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Controlled and targeted tumor chemotherapy by ultrasound-activated nanoemulsions/microbubbles

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Abstract

The paper reports the results of nanotherapy of ovarian, breast, and pancreatic cancerous tumors by paclitaxel-loaded nanoemulsions that convert into microbubbles locally in tumor tissue under the action of tumor-directed therapeutic ultrasound. Tumor accumulation of nanoemulsions was confirmed by ultrasound imaging. Dramatic regression of ovarian, breast, and orthotopic pancreatic tumors was observed in tumor therapy through systemic injections of drug-loaded nanoemulsions combined with therapeutic ultrasound, signifying efficient ultrasound-triggered drug release from tumor-accumulated nanodroplets. The mechanism of drug release in the process of droplet-to-bubble conversion is discussed. No therapeutic effect from the nanodroplet/ultrasound combination was observed without the drug, indicating that therapeutic effect was caused by the ultrasound-enhanced chemotherapeutic action of the tumor-targeted drug, rather than the mechanical or thermal action of ultrasound itself. Tumor recurrence was observed after the completion of the first treatment round; a second treatment round with the same regimen proved less effective, suggesting that drug resistant cells were either developed or selected during the first treatment round.

Keywords

nanoemulsions; microbubbles; ultrasound; chemotherapy; ovarian cancer; breast cancer; pancreatic cancer

1. Introduction

During the last decade, nanomedicine has evolved as a new field of medicine that holds promise for solving some problems of current chemotherapy, such as severe side effects of chemotherapeutic drugs and drug resistance. Various nanocontainers and drug delivery modalities have been suggested for decreasing systemic toxicity caused by chemotherapeutic drugs. They are commonly based on drug encapsulation in liposomes, polymeric micelles, hollow particles, emulsion droplets etc. The encapsulation may dramatically increase drug

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concentration in an aqueous environment and offer new life to bioactive compounds previously abandoned due to low aqueous solubility. Properly designed nanoparticles avoid extravasation to normal tissues and recognition by reticulo-endothelial system cells, which prolongs their circulation time after systemic injection. This in turn allows passive targeting of cancerous or inflamed tissues. Passive targeting is based on the enhanced permeability of defective tumor microvasculature that allows extravasation of drug-loaded nanoparticles through large inter-endothelial gaps; it was shown that in a variety of tumors, characteristic pore cutoff size ranges between 380 and 780 nm, though in some tumors it may reach up to 2 μm [1,2]. In contrast to tumors, blood vessels in normal tissues have tight inter-endothelial junctions that don't allow extravasation of nanoparticles. In addition to enhanced vascular permeability, tumors demonstrate poor lymphatic drainage; this positive effect provides for a long retention of the extravasated particles in tumor tissue. This effect is called enhanced permeability and retention, or EPR [3]. The potent tumor accumulation of the nanoparticles via the EPR effect requires sufficient particle residence time in circulation; to provide for this, nanoparticles are commonly coated with poly(ethylene oxide) chains that suppress blood protein adsorption and prevent particle recognition by reticulo-endothelial system cells. However, tumors demonstrate spatial heterogeneity in the distribution of inter-endothelial gaps, which results in a focal distribution of delivered nanoparticles [4]. This may have important implications for the outcome of tumor nanotherapy, which is discussed below.

An alternative approach to tumor targeting consists of developing stimuli-responsive nanoparticles that release their drug load only in response to environmental or physical stimuli, such as pH, hyperthermia, light, or ultrasound [5]. Developing passively targeted stimuli-responsive delivery systems offers the advantage of double-targeting. In our previous work, we have developed polymeric micelles that combined passive tumor-targeting ability with ultrasound responsiveness [6–13]. We have demonstrated that drug-loaded polymeric micelles passively accumulated in tumor tissue; ultrasonic irradiation of the tumor triggered drug release from the micelles and transiently altered cell membrane permeability, resulting in effective intracellular drug uptake by tumor cells. Ultrasound irradiation also enhanced drug diffusion throughout the tumor volume, thus reducing drug concentration gradients [9,13,14]. Substantial reduction of the tumor growth rate was achieved for drug-sensitive ovarian carcinoma and multidrug-resistant breast cancer tumors in nude mice [9,13,15].

Ultrasound as a component of a drug delivery modality may be applied with a variety of drug carriers. Development of ultrasound-responsive stable liposomes that manifested prolonged circulation time and effective tumor targeting was recently reported [16]. Ultrasound-induced heating triggered phase transition in the phospholipid membrane and released the drug in the target region. Ultrasound-triggered delivery of paclitaxel (PTX) to the monolayers of prostate cancer cells from a phospholipid-coated perfluorohexane nanoemulsion developed by ImaRx was also reported [17].

