Ultrasonically Activated Chemotherapeutic Drug Delivery in a Rat Model


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ABSTRACT

Systemic delivery of anticancer agents is accompanied by many unwanted side effects that can be mitigated by encapsulation of antineoplastic agents. However, encapsulation necessitates a technique for controlled delivery to the cancerous tissue. We have developed a novel drug delivery system that releases drug from stabilized micelles upon application of low-frequency ultrasound and that demonstrates efficacy using doxorubicin (Dox) to treat tumors in vivo. Forty-two BDIX rats were inoculated in each hind leg with a DHD/K12/TRb tumor cell line. Dox was encapsulated within stabilized Pluronic micelles and administered weekly i.v. to the rats starting 6 weeks after the tumor inoculations. One of the two tumors was exposed to low-frequency ultrasound for 1 h. Dox concentrations of 1.33, 2.67, and 8 mg/kg and ultrasound frequencies of 20 and 70 kHz were used for treatment. Tumor volume was measured with calipers and observed over the treatment time. Administration of encapsulated Dox at concentrations of 1.33 and 2.67 mg/kg was not lethal to the rats. Application of low-frequency ultrasound (both 20 and 70 kHz) significantly reduced the tumor size when compared with noninsonated controls (P = 0.0062) in the other leg for rats receiving encapsulated Dox. Significant tumor reduction was also noted for those rats receiving ultrasound and encapsulated Dox at 2.67 mg/kg (P = 0.017) and rats receiving Dox and ultrasound at 70 kHz (P = 0.029). We postulate that ultrasound releases the Dox from the micelles as they enter the insonated volume, and ultrasound could also assist the drug and/or carriers to extravasate and enter the tumor cells. Encapsulation of Dox using stabilized Pluronic micelles and localized release using low-frequency ultrasound show promise in offering controlled drug delivery in the treatment of tumors in a rat model.

INTRODUCTION

Currently, chemotherapy remains one of the most effective treatments of cancerous tumors. However, the high doses often necessary to successfully eliminate the tumors also adversely affect healthy tissues in the host. The side effects of many antineoplastic agents include cardiotoxicity, immune suppression, nephrotoxicity, and more (1–3).

Our laboratory has shown that hydrophobic drugs can be incorporated into micelles of the polymer Pluronic P105 and released on demand with low-frequency ultrasound (4, 5). Stabilization of the micelles with a penetratating network of NNDEA prevents the micelles from dissociating upon dilution (6). These stabilized micelles, named Plurogels, spontaneously absorb many hydrophobic drugs. We have previously determined that these micelles are not toxic to rats at blood concentrations below 0.3 wt% (7). Although much work has been done in vitro, no previous testing had been performed to determine the system’s efficacy in vivo.

The use of ultrasound to control release of Dox from Plurogels offers many benefits in improving chemotherapeutic treatments. Transdermal application of ultrasound is noninvasive and offers both temporal and spatial control of drug release. Ultrasound-controlled drug delivery also minimizes adverse side effects throughout the parts of the body where ultrasound is not applied. This research shows that ultrasound can be used to selectively release Dox at a tumor site and reduce tumor volume compared with noninsonated tumors in a rat model of a colon carcinoma.

MATERIALS AND METHODS

Forty-two (42) 6-week-old BDIX rats (Charles River Laboratories, Wilmington, MA) were anesthetized with a combination of ketamine HCl (50 mg/kg, i.p.; Fort Dodge Animal Health, Fort Dodge, IA) and medetomidine HCl (0.3 mg/kg, i.p.; Pfizer Animal Health, Exton, PA). They were inoculated in each upper hind leg with a 50-μl s.c. injection of a colon tumor cell suspension (DHD/K12/TRb; 2 × 10⁶ cells/ml; reference number 900629011; European Collection of Cell Cultures, Salisbury, United Kingdom) and allowed to recover. DHD/K12/TRb is a metastatic colorectal tumor cell line originating from a 1,2-dimethylyazine-induced colon adenocarcinoma in BDIX rats, and it has been shown to spontaneously generate tumors at the injection site (8, 9). In our studies, the cell inoculation grew into a solid, dense s.c. tumor at the injection site to model colon cancer and metastasis. Rats whose tumor(s) did not develop were not included in the sample. All animals were handled in accordance with Brigham Young University’s Institutional Animal Use and Care Committee guidelines. Tumor volume was estimated by making two perpendicular measurements (a and b, where a > b) with a caliper and by using the following formula (9).

\[ TV = \frac{a \times b^2}{2} \]

