statistical Analysis

Treatment groups were compared for mean differences in cell survival, drug release and ROS production using analysis of variance (ANOVA) followed by Turkey's multiple comparison post-hoc test. All analyses were performed using Graph Pad Prism 6.0 (Graph Pad Software, Inc., San Diego, CA). A p-value less than 0.05 indicated statistical significance. Values are reported as Mean ± Standard Error of Mean (SEM) unless otherwise indicated.

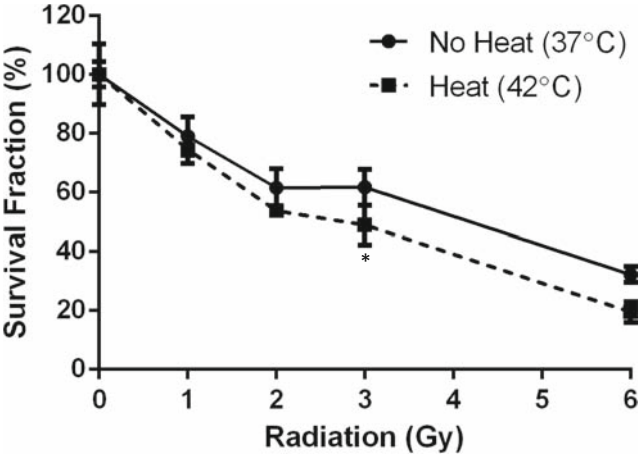

Fig. 2 Hyperthermia sensitization of the cells to PBRT. The *solid back line* represents the survival curve of monolayer cells exposed to radiation treatment without mild hyperthermia treatment, while the *dashed line* represents the survival curve of cells subjected to mild hyperthermia (incubated to 42°C for 30 min) just prior to exposure to each radiation dose. The asterisk (*) indicates the first radiation dose with a significantly reduced survival fraction ($p < 0.05$).

RESULTS

LTSL Characterization

The hydrodynamic diameter of LTSLs measured by dynamic light scattering was approximately 106.4± 0.5 nm. Active loading of Dox in LTSLs by transmembrane pH gradient yielded an encapsulation efficiency of >95%. Release of Dox in PBS was minimal (10–15%) at 37°C, and nearly complete release (>90%) was observed at ~42°C.

Hyperthermia-Induced Sensitization to PBRT

In the absence of hyperthermia, the SF decreased from an average of 100–32.3% as the dose of PBRT increased from 0–6 Gy (Fig. 2, solid line). In contrast, adding heat prior to irradiation decreased viability by more than 9–10% compared to the unheated group (Fig. 2, dashed line). Significant differences in viability were observed between the heat and no heat groups at 3–6 Gy.

Cell Survival upon LTSL-PBRT Treatment

The SFs of cells treated with Dox-LTSL and Dox alone in the absence of hyperthermia were 93.9 and 76.9%, respectively for unirradiated samples (0 Gy PBRT dose) (Fig. 3a). The mean SF upon concurrent hyperthermia treatment (40–42°) for Dox-LTSL was 49.5%, which was similar to the mean SF of the group subjected to 100 nM free Dox and hyperthermia indicating a near complete release of Dox from Dox-LTSL at 42°C (Fig. 3b). At higher doses (2–6Gy), the SF reduction achieved by using the combination of PBRT and LTSL-Dox was significantly different from that of radiation alone. Furthermore, at a dose of 3 Gy or greater, the combination treatment (Free Dox or Dox LTSL ± hyperthermia) showed synergistic effects, killing more cells (19.7±4.5, 10.9±1.8, 22± 0.4, and 17.8±1.1% with Free Dox, Free Dox + hyperthermia, LTSL, LTSL + hyperthermia respectively) than radiation alone (SF ~70%).

Infrared Thermometry and HPLC Quantification of PBRT-Triggered Dox Release from LTSLs

Temperature increase during proton irradiation measured using an infrared camera did not reveal significant changes and the mean temperature stayed close to the room temperature (~23°C, Fig. 4c-d). HPLC quantification of irradiated samples demonstrated ~30% greater Dox release compared to the representative control (n=5, 3 replicates; significant at P<0.01, Fig. 5).

ROS Production in LTSL-PBRT Treated Cells

To measure the ability of the LTSL-PBRT combination treatment to generate ROS and initiate cytotoxic effects, the amount of ROS in the cells was measured 15 h after treatment. High cellular ROS concentrations (~1.3–1.6 fold, Fig. 6) were observed in the LTSL-Dox and PBRT treatment compared to representative controls (radiation, bland liposome). Furthermore, ROS generation was similar to that of the positive control (H_2O_2), indicating significant cytotoxic effects (data not shown).