Ultrasound has a number of attractive features as a drug delivery modality. Tumor sonication with millimeter precision is feasible and ultrasound may be directed toward deeply located body sites in precise energy deposition patterns. Sonication may be performed non-invasively or minimally invasively through intraluminal, laparoscopic or percutaneous means. For extracorporeal sonication, the transducer is placed in contact with a water-based gel or a water layer on the skin, and no insertion or surgery is required.

All energy-based tumor treatment modalities including ultrasound-mediated drug delivery require tumor imaging prior to treatment. Although imaging can be performed in a variety of ways, combining diagnostic and therapeutic ultrasound for tumor imaging and treatment is especially attractive for reasons of simplicity and cost-effectiveness. For many decades, microbubbles have been used in clinical practice as ultrasound contrast agents in ultrasound

imaging. During the last decade, microbubbles have attracted attention as drug carriers and enhancers of drug and gene delivery and are now being widely investigated for this application [13,18–24].

The use of microbubbles as drug carriers is very attractive because it would combine cost-effective ultrasound imaging with ultrasound-mediated therapy. Several research groups have concentrated their efforts on developing microbubble-based drug delivery systems [25–29]. However, these systems present difficult, inherent problems. Their very short circulation time (minutes) and micron size do not allow effective extravasation into tumor tissue, which is an essential prerequisite for effective drug targeting.

The way to solve above problems may consist in developing drug-loaded, nano-scaled microbubble precursors that would effectively accumulate in tumor tissue by passive or active targeting and then convert into microbubbles *in situ* after tumor accumulation. With this in mind, we have recently developed block copolymer stabilized echogenic (i.e. ultrasound contrast generating) perfluoropentane (PFP) nanoemulsions that convert into microbubbles under heating to hyperthermia conditions or ultrasound irradiation [13,24,30,40]. The nanoemulsions are produced from drug-loaded poly(ethylene oxide)-co-poly(L-lactide) (PEG-PLLA) or poly(ethylene oxide-co-polycaprolactone (PEG-PCL) micelles. Their important properties combine drug carrying, tumor-targeting, enhancing intracellular drug delivery, and enhancing the ultrasound contrast of tumors. Here, we describe therapeutic and ultrasound-imaging properties in these nanoparticles that were explored using three human tumor xenografts inoculated in nu/nu mice, namely A2780 ovarian carcinoma, MDA MB231 breast cancer, and red fluorescent protein labeled MiaPaCa-2 pancreatic cancer.

2. Materials and Methods

2.1. Block copolymer

The block copolymers used in this study were obtained from Polymer Source Inc. (Montreal, Quebec, Canada). The PEG-PLLA copolymer had a total molecular weight of 9,700; the molecular weights of a hydrophilic PEG block and a hydrophobic PLLA block were 5,000 D and 4,700 D respectively. The PEG-PCL copolymer had a total molecular weight of 4,600 D; the molecular weights of a PEG block and PCL block were 2000 D and 2600 D respectively.

2.2 Micellar solutions and drug loading

Micellar solutions of the block copolymers were prepared by a solvent exchange technique as described in detail previously [13]. Genexol PM (GEN) was bought from Samyang Corp. (Daejeon, South Korea). A desired weight of the Genexol PM powder was dissolved in the PEG-PLLA micellar solution.

2.3. Formulations

PTX-loaded nanoemulsions were prepared as follows: micellar-encapsulated PTX, Genexol PM was dissolved in 0.25% PEG-PLLA micelles; 1% vol. PFP was added to this solution and samples were sonicated in ice-cold water by 20-kHz ultrasound (VCX 500, Sonics & Materials, Newtown, CT) until all PFP was transferred into an emulsion. In what follows, this formulation is called nbGEN.

2.4. Particle size distribution

Size distribution of nanoparticles was measured by dynamic light scattering at a scattering angle of 165° using Delsa Nano S instrument (Beckman Coulter, Osaka, Japan) equipped with a 658 nm laser and a temperature controller. Particle size distribution was analyzed using Non-Negative Least Squares (NNLS) method.