Plurogel was made by polymerizing NNDEA (Polysciences, Warrington, PA) in the presence of 10% Pluronic P105 (BASF Corp., Mount Olive, NJ) in water using 2,2′-azobisisobutyronitrile (Aldrich, Milwaukee, WI) as an initiator and N,N′-bis(acryloyl)cysteamine (Fluka, Milwaukee, WI) as a cross-linking agent. After a nitrogen purge for 2–3 h at room temperature, the polymerization was conducted at 65°C overnight in the presence of nitrogen gas and magnetic stirring to create an intrapermeating cross-linked network of poly(NNDEA) in the core of the P105 micelles (6, 10). At 37°C, these stabilized micelles are between 50 and 100 μm in diameter and do not dissolve immediately upon dilution because the polymerized poly(NNDEA) network entangles the Pluronic chains. However, there are no covalent bonds between the network and the Pluronic chains, so the latter can slowly diffuse away over time and be cleared from the circulatory system. The biodegradable cross-linker allows the network to degrade and be cleared. The Plurogels used herein have an in vitro half-life of about 17 h (10).

Dox (Adriamycin RFD, Pharmacia & Upjohn Co., Kalamazoo, MI) was encapsulated by simple mixing of the drug with the Plurogel. Stock solutions of encapsulated Dox were prepared to provide upon injection varying concentrations of Dox (1.33, 2.67, and 8 mg/kg body weight) and a Plurogel concentration in the blood of 0.15 wt%.

The rats were randomly divided into nine groups including two control groups. Selection of right or left tumor for treatment with ultrasound was also randomized. Three weeks after tumor inoculation, rats were preanesthetized with i.p. injection of 50 mg/kg ketamine and pretreated with s.c. injections of 4 mg/kg dexamethasone (Phoenix Scientific, Inc., St. Joseph, MO) and 5 mg/kg diphenhydramine (Benadryl; 50 mg/ml; Parke-Davis, Morris Plains, NJ) to reduce incidence of anaphylactic shock (11). I.v. administration of Dox occurred via the tail vein. Immediately after injection, the anesthetic regimen was completed with an i.p. injection of 0.3 mg/kg medetomidine HCl, and the rats were placed in a special restraint device to submerge one depilated leg and...
its tumor in an ultrasonic bath. The rest of the body (including the other leg) was not exposed to ultrasound.

Parameters. The variables investigated in this experiment included power density (1 and 2 W/cm²), frequency (70 and 20 kHz), Dox concentration (1.33, 2.67, and 8 mg/kg), power train (continuous and pulsed), and ultrasound application frequency (once and twice weekly). Ultrasound was applied for 1 h to one leg of the animal, and treatment was repeated once weekly (except for one group that received an additional 1-h treatment 24 h after the initial administration of both drug and ultrasound) for 4 weeks on the same leg. Seventy-kHz ultrasound was generated using SC-100 baths (Sonicon Instrument Co., Copiaque, NY) operating at power densities of 1 and 2 W/cm². Power density was controlled with variable AC voltage transformers (12) and measured with a calibrated hydrophone (Bruel & Kjær, Nierum, Denmark). Pulsed ultrasound was delivered by insonation for 0.2 s, followed by no insonation for 1.8 s, producing a 1:10 pulsed duty cycle. Ultrasound at 20 kHz was generated with an ultrasonic probe (Vibra-Cell; Sonics & Materials, Inc., Newtown, CT) placed in a water bath adjacent to the rat leg. The negative control in this experiment received neither drug nor ultrasound treatments, whereas a positive control received both free Dox (nonencapsulated) and ultrasound treatment to one leg. The remaining seven groups received both drug and ultrasound treatments listed in the experimental matrix (Table 1). In this exploratory research, a full factorial design was not feasible due to the number of parameters. The partial design implemented in this study used combinations of the parameters suggested to be of greatest significance based on literature and previous research. As noted in the table, each group contained at least five animals. Power density was maintained at 2 W/cm² for all groups except group 4 because higher powers cause greater release. Although lower frequencies effectively release Dox from micelles (5), tissue damage due to acoustic cavitation is more likely at lower frequencies (13). Therefore, all groups except groups 5 and 6 received 70-kHz ultrasound.

The rats were humanely euthanized a few days after the fourth treatment. Heart, kidney, liver, and tumor tissues were collected for histopathological examination. Tumor growth was normalized by dividing the final tumor volume. Treatment for this group consisted of encapsulated Dox at 2.67 mg/kg and ultrasound at 70 kHz and 2 W/cm² administered once weekly for 1 h. The normalized growth data for rats from group 7. Although there is much scatter, the mean average size of the insonated tumor (indicated by the dotted line) was less than the other noninsonated side (solid line). The other insonated groups (groups 1–6) showed similar patterns in the data (with similar scatter), but the mean and median of the insonated tumor sizes were less than the noninsonated tumor sizes.