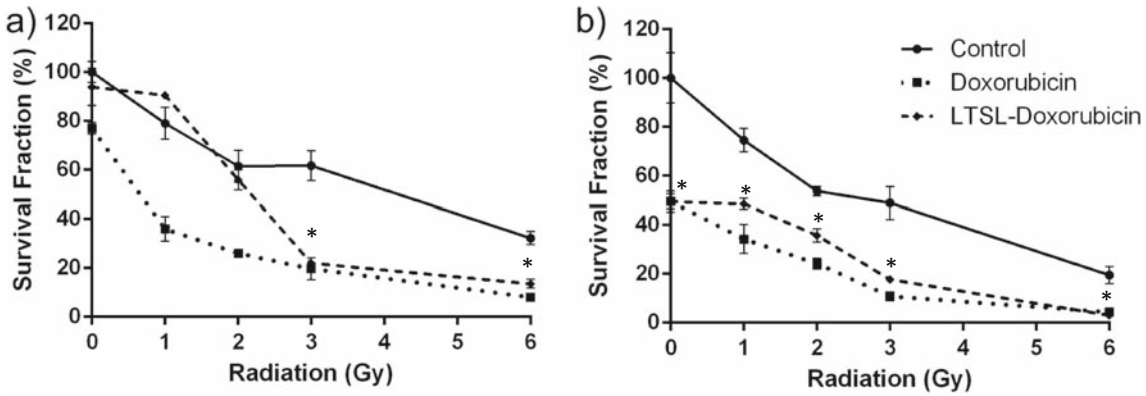

Fig. 3 (**a**) Survival fraction (SF) of monolayer cells treated with 100 nM Dox-LTSL (*dashed line*), 0 nM Dox (*solid line*; control), and 100 nM free Dox (*dotted line*) with PBRT and without mild hyperthermia. At low doses of PBRT, 100 nM Dox-LTSL samples and 0 nM Dox samples had a similar SF. Similarly, at higher doses of PBRT, the 100 nM Dox-LTSL group and the 100 nM free Dox group had a similar SF. (**b**) Mild hyperthermia-induced drug release from LTSLs resulted in a similar SF for the 100 nM free Dox and 100 nM Dox-LTSL groups at all radiation doses. Points on each curve significantly different from the controls at the same radiation dose are marked with an asterisk (*$p < 0.05$).

DISCUSSION

The goal of this *in vitro* study was to determine if the combination of PBRT and Dox-LTSL is an effective concurrent treatment modality for solid tumor therapy. The findings from this study provide background data and a preliminary foundation for future preclinical studies of the use of this radiation- and hyperthermia-guided drug delivery approach to improve the spatial targeting of therapies to tumors.

Hyperthermia Sensitization

Many X-ray-based radiotherapeutic regimens have been shown to work synergistically with mild hyperthermia (19, 20). This occurs because hyperthermia treatment inhibits sub-lethal DNA damage repair, causing significantly higher tumor killing (21). We hypothesized that hyperthermia can also potentiate the therapeutic response of proton therapy. Our data indicate that exposing cells to mild hyperthermia prior to proton irradiation increased their kill rates (~10% greater) compared to unheated cells. Although the extent of the interaction between heat and ionizing radiation will need to be determined empirically in an animal tumor model, our findings provide interesting insight about hyperthermia potentiation. For maximal benefit, translation of the treatment to an *in vivo* model should aim for spatial accuracy of the hyperthermia treatment in the targeted site. This can be facilitated by recent advances in MR-HIFU technology, which can provide real-time control of mild hyperthermia in tumor tissues (18). In summary, the *in vitro* results presented herein are consistent with those for X-ray irradiation and demonstrate the feasibility of using hyperthermia as an adjunct to particle therapy.

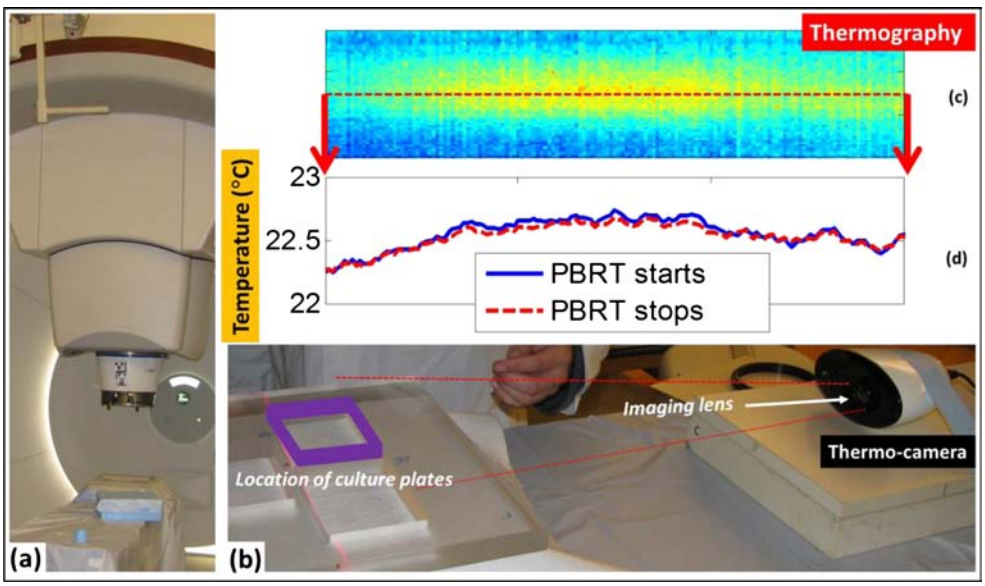

Fig. 4 Infrared thermometry. (**a**) Experimental set-up for cell irradiation delivered using a clinical proton treatment beam with energy of 150 MeV with a 25 cm diameter lateral irradiation field size. (**b**) For Infrared thermometry, culture plates were kept under a 11 cm depth of solid water phantom, and readings were taken from the transparent side walls during irradiations. (**c**-**d**) Mean temperature in the culture media immediately before and after 10 s of irradiation.

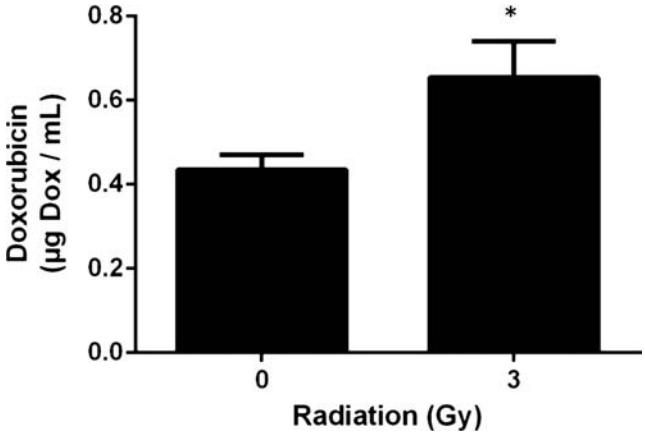

Fig. 5 The release of Dox from Dox-LTSL as a function of radiation dose. At the PBRT dose of 3 Gy, 30% more Dox was released compared to the 0 Gy dose (*$P < 0.1$).

Benefits of LTSL and PBRT Combination Therapy

At clinically relevant dosages, the effectiveness of concurrent chemoradiation in our monolayer cell culture models was remarkable. In general, the effects were largely additive, although they demonstrated synergism at certain dose combinations. For example, individual treatment with 100 nM free Dox or 3 Gy irradiation resulted in a mean SF of 60–80%. In contrast, a combination therapy of free Dox and 3Gy PBRT dose significantly lowered the SF rates by up to 20%. Currently, a major research goal is to reduce treatment toxicity by discovering combinations of drug and radiation treatments that show synergism in tumor killing. Synergistic responses are achieved by enhancing the sensitivity of tumor cells to radiotherapy upon systemic exposure to anticancer drugs (*e.g.*, cisplatin, taxanes, gemcitabine). To achieve true synergism and non-toxic outcomes, the agents under investigation should have non-overlapping toxicity profiles. This will promote the use of both modalities at effective doses without increasing normal tissue effects. For this to occur, the agents need to have a higher effective concentration in the tumor relative to normal tissues. LTSLs are modified stealth liposomes that have lysolipids incorporated into the liposomal membrane. Lysolipids allow rapid release of their contents in response to mild hyperthermia (>41°C) and their use is being widely explored in Phase III studies of energy deployed drug delivery (22). Many previous reports suggest that drug delivery from LTSLs *in vivo* is highly target specific and superior to Dox only therapy, and this could have important implications for the LTSL-PBRT combination treatment paradigm. The *in vitro* data for the proposed combination therapy point towards a new technology that removes multiple barriers to progress in concurrent or sequential chemoradiotherapy and plans to conduct *in vivo* studies using this system in a mouse tumor model are under way.

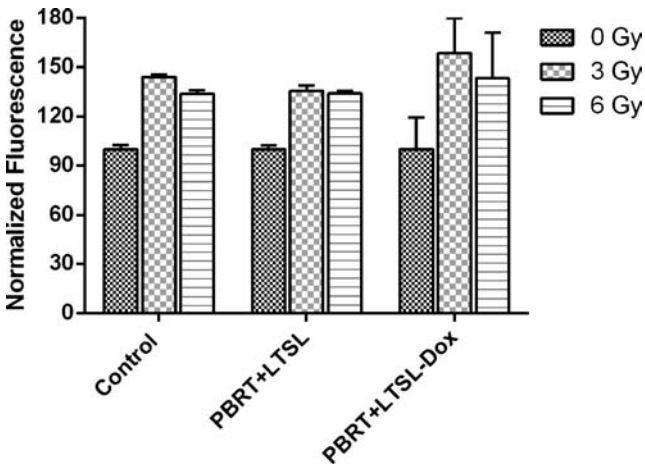

Fig. 6 A549 cells incubated at 37°C with 5 μM CM-H2DCFA exposed to 0, 3, and 6 Gy of proton irradiation in the presence or absence of LTSLs. Normalized fluorescence indicates 1.3–1.6 fold greater ROS production in the presence of LTSL-Dox and PBRT.

Proton Beam Guided Drug Delivery

An important finding in this study was that the Dox-LTSL and Dox alone treated cells had similar SFs at higher radiation dose (3 Gy). Two different scenarios may explain this finding: 1) release of Dox from Dox-LTSL (up to 15–20% Dox) in serum containing medium at 37°C caused a lower SF than expected and 2) PBRT induced drug delivery from LTSLs. Our HPLC data showed that 20–30% of the Dox was released from LTSLs at the 3 Gy dose. Triggered drug release from liposomes upon X-ray irradiation was reported recently when a radiation-sensitive material, di-(1-hydroxylundecyl) diselenide, was incorporated into liposomes. Release plots of Dox from X-ray-sensitive Dox-loaded liposome exposed to X-rays at doses of 0, 15, and 30 Gy in this study indicated a dose-dependent response (23). Similarly, dose-dependent release of Dox was reported in diselenide-containing block co-polymer aggregates (24). However, measurable drug release from these nanoparticles required a large radiation dose. In contrast, the drug release observed in our study occurred at a PBRT that is within the range of doses used for clinical treatment. Although interesting, data from our study are largely speculative and need to be confirmed by multiple laboratories to determine the feasibility of particle beam guided drug release. However, we can safely conclude that the release pattern observed in our study was independent of the temperature increase. As shown in Fig. 4(c-d), we did not observe significant increase in temperature of the liposome suspended culture media by infrared thermometry. We speculate the energy deposition mechanisms of the clinical proton beam (150 MeV) used in this study are more likely to create deformation/pore formation in the liposomes and induce drug release than those of the x-ray beams used in previous studies (23). This premise is supported by the 1.3–1.6 fold greater ROS production in the LTSL +

PBRT treatment and the HPLC data (Figs. 4 and 5). It is noteworthy that LTSLs are unlikely to be applicable in cases for which deep tissue hyperthermia is not possible (10). PBRT-mediated triggered drug release can trade off the benefits provided by mild hyperthermia and minimize need for additional equipment such as HIFU or laser hyperthermia applicators.

CONCLUSION

This work demonstrated the feasibility of combining clinical PBRT and clinical-grade LTSLs in a monolayer *in vitro* culture model. The data suggest that mild hyperthermia treatment of tumor cells can enhance the therapeutic response to PBRT therapy and enhance the effectiveness of the PBRT and Dox-LTSL combination therapy. Based on the results of this initial study, we hypothesize that PBRT may also induce drug release from LTSL that may allow localized targeting in the absence of hyperthermia.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by a multidisciplinary team grant from the Center for Veterinary Health Sciences and the College of Arts and Sciences, Oklahoma State University, and a collaborative research agreement with ProCure Proton Therapy Center, Oklahoma City.

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