Ultrasound-enhanced localized chemotherapy of drug-sensitive and multidrug resistant tumors

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Abstract. A new modality of targeted tumor chemotherapy is based on the drug encapsulation in polymeric nanoparticles followed by a localized release at the tumor site triggered by focused ultrasound. Effect of 1 MHz and 3 MHz unfocused ultrasound applied locally to the tumor on the Doxorubicin (DOX) biodistribution and tumor growth rates was measured for ovarian carcinoma tumors in nu/nu mice. The bioeffects of ultrasound were investigated on the systemic and cellular levels. Growth rates of A2780 ovarian carcinoma tumors were substantially reduced by combining micellar drug delivery with tumor irradiation. Ultrasound effect was not thermal as manifested by intratumoral temperature measurements during sonication. Biodistribution studies showed that ultrasound did not enhance micelle extravasation. Main mechanisms of the ultrasound-enhanced chemotherapy included (i) passive targeting of drug-loaded micelles to the tumor interstitium; (ii) ultrasound-triggered localized drug release from micelles in the tumor volume; (iii) enhanced micelle and drug diffusion through the tumor interstitium; and (iv) ultrasound-triggered cell membrane damage resulting in the enhanced micelle and drug uptake by tumor cells.

INTRODUCTION

A new modality of targeted chemotherapy that we are developing is based on the encapsulation of drug within polymeric nanoparticles followed by a localized release at the tumor site triggered by focused ultrasound. The rationale behind this approach is that drug encapsulation in nanoparticles decreases systemic drug exposure, diminishes intracellular drug uptake by normal cells, and provides for a passive drug targeting to tumors. Upon passive accumulation of drug-loaded nanoparticles in the tumor interstitium, tumor is irradiated by focused ultrasound, which triggers drug release from carriers and enhances the intracellular uptake of both released and encapsulated drug. Effect of local tumor sonication on drug biodistribution and tumor growth rates as well as mechanisms involved in the ultrasound action on the systemic and cellular levels are reported.

MATERIALS AND METHODS

Cells and animals. Human xenografts of ovarian carcinoma A2780 and colon cancer HCT116 tumors were inoculated subcutaneously (s.c.) in nu/nu mice. Biodistribution of drug (Doxorubicin, DOX) and tumor growth rates were measured *in vivo* for various drug delivery systems with and without tumors sonication.

Polymeric Micelles. Micelles formed by various block copolymers were studied. Pluronic P-105 is a triblock copolymer poly(ethylene oxide)-co-poly(propylene oxide)-co-poly(ethylene oxide), with monomer unit ratio of 37/56/37. PEG-PBLA is a diblock copolymer of poly(ethylene oxide)-co-poly(β-benzyl-L-aspartate); PEG2000-diacylphospholipid is a PEGilated phospholipid used to stabilize Pluronic P-105 micelles against degradation upon i.v. injection. For studying micelle biodistribution, Pluronic P-105 molecules were fluorescently labeled as described in ref. [1].

Sonication. Unfocused 1-MHz or 3-MHz ultrasound was generated by Omnisound 3000 instrument. Ultrasound was applied through Aquasonic coupling gel for 30 s locally to s.c. tumors; for 1-MHz ultrasound, power density was 3.4 with 50% or 33% duty cycle; for 3-MHz ultrasound, power density was 1.8 W/cm^2 with 100% duty cycle. Tumor sonication by focused 1.1 MHz ultrasound was performed using a submersible focused piezoceramic transducer (model H-101 S/N-29, Sonic Concepts, Woodinville, WA) producing a beam width of 1.5 mm at the focal site.

Tumor growth rates

A2780 cells were s.c. inoculated and tumors were allowed to develop and grow. Treatment was initiated when tumor volume reached at least 50 mm^3. DOX was injected intravenously through the tail vein of a mouse at a dose of 3 mg/kg or 1.5 mg/kg. Three consecutive treatments were applied on days 1, 3, and 5. Ultrasound was applied locally to the tumor. Tumor volume was calculated based on the equation: $V = (w)^2 \times (l)/2$, where (w) and (l) are width and length of the tumor measured by a caliper. Between various treatment groups, growth rates were compared for the same initial tumor volumes at the start of the treatment.
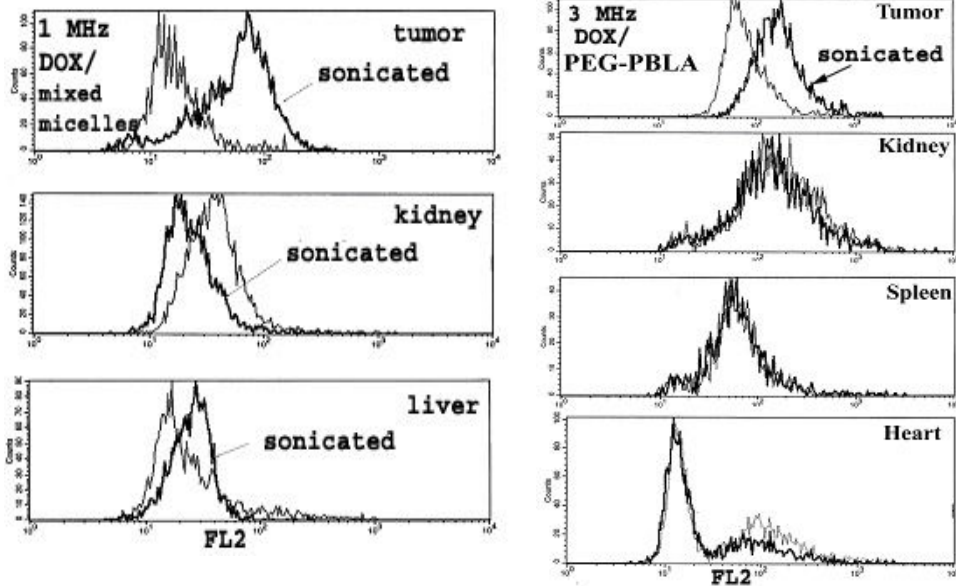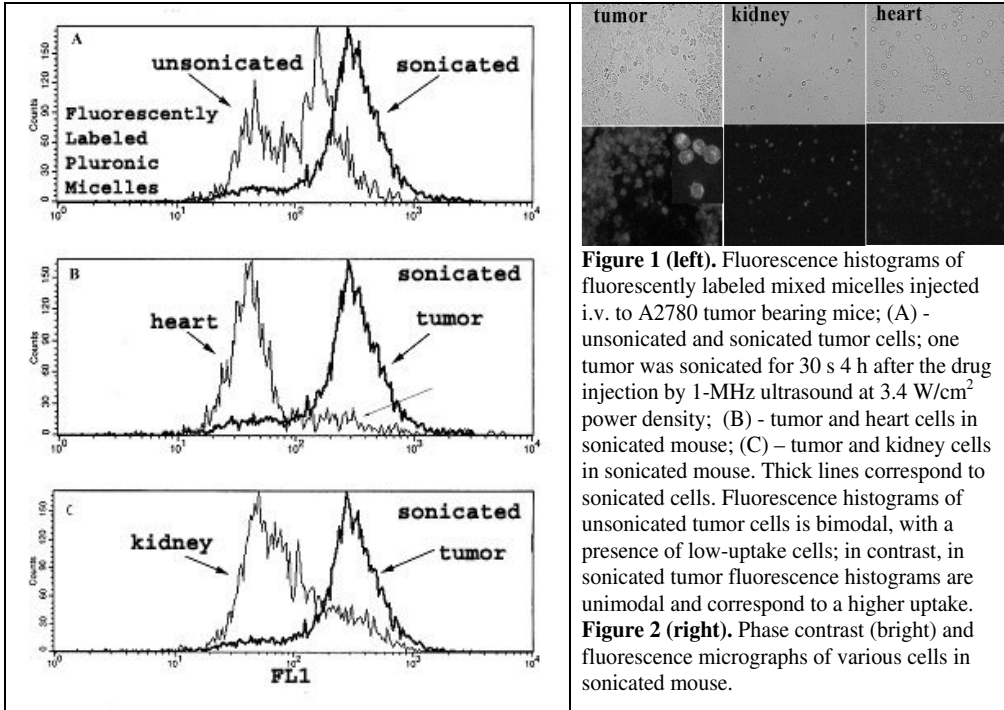
Micelle and DOX biodistribution. In micelle biodistribution experiments, fluorescently labeled mixed Pluronic/PEG2000-diacylphospholipid micelles were used. In DOX biodistribution studies, drug was encapsulated in unlabeled micelles. The time between drug injection and ultrasound application varied between 30 min and 12 hours. Ten minutes after the sonication, animals were sacrificed; tumors and various organs were excised, dried by filter paper, digested by trypsin, and fixed with 2.5% glutaraldehyde; after filtering through nylon mesh, cell fluorescence was measured by flow cytometry (FACSCAN, Becton Dickinson).