When all tumors from the survivors of groups 1–8 were analyzed statistically (both encapsulated and free Dox, n = 31), there was no age, grew less than the noninsonated tumors. For example, Fig. 2 shows the normalized growth data for rats from group 7. Although there is much scatter, the mean average size of the insonated tumor (indicated by the dotted line) was less than the other noninsonated side (solid line). The other insonated groups (groups 1–6) showed similar patterns in the data (with similar scatter), but the mean and median of the insonated tumor sizes were less than the noninsonated tumor sizes.

### Table 1 Experimental matrix for ultrasound and doxorubicin treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Dox dosage (mg/kg)</th>
<th>Frequency (kHz)</th>
<th>Power density (W/cm²)</th>
<th>Power train</th>
<th>Treatment regimen</th>
<th>Survival no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.33 (encaps.)</td>
<td>70</td>
<td>2</td>
<td>Continuous</td>
<td>Once weekly</td>
<td>3/5</td>
</tr>
<tr>
<td>2</td>
<td>2.67 (encaps.)</td>
<td>70</td>
<td>2</td>
<td>Continuous</td>
<td>Twice weekly</td>
<td>5/5</td>
</tr>
<tr>
<td>3</td>
<td>2.67 (encaps.)</td>
<td>70</td>
<td>2</td>
<td>Continuous</td>
<td>Once weekly</td>
<td>5/5</td>
</tr>
<tr>
<td>4</td>
<td>2.67 (encaps.)</td>
<td>70</td>
<td>1</td>
<td>Continuous</td>
<td>Once weekly</td>
<td>5/5</td>
</tr>
<tr>
<td>5</td>
<td>8.00 (encaps.)</td>
<td>20</td>
<td>0.048</td>
<td>Continuous</td>
<td>Once weekly</td>
<td>5/5</td>
</tr>
<tr>
<td>6</td>
<td>2.67 (encaps.)</td>
<td>20</td>
<td>0.048</td>
<td>Continuous</td>
<td>Once weekly</td>
<td>5/5</td>
</tr>
<tr>
<td>7</td>
<td>2.67 (encaps.)</td>
<td>70</td>
<td>2</td>
<td>1:10 pulse</td>
<td>Once weekly</td>
<td>4/4</td>
</tr>
<tr>
<td>8</td>
<td>2.67 (free)</td>
<td>70</td>
<td>2</td>
<td>Continuous</td>
<td>Once weekly</td>
<td>3/3</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* encaps., encapsulated.  
*b* Died prematurely of bronchial pneumonia.  
*c* Died immediately upon injection with Dox solution (probably air embolus or anaphylactic shock).  
*d* Died within 2 weeks of first injection with 8 mg/kg encapsulated Dox.

### Results

A Dox concentration of 8 mg/kg was lethal within 2 weeks of the first ultrasound/Dox treatment. Lower concentrations of 1.33 and 2.67 mg/kg were not fatal; however, two rats died prematurely from bronchial pneumonia. The growth of the bilateral tumors in the negative control rats was relatively similar, increasing approximately exponentially over time. Fig. 1 shows that the normalized growth of tumors in the nontreated group (group 9) was very consistent on each leg (left compared with right).

In rats receiving encapsulated Dox, the insonated tumor, on average, grew less than the noninsonated tumors. For example, Fig. 2 shows the normalized growth data for rats from group 7. Although there is much scatter, the mean average size of the insonated tumor (indicated by the dotted line) was less than the other noninsonated side (solid line). The other insonated groups (groups 1–6) showed similar patterns in the data (with similar scatter), but the mean and median of the insonated tumor sizes were less than the noninsonated tumor sizes.
significant difference between the ultrasound-treated and noninsonated tumors. However, when the rats with free Dox (group 8) were eliminated from the sample set, the remaining data revealed that the insonated tumors were significantly smaller than noninsonated tumors \( (P = 0.0062; \ n = 27) \). A similar analysis of the subset of rats that received encapsulated Dox at 2.67 mg/kg (groups 2–4, 5, and 7) revealed that insonated tumors were again significantly smaller than their noninsonated pairs \( (P = 0.017; \ n = 24) \). The tumors from the subset of rats exposed to 70-kHz ultrasound and encapsulated Dox (either concentration) during their treatment (groups 1–4 and 7) were significantly smaller than the noninsonated tumors \( (P = 0.029; \ n = 23) \). None of the individual treatment group differences proved to be statistically significant because of the scatter in tumor growth patterns. However, when taken together as a group, ultrasound is correlated with less tumor growth when using encapsulated Dox. For group 8, there was no statistically significant difference in normalized tumor size for these rats that received nonencapsulated Dox \( (P > 0.1; \ n = 4) \).

