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High-intensity Focused Ultrasound-Mediated Doxorubicin Delivery with Thermosensitive Liposomes

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Abstract. Local drug delivery of doxorubicin holds promise to improve the therapeutic efficacy and to reduce toxicity profiles. Here, we investigated the release of doxorubicin from thermosensitive liposomes (Dox-TSL) into human glioblastoma (U-87MG) cells. Using Dox-TSL, experiments were carried out in a water bath and showed that 15 min incubation of TSL at 43°C induced the release of 80% doxorubicin loaded TSL compared to the release at 37°C. The cytotoxicity of a range of concentrations of Dox-TSL was also evaluated on U-87MG cells. At 37°C, no cytotoxicity was observed, whereas at 43°C the results showed that the cytotoxicity is dose dependent. At maximal dose of doxorubicin (30 µg/mL), the cell viability was less than 20%. Application of 15 min of HIFU at 1 MHz, 1.5 MPa and 50% duty cycle induced the release of 100% of doxorubicin from Dox-TSL. In the same experimental condition, the cell viability decreased to 40% and 20% at 12h and 48h, respectively, in comparison to that obtained during the incubation of cells with Dox-TSL alone without HIFU. In conclusion, a significant release of doxorubicin from temperature-sensitive liposomes can be achieved leading to an efficient treatment and cell death of tumor cells using HIFU.

Keywords: Drug delivery; Temperature-sensitive liposomes; Focused ultrasound; Hyperthermia

INTRODUCTION

Doxorubicin, also known as adriamycin, is one of the most powerful anti-neoplastic drugs prescribed on its own or in a combination with other agents. This anthracycline is widely used for the treatment of solid tumors and hematological malignancies. In conventional cancer therapy, the therapeutic window for chemotherapy using doxorubicin is limited by the undesired high drug uptake in healthy organs compared to tumor uptake [1]. To overcome this problem, the development of new nanomedicines, where the doxorubicin is encapsulated into liposomal nanoparticles, shows significantly reduced acute toxicity by preventing drug uptake in normal tissue. Currently, liposomal doxorubicin formulations (e.g. Doxil[®], Caelyx[®]) have shown to significantly reduce cardiotoxicity in comparison to unencapsulated doxorubicin [2,3]. Moreover, preclinical and clinical studies comparing free doxorubicin and doxorubicin-encapsulated liposomes showed an increase of anti-tumoral activity for

liposomal formulations [4-7]. However, others studies demonstrated a comparable effectiveness for liposomal versus free doxorubicin [8-10]. Clearly, the increased doxorubicin concentration does not directly correlate to an improved anti-tumor effectiveness due to the limited bioavailability of doxorubicin within the liposomal nanoparticles.

Targeted triggered drug delivery from responsive liposomal nanoparticles hold great promise to further increase the drug concentration and its bioavailability in the tumor. Novel liposomal formulations have emerged that allow the temperature-triggered release of drugs [11]. Temperature-sensitive liposomes (TSLs) are able to lead to a more rapid release of the encapsulated drugs through transient nanopores formed at their phase transition temperature (T_m) [12-15]. Preclinical studies with doxorubicin-loaded TSLs in combination with an externally applied regional temperature increase showed an improved effectiveness of temperature-induced drug delivery [16,17]. Ideally, the doxorubicin should remain stably encapsulated in the TSLs at body temperature, whereas a rapid and quantitative drug release should occur at mild hyperthermia (39-43°C) [18,19]. For cancer therapy, hyperthermia can be locally applied at the tumor site for controlled drug delivery with TSLs. High intensity focused ultrasound (HIFU) has emerged as a promising technique that allows local hyperthermia and ablation of pathological tissue [19,20].

In the present work, we investigated the effectiveness of HIFU to induce the doxorubicin release from TSLs and the death of human glioblastoma cells.