RESULTS AND DISCUSSION

A very significant degree of micelle targeting to tumor cells was observed for sonicated tumors (Figures 1 and 2). Ultrasonic treatment made micelle distribution in the tumor volume much more uniform (Figures 1).

Biodistribution of micellar-encapsulated DOX followed that of micellar carrier, with a high degree of drug targeting to tumor cells (Figure 3).

Ultrasound energy spreading beyond the tumor volume. As shown for the mixed micelles in Figure3 (left column), sonication of the tumor by unfocused 1-MHz ultrasound slightly increased DOX uptake by the other organ cells. This unwanted effect

Figure 1 (left). Fluorescence histograms of fluorescently labeled mixed micelles injected i.v. to A2780 tumor bearing mice; (A) - unsonicated and sonicated tumor cells; one tumor was sonicated for 30 s 4 h after the drug injection by 1-MHz ultrasound at 3.4 W/cm^2 power density; (B) - tumor and heart cells in sonicated mouse; (C) – tumor and kidney cells in sonicated mouse. Thick lines correspond to sonicated cells. Fluorescence histograms of unsonicated tumor cells is bimodal, with a presence of low-uptake cells; in contrast, in sonicated tumor fluorescence histograms are unimodal and correspond to a higher uptake. **Figure 2 (right).** Phase contrast (bright) and fluorescence micrographs of various cells in sonicated mouse.

Figure 3. DOX biodistribution in ovarian carcinoma tumor bearing mice upon injection of micelle-encapsulated DOX (6 mg/kg). Ultrasound frequency: left –1-MHz; right – 3-MHz .

was caused by spreading ultrasound energy beyond the tumor volume. Direct measurements by a hydrophone confirmed that during a localized irradiation of the tumor with unfocused 1-MHz ultrasound, a significant fraction of the ultrasonic energy was delivered to other organs. The cause of this effect was that at the site opposite to the transducer, ultrasound waves were reflected from a skin/air interface back into the interior of a mouse body; plus, interior interfaces and bones located on the ultrasound path acted as reflection centers. This was not observed for 3-MHz ultrasound that was much more localized in the tumor volume (Figure 3, right column). Ultrasound spreading beyond the tumor volume due to scattering on interfaces and bones should be taken into consideration in clinical application of ultrasonically-enhanced drug delivery. Ultrasound focusing on the tumor would substantially reduce this problem.

Effect of the time of ultrasound application. The extent of the ultrasonic enhancement of the intracellular DOX uptake depended on the time of ultrasound application after the drug injection. For DOX injected in PBS, the maximal effect of ultrasound was observed two to four hours after the drug injection. The effect was completely eliminated twenty four hours after the drug injection suggesting that little, if any DOX remained in the tumor interstitium.

For DOX encapsulated in PEG-PBLA micelles, the effect of ultrasound constantly increased with increasing time between drug injection and ultrasound application. Thirty minutes after the drug injection, drug accumulation in the tumor cells was very low for both unsonicated and sonicated tumors, fluorescence of the tumor cells being close to the autofluorescence of the tumor cells in the control non-injected mice. With increasing time after the drug injection, fluorescence of the tumor cells and a difference between sonicated and non-sonicated tumors constantly increased; the ultrasound effect was very pronounced twelve hours after the drug injection; tumor sonication by 3-MHz ultrasound resulting in about 3-fold increase of DOX uptake by the tumor cells, as illustrated in Figure 3 (right). A similar effect was observed for mixed micelles. This ruled out ultrasonic enhancement of micelle extravasation at the experimental conditions used here. If ultrasound enhanced micelle extravasation, some ultrasonic enhancement of the drug uptake by the tumor cells should have been observed at a short time after the drug injection, when a significant portion of the injected drug was still circulating. However, no effect of ultrasound was observed thirty minutes after the injection of micellar-encapsulated DOX, while a very strong ultrasound effects were observed for these micelles at later times, which was presumably associated with the effect of ultrasound on the micelles that had already accumulated in the tumor interstitium *via* the EPR effect.

Thermal effects. Thermal effects could play a role in enhancing the intracellular drug uptake. However, at the experimental conditions used in experiments described above, a significant role of the thermal effects was ruled out by the direct intratumoral temperature measurements. In addition, for DOX encapsulated in mixed micelles, the effect of 60-s tumor sonication was compared to that of a 60-s tumor heating by "ironing", to maintain a temperature of about 35°C for 30-s in the center of the tumor (this temperature is several degrees higher than the final tumor temperature reached

under ultrasound); even at these harsh heating conditions, the effect of ultrasound on the DOX intracellular uptake was significantly stronger than that of the tumor heating

Effect of micellar delivery and ultrasound on the growth rates of s.c. ovarian carcinoma tumors is shown in Figure 4 [2].
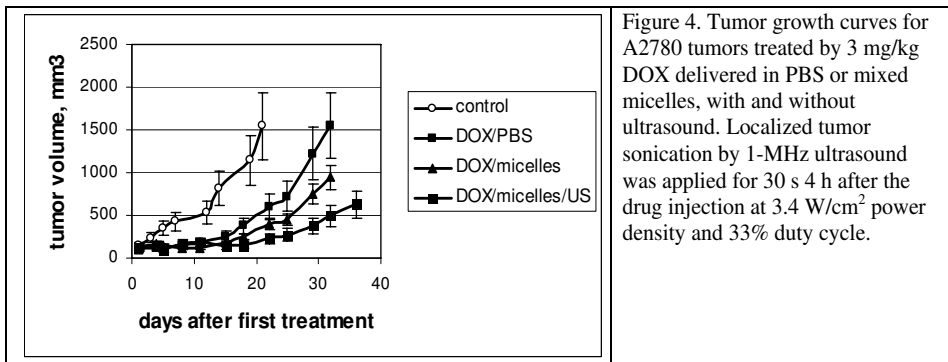

Figure 4. Tumor growth curves for A2780 tumors treated by 3 mg/kg DOX delivered in PBS or mixed micelles, with and without ultrasound. Localized tumor sonication by 1-MHz ultrasound was applied for 30 s 4 h after the drug injection at 3.4 W/cm^2 power density and 33% duty cycle.

Mechanisms of ultrasound bioeffects: systemic level. The study revealed following mechanisms involved in the ultrasound-enhanced chemotherapy i) passive targeting of drug-loaded micelles to the tumor interstitium; ii) ultrasound-triggered localized drug release from micelles in the tumor volume [3]; iii) enhanced micelle and drug diffusion through the tumor interstitium resulting in a more uniform drug distribution in the tumor volume; and iv) ultrasound-enhanced micelle and drug uptake by the tumor cells. At the experimental conditions, ultrasound did not enhance micelle extravasation. A significant role of the thermal effects was ruled out by direct intratumoral temperature measurement and by stronger effect of tumor sonication compared to tumor heating to approximately same final temperature [2].

Limitations of the intravenous drug delivery. The same micelle/ultrasound drug delivery technique failed in treating s.c. HCT116 colon cancer tumors. The reason of the treatment failure was a very poor tumor vascularization resulted in insufficient drug supply to tumor cells. For this tumor, successful treatment was achieved by direct intratumoral injections of micellar-encapsulated DOX combined with tumor sonication that enhanced drug diffusion from the injection site over the tumor volume.

Conclusions

Combining micellar drug delivery with localized tumor sonication allows a high degree of drug targeting to tumor cells and uniform drug distribution over the tumor volume, which in turn, results in successful tumor chemotherapy.

REFERENCES

1. Z-G. Gao, H. D. Fain, N. Rapoport, *Molecular Pharmaceutics*, **1**: 317-330 (2004)
2. Z-G. Gao, H. D. Fain, N. Rapoport, *J. Control. Release* **102**, 203-222 (2005).
3. G. Husseini, W.G. Pitt, D.A. Christensen, N. Rapoport, J. Controlled Release **69**: 43-52 (2000).