**DISCUSSION**

Previous in vitro work established that low frequency ultrasound caused release of Dox from these micellar carriers (5, 14). Furthermore, in vitro cancer cells were protected from the effects of Dox when it was encapsulated but were toxicologically affected once ultrasound was applied to the encapsulated Dox (4). Other studies showed that ultrasonic exposure increased the uptake of labeled and unlabeled Dox into cells (15). These observations led to the hypothesis that ultrasound release of Dox from Plurogels would also be effective in vivo against a targeted tumor, whereas a nontargeted tumor would be less affected by the encapsulated drug. This hypothesis is supported by the data reported herein.

Exposure of systemic encapsulated Dox to low-frequency ultrasound resulted in a statistically significant reduction in tumor size when compared with noninsonated tumors. Although the tumors that were exposed to ultrasound did not completely regress during the time investigated, the decrease in tumor volume suggests that the combination of ultrasound and encapsulated Dox changed the local conditions at the tumor site in a manner to slow tumor growth. Based on our in vitro work, we postulate that a higher concentration of Dox is realized in the vicinity of the tumor due to ultrasonically activated drug release from the carriers. However, drug release in vivo may be very different from that in vitro, and thus we must consider other possible scenarios consistent with the data that indicate that tumor growth was slowed either by increased drug uptake or by some uncharacterized interaction between the ultrasound, the tumor, and free drug that leached from the micelle.

There are at least two other scenarios that should be considered. The first is that ultrasound changes the uptake of Dox into the tumor cells. This could happen if ultrasound renders the cell membrane more permeable or triggers an increase in active import or a decrease in active export of the drug. Rapoport et al. (15) have reported that low-frequency ultrasound (20 kHz) increases the membrane permeability of HL-60 cells in vitro, attributed to formation of defects in the cell membrane (15, 16). If such is also the case in vivo, then ultrasound could enhance the uptake of drug into the targeted tumor, irrespective of whether the drug arrived by ultrasonic release from a nearby Plurogel or whether the drug was present in the fluid, having previously diffused out of the Plurogel.

The second scenario is that ultrasound promotes the extravasation into the tumor capillaries of any Plurogel drug carriers or released Dox. Kruskal et al. (17) have reported that higher frequency ultrasound (commonly used in imaging) increased the permeability of angiogenic vessels and increased the quantity of Dox delivered by stable liposomes to hepatic colorectal metastases in a mouse model. Similar extravasation of Plurogel or Dox may occur at the lower frequencies used in our technique.

The observed decrease in tumor volume could be attributed to our main hypothesis or to any number of other scenarios. Indeed it is probable that more than one mechanism (ultrasonic-triggered extravasation of the drug carrier, ultrasonic-activated release of Dox from the micelle, and ultrasonic-enhanced uptake into the cells) may occur in vivo. Because we could not directly measure release from the Plurogels in vivo, we cannot determine which, if any, of these mechanisms predominates.

This tumor-bearing rat model, developed to study ultrasonically controlled release of Dox from Plurogels, offers many benefits for the in vivo investigation of this drug delivery technology. The tumors grow well in the rat legs, and placement of the tumor allows both bilateral and differential treatment. Another benefit in using a larger rodent is the ability to monitor other physiological parameters of the treatment such as cardiac function. Rats are sufficiently large to enable echocardiographic evaluation of whether encapsulation of Dox by the Plurogel reduces cardiotoxic effects (18).

Our strategy of using ultrasound to deliver a chemotherapeutic agent to a specific target site may not be applicable to treatment of disseminated disease or to avoid metastatic spread. We envision that this therapy could be used in early treatment of a localized tumor in a tissue that is less suitable for surgical removal due to physiological or cosmetic considerations. This therapy could also be combined with other adjuvant therapy to avoid or reduce metastatic spread. Indeed, a pressing question that we are currently addressing is whether the ultrasound may contribute to metastasis.

In theory, the ultrasound could be focused on superficial or deep tumors, with the possible exception of tumors within tissues that block ultrasound, such as bone and lung. For example, tumors of the breast, colon, ovaries, uterus, larynx, or other internal structure may be candidates for this type of therapy. Our current research is directed toward application in colon, breast, and ovarian tumor models.

Until new treatments are developed that can distinguish between cancer cells and the normal host’s cells, chemotherapy will carry with it many unwanted and dangerous side effects. Although more efficient surgical techniques are emerging for treatment removal, many forms of cancerous growths still require systemic treatment in an attempt to eradicate, thwart, or simply prevent additional development. This delivery vehicle and release mechanism provides a means of targeting specific areas supplied by the host’s circulatory system. Because the Plurogels potentially protect tissues from the drug where ultrasound is not applied, side effects can be mitigated through carefully controlled application of ultrasound to targeted areas. Future studies should include more rigorous models to test the robustness of this controllable delivery system.

**REFERENCES**


Ultrasonically Activated Chemotherapeutic Drug Delivery in a Rat Model