MATERIAL AND METHODS

Cell Culture

Human glioblastoma astrocytoma cells (U-87 MG) were derived from a malignant glioma. Cells were grown as a monolayer in DMEM-GlutaMAX-I supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco®-Invitrogen™, Carlsbad, CA). The cells were routinely sub-cultured every 4 days and incubated at 37°C in humidified atmosphere with a 5% CO₂ incubator.

Doxorubicin-loaded TSLs

TSLs were obtained by the lipidic film re-hydration method. The following lipidic composition was used: dipalmitoylphosphatidylcholine (DPPC), hydrogenated soybean phosphatidylcholine (HSPC), cholesterol (Chol) and pegylated distearylphosphatidylethanolamine (DSPE-PEG2000) (Lipoid) in the molar ratio of DPPC:HSPC:Chol:DSPE-PEG 100:33:27:7. Lipids were solubilized in chloroform in a Pyrex tube. Chloroform was slowly evaporated under a nitrogen flow to form a lipidic film on the inner surface of the tube, which was finally dried in a dessicator under vacuum during 2 hours. The lipidic film was re-hydrated at 55°C with a solution of ammonium hydrogen phosphate (NH₄)₂HPO₄ 300mM, at pH 5. Freeze-thaw cycles were then performed by successively plunging the sample into liquid nitrogen and a water bath at 55°C (7 cycles). In order to calibrate liposomes size, series of extrusions were then performed at 55°C and at 5 bars, first on a 0.45µm membrane

filter (5 passages) and then on a 0.22 μ m membrane filter (10 passages), by using a thermobarrel extruder (LIPEXTM Extruder, Northern Lipids). Dialysis steps were then performed in buffer saline (HEPES 20mM, NaCl 140mM, pH 7.4) to eliminate external (NH₄)₂HPO₄: once over 1 night at 4°C, then twice during 3 hours at ambient temperature (final dilution factor of 250*250*250). Doxorubicin (Dox) was then added at 37°C and incubated with TSLs during 1 night at 37°C. Non-encapsulated doxorubicin was eliminated by size-exclusion chromatography on a Sephadex G50 filtration gel (column height: 5 cm; eluant: HEPES 20mM, NaCl 140mM, pH 7.4).

Ultrasound Setup

Ultrasound waves were generated from a focused single element transducer with a center frequency of 1 MHz. The transducer had a diameter of 45 mm and was focused at 50 mm. The transducer was driven with an electrical signal generated by an arbitrary waveform generator (Agilent, Santa Clara, CA, USA) and amplified with a power amplifier (ADECE, Artannes sur Indre, France). The peak negative pressure of the acoustic wave was measured in a separate setup using a calibrated PDVF needle hydrophone (0.2 mm diameter, Precision Acoustics, Dorchester, UK) at the focal distance of the transducer.

Characterization of Dox-TSLs

One day after seeding the cells in 24-wells plates (100 000 cells/well), incubation experiments with solutions of a range concentration of Dox-TSLs were performed. The Dox-TSLs were diluted in OptiMEM-1%FCS to obtain a range of doxorubicin concentrations from 0 to 30 μ g/mL. All samples were kept for 15 min at either 37°C or 43°C respectively before they were added to the cells. After removal of the cell medium, the cells were incubated with 1 mL of the preheated samples for 4h at 37°C. After the incubation, the samples were removed and the cells were gently washed with PBS and incubated in 1 mL fresh medium for 2 days at 37°C. The cell viability was evaluated by a Trypan blue dye exclusion assay 48 h after treatment.

HIFU-mediated Doxorubicin Release

The doxorubicin release from TSL was determined by measuring the fluorescence intensity (λ_{ex} : 485 nm, λ_{em} : 590 nm). At the end of each measurement, 5 μ L of a 10% v/v solution of Triton X-100 was added to 2 mL of the solution, to afford quantitative release of doxorubicin. The percentage of doxorubicin release was calculated according to: $(IHIFU-I0)/(I100-I0) \times 100\%$, in which IHIFU is the fluorescence intensity after HIFU treatment, I0 is the fluorescence intensity before HIFU treatment (i.e. negative control), I100 is the fluorescence intensity after the addition of Triton X-100 (i.e. positive control). The doxorubicin release from the TSLs (23 μ L) in 2 mL OptiMEM-1%FCS was determined before and after HIFU treatment using 1 MHz sinusoidal waves of 1.5 MPa, 500 cycles, repeated every 1 ms during 15 min.

HIFU-mediated Doxorubicin Delivery

Cells were trypsinized, washed once and resuspended in OptiMEM supplemented with 1% FCS. During the procedure, the cell suspension was maintained at 37°C in a water bath (Grant Instruments Ltd, Cambridge, UK). Then, the cell suspension (5.105 cells in 2 mL) was placed in a polystyrene cuvette (45 mm height, 10 mm internal width and 12 mm external width). A volume of 23 μL of Dox-TSLs was added to obtain a final doxorubicin concentration of 5 μM . The center of the plastic cuvette was positioned at the focal distance of the transducer in a de-ionized water bath maintained at 40°C. Subsequently, the cell suspension was exposed to 1 MHz sinusoidal ultrasound waves with a pulse repetition frequency of 1 kHz, 500 cycles per pulse and for 15 min. Acoustic pressure of 1.5 MPa was applied. After ultrasound application, 500 μL of cells were cultured in 24-wells cell culture plate and incubated at 37°C in humidified atmosphere with a 5% CO₂ incubator. Four hours later, 1 mL of OptiMEM-10% FCS medium was added to each well and incubated at 37°C in humidified atmosphere with a 5% CO₂ incubator for 12 and 48 hours. The cell viability was evaluated by a Trypan blue dye exclusion assay 12h and 48 h after treatment.

RESULTS

Characterization of Dox-TSLs

The heating of Dox-TSLs at 37°C in water-bath system did not result in a significant cell death for all concentrations of Dox-TSLs. However, the temperature increase from 37°C to 43°C during 15 min induced the release of 80% of doxorubicin from Dox-TSLs (data not shown). At 43°C, the cytotoxicity of Dox-TSLs increased with the dose of Dox-TSLs. At maximal dose of doxorubicin (30 $\mu\text{g/mL}$), the cell viability was less than 20% (Fig. 1).

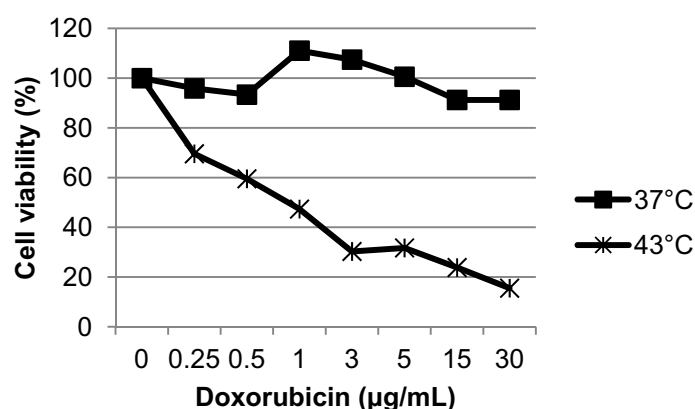


FIGURE 1. Doxorubicin release – The doxorubicin release from TSLs was performed in OptiMEM-1% FCS at 37°C and 43°C.

HIFU-mediated Doxorubicin Release

Due to the high intra-liposomal doxorubicin concentration, the doxorubicin fluorescence is quenched. The release of doxorubicin from the aqueous compartment of the liposomes will result in a dilution of this drug in the cell medium to concentrations below self-quenching concentration leading to an increase in the fluorescence intensity. The latter is proportional to the concentration of doxorubicin in the solution. The doxorubicin release from the TSLs after Triton X-100 or HIFU treatment was determined by measuring the increase of the fluorescence intensity of the TSLs in OptiMEM-1% FCS (Fig. 2). As shown in the Fig. 2, the application of 15 min of HIFU at 1 MHz, 1.5 MPa and 50% duty cycle induced the release of 100% of doxorubicin from doxorubicin-loaded TSLs.

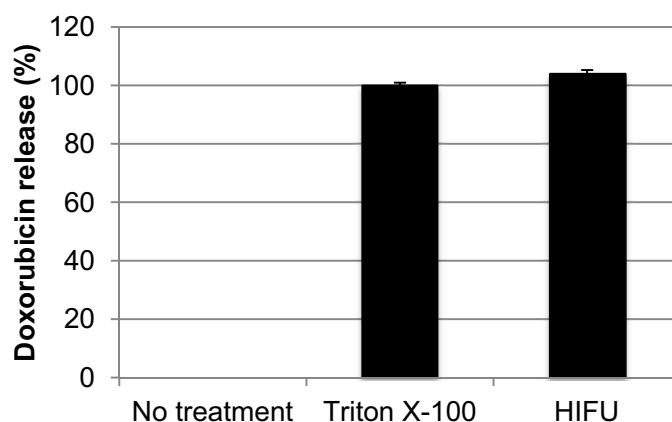


FIGURE 2. Doxorubicin release – The doxorubicin release from TSLs was performed in OptiMEM-1% FCS without treatment, after Triton X-100 treatment and after HIFU treatment (N=3).

HIFU-mediated Doxorubicin Delivery

Cell viability was assessed by the Trypan blue dye exclusion test at 12h (Fig. 3a) and 48h (Fig. 3b) after Dox-TSLs in combination with HIFU. When the cells were incubated with Dox-TSLs alone, the cell viability was not affected, whereas after HIFU treatment alone, the cell viability was $59\pm 26\%$ at 12h (Fig. 3a). The combination of HIFU and Dox-TSLs induced a 40% decrease in the cell viability compared to Dox-TSL treatment alone at 12h. However this induced cell viability was similar to that obtained with HIFU alone at 12h (Fig. 3a). At 48h, HIFU or Dox-TSLs treatment alone induced similar cell viability. The combination of Dox-TSLs with HIFU led a 2-fold decrease in the cell viability compared to Dox-TSLs treatment alone (Fig. 3b).

CONCLUSIONS

In this present study, we showed that the HIFU treatment caused the release of 100% doxorubicin from Dox-TSLs. Moreover, we reported the direct evidence that the combination of HIFU and Dox-TSLs induced a high cell death level by a synergistic effect.

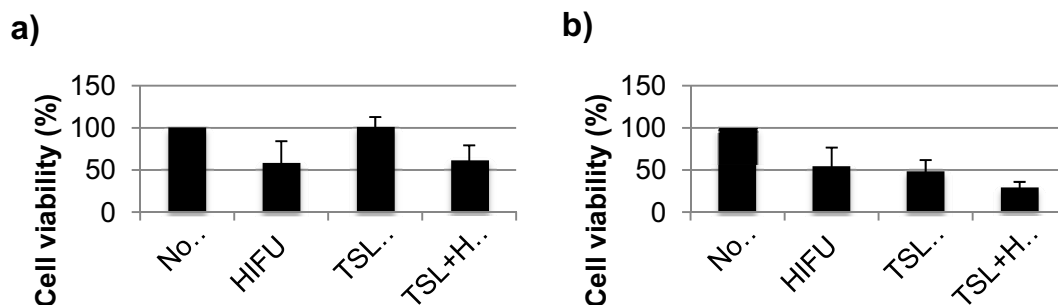


FIGURE 3. Doxorubicin cytotoxicity – Trypan blue viability assay is performed on U-87MG cells, without treatment (No treatment), after HIFU treatment alone (HIFU), after incubation with TSL alone (TSL alone) and after HIFU treatment (TSL+HIFU) in OptiMEM-1% FCS at 12h (a) and 48h (b) (N=3).

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