2.5. Nanodroplets introduction into gels

To introduce nanoemulsion into plasma clots, equal volumes (200 μL each) of a nanodroplet emulsion and bovine plasma (Innovative Research, Novi, MI, USA) were gently mixed. The clotting was initiated by adding 10 μL of 20 IU/mL bovine thrombin (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 mol/L calcium chloride. The mixture was drawn into a Samco transfer pipette (5-mm inner diameter, 0.3-mm wall thickness, 2 mm diameter of the narrow bottom part) (Fisher Scientific, Pittsburg, PA, USA) and stored in the refrigerator (4 $^{\circ}\text{C}$). For nanodroplet introduction into agarose gel, 0.2% agarose in phosphate buffered saline (PBS) was used.

2.6. Sonication

Unfocused 1-MHz or 3-MHz ultrasound was generated by an Omnisound 3000 instrument (Accelerated Care Plus Inc, Sparks, NV). To generate 90-kHz ultrasound, a box type sonicator (SC-100, Sonicor Instrument Co., Copiague, NY) was used.

2.7. Cells

A2780 ovarian cancer cells were obtained from Dr. E. Batrakova (Omaha Medical Center). Breast cancer MDA MB231 cells and pancreatic cancer MiaPaCa-2 cells were obtained from American Type Culture Collection (Manassas, VA). Ovarian and breast cancer cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY); pancreatic cancer cells were maintained in DMEM supplemented with 10% FBS. MiaPaCa-2 cells were transfected with red fluorescence protein (RFP) according to the procedure described in refs. [31,32]. Cells were cultured at 37 $^{\circ}\text{C}$ in humidified air containing 5% CO_2 .

2.8. In vivo ultrasound imaging

Ultrasound imaging was performed using a 14-MHz linear transducer (Acuson Sequoia 512, Siemens, Mountain View, CA).

2.9. Animal Procedures

Four to six weeks old nu/nu mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Utah (Protocol 08-01001). For inoculation, ovarian carcinoma A2780 or breast cancer MDA MB231 cells were suspended in serum-free RPMI-1640 medium and inoculated subcutaneously to the flanks of unanaesthetized mice (1×10^6 cells/100 μL /mouse). Red Fluorescence Protein (RFP) transfected MiaPaCa-2 pancreatic cells were inoculated orthotopically. The details of animal experiments are described in Supplementary Materials.

For ovarian and breast cancer, tumor sizes were measured twice weekly with a caliper. Tumor volume (V) was calculated as follows:

$$V = L \times W^2 / 2, \quad (1)$$

where L and W are the length and the width of the tumor, respectively.

The normalized tumor size (D_n) was calculated according to the following formula:

$$D_n = \sqrt[3]{\frac{V}{V_o}}, \quad (2)$$

where V and V_o are current and initial tumor volumes (V_o is a tumor volume at the start of the treatment). For pancreatic cancer, projected tumor areas were measured in whole body images of RFP.

The upper limit of the injected nanodroplet dose (N_m) was estimated based on the concentration of the introduced PFP and the measured nanodroplet size distribution according to the following equation:

$$N_m = \frac{v \times f \times 10^9}{4\pi r^3 / 3} \quad (3)$$

where $v = 2 \mu\text{L}$ is the volume of PFP in 200 μL of the injected nanoemulsion, f is a volume fraction of a selected droplet population, and r is the droplet radius in micrometers at the peak of a selected population (in this estimation, the loss of the PFP at the sample preparation and the thickness of the droplet shell were neglected). The estimated upper limit of the injected nanodroplet dose was about 7×10^9 per mouse for nanodroplets with a peak diameter of 0.7 μm .

3. Results and Discussion

3.1. Particle size distributions

The size distribution parameters for PEG-PLLA micelles and nanodroplets at room temperature are presented in Table 1. The size distribution of micelles was bimodal, with smaller particles corresponding presumably to individual spherical micelles while larger particles represent either worm-like micelles or micellar aggregates. For both types of particles, GEN-loaded micelles were larger than empty micelles. Interestingly, the fraction of larger particles significantly dropped after GEN loading. Figure 1 of Supplementary Material shows corresponding size distributions.

After the PFP introduction into the solution of empty micelles, the formation of nanoemulsion resulted in a disappearance of small micelles (22.2 nm) and generation of nanodroplets (592.6 nm, 73%); a fraction of larger micelles (117.5 nm, 27%) remained in emulsion. For GEN-loaded systems, introduction of PFP resulted in a tri-modal size distribution. The size of the nanodroplets (718.4 nm, 62%) was significantly larger than that of empty droplets (592.6 nm); some fraction of micelles (19.3 nm, 7% and 122.9 nm, 31%) remained in emulsion. Comparison of micelle and droplet sizes for empty vs. GEN loaded systems suggests that upon PFP introduction, PTX was transferred from micelles to nanoemulsion droplets, leaving behind empty micelles. Based on this information, in the formulation used *in vivo*, the entirety of the drug may be considered located in nanodroplets.

3.2. Droplet-to-bubble transitions in liquid systems

Due to large differences in acoustic impedance values for water (1.4 MRayl) and PFP droplets (~ 0.3 MRayl) or bubbles ($\ll 0.3$ MRayl), both PFP droplets and bubbles manifest echogenic properties in biological tissues [33]; however bubbles manifest much higher echogenicity than droplets, which creates better contrast in ultrasound images. Even more importantly, only bubbles undergo inertial cavitation (growth and collapse of bubbles in an ultrasound field),

which concentrates ultrasound energy and substantially enhances ultrasound-mediated drug delivery. Though drug delivery from micelles, liposomes, or emulsions may be ultrasonically enhanced even without microbubbles [5,7,10,11,17,34], presence of microbubbles dramatically increased intracellular uptake of drugs or genes [13,29,35,36].

Therefore nanodroplet vaporization to generate bubbles stabilized by block copolymer shells would be highly desirable for both ultrasonography and drug delivery. The core of nanodroplets used in this study is formed by PFP that has a boiling temperature of 29 °C at atmospheric pressure and therefore manifests high propensity for vaporization at heating. However, for small droplets stabilized by elastic copolymer shells, the Laplace pressure may substantially increase boiling temperature. The Laplace pressure is the pressure difference between the inside and the outside of droplet or bubble. This effect is caused by the surface tension at the interface between bulk liquid and droplet liquid.

The Laplace pressure is given as

$$\Delta P = P_{\text{inside}} - P_{\text{outside}} = \frac{2\sigma}{r} \quad (4)$$

where P_{inside} is the pressure inside a droplet, P_{outside} is the pressure outside a droplet, σ is the surface tension, and r is droplet radius.

Excessive pressure inside a droplet results in increase of PFP boiling temperature. This phenomenon has important consequence for drug delivery. Because Laplace pressure is reversely proportional to droplet size according to eq. 4, smaller droplets have higher boiling temperatures than larger droplets. The surface tension at the PFP/water interface for “naked” (i.e. not surfactant coated) PFP droplets is 56 ± 1 mN/m [37]. This is the upper limit of a possible surface tension. To the best of our knowledge, the surface tension at the PFP/water interface in the presence of polymeric surfactants PEG-PLLA or PEG-PCL has not been reported in the literature. Some rough ideas may be derived from the published values on surface tensions for a family of the poly(ethylene oxide)-co-poly(propylene oxide)-co-poly(ethylene oxide) triblock copolymers (PEO-PPO-PEO, Pluronics) presented in the review by Alexandridis and Hatton [38]. Surface tension depended on the hydrophobic/hydrophilic balance of copolymer molecules and varied from 33 mN/m (for copolymers with a large fraction of a hydrophobic PPO block) to 52 mN/m (for copolymers with a large fraction of a hydrophilic PEO block). Figure 1 illustrates the effect of droplet size on the vaporization temperature calculated for surface tension values of $\sigma = 50$ mN/m and $\sigma = 30$ mN/m using the Antoine equation for the pressure dependence of vaporization temperature; the values of the Antoine equation parameters A , B , and C for the PFP were reported in the literature [39].

$$\log_{10} P = A - \left(\frac{B}{T+C} \right)$$

As shown in Figure 1, at physiological temperature of 37 °C, the borderline droplet size is about 6.4 μm for $\sigma = 50$ mN/m and about 4 μm for $\sigma = 30$ mN/m. At physiological temperature, droplets smaller than 4 μm will remain in the liquid state while larger droplets will evaporate. However, droplets of these sizes are not present in initial nanoemulsions (see Table 1). Therefore nanodroplets are expected to remain in circulation as liquid droplets rather than form microbubbles at physiological temperatures, which is beneficial for extravasation into tumor tissue. However after extravasation, droplet-to-bubble transition is desirable.

We have detected three factors that induced droplet-to-bubble transition in block copolymer stabilized PFP nanodroplets: heating (thermal factor) [13,24]; sonication (thermal and/or mechanical factor) [40]; and injection through a thin needle (mechanical factor) [40]. Among these factors, ultrasound was the most powerful [40].

Ultrasound-induced droplet-to-bubble transition is called acoustic droplet vaporization, or ADV; for albumin-coated perfluorocarbon nanoemulsions, this effect was studied in a series of publications [33,41–43]. Droplet-to-bubble transition occurs when ultrasound pressure increases above a certain threshold [33,41–43]. In the present work, we studied the ADV effect for the two types of the droplet-stabilizing copolymers (PEG-PLLA and PEG-PCL) with 1-MHz or 3-MHz ultrasound at room temperature and 37 °C, in liquid emulsions and gels.

In liquid systems, the ADV threshold depended on the type of the droplet-stabilizing copolymer and was lower for PEG-PCL stabilized droplets compared with PEG-PLLA stabilized droplets. At the same delivered ultrasound energy, the ADV threshold was lower for CW ultrasound compared with pulsed ultrasound. Vaporization thresholds were lower at 37 °C than at room temperature. These data are presented in Table 1 of Supplementary Material.

The droplet-to-bubble transition upon injection through needles is not as efficient as ADV, however it may have clinical relevance. This effect was first described for the Echogen microemulsions; its clinical implications have been discussed in refs. [44,45]. In our work, we studied injection-induced droplet-to-bubble transition by ultrasound imaging. As mentioned above, the echogenicity of bubbles is much higher than that of droplets which allows discrimination between droplets and bubbles using ultrasound images. The generation of bubbles upon nanoemulsion injection into PBS or agarose gels is illustrated in Figure 2A and B and in the video clip presented in Supplementary Material. Figures show that injection through a thin needle (26 G) is much more potent in inducing droplet-to-bubble transition than that through a thicker needle (18 G). For the same needle gauge, injection into gel is more potent in inducing droplet-to-bubble transition than injection into liquid. These results may have important implications for therapeutic application of the developed nanoemulsions. Upon nanoemulsion injection, some generation of microbubbles in the vascular bed may be beneficial because upon local sonication of tumor blood vessels, microbubble/ultrasound interaction may result in increased vascular permeability, which can be achieved without excessive hemorrhage or endothelial cell death as was demonstrated in rats' muscle, brain, and other tissues [46–50]. Recently, Caskey et al. [51] showed that under the action of ultrasound, the microbubbles in small blood vessels experience coalescence into bubbles larger than 4 µm; the latter undergo asymmetrical collapse and repeated expansion beyond the blood vessel boundaries, which may result in increased vascular permeability.

The droplet-to-bubble transition in our systems was also monitored by changes of cavitation activity that was assessed by measuring subharmonic and broadband noise amplitudes in Fast Fourier Transform spectra in the scattered ultrasound beam. The generation of subharmonic frequency indicates the onset of stable cavitation while broadband noise characterizes inertial cavitation; in more detail, these effects were described for our systems in ref. [30]. The data for a subharmonic frequency are presented in Figure 2 of Supplementary Material. The thresholds for subharmonic and broadband noise generation were close to that for acoustic droplet vaporization indicating that subharmonic and broadband noise was generated by cavitating microbubbles. As discussed above, this effect may increase vascular permeability.

However for effective drug delivery, massive transition of drug-loaded PFP nanodroplets into microbubbles inside blood vessels should be prevented because only nanodroplets extravasate into tumor tissue. To prevent excessive droplet-to-bubble transition in vasculature, systemic injection of PFP nanoemulsions should be performed either by infusion or injection through

low-gauge needles. On the other hand, after extravasation into tumor tissue, massive droplet-to-bubble transition is very beneficial. The latter may be initiated by tumor sonication with therapeutic ultrasound.

The data obtained for liquid nanoemulsions may be relevant to the bubble behavior in circulation. However after extravasation into the tumor tissue, the droplets or bubbles are surrounded by a much more viscous extracellular matrix of the tumor interstitium. To account for this, we introduced droplets into a 0.2% agarose gel or a plasma clot.

3.3. Ultrasound-triggered droplet-to-bubble transition in gel matrices

After extravasation into tumor tissue, nanodroplets are surrounded by a viscous extracellular matrix where their behavior including response to ultrasound irradiation may be different from that in liquids. To account for the increased viscosity, we introduced nanodroplets in plasma clots or agarose gel and monitored nanodroplet vaporization under the action of ultrasound of various frequencies. An example of ultrasound-induced droplet-to-bubble transition triggered by 90-kHz ultrasound is presented in Figure 3 for PFP/PEG-PLLA nanoemulsion inserted in plasma clot. Figure 3 shows that pre-existing bubbles catalyze droplet-to-bubble transition in the nanoemulsion. Under 1-MHz ultrasound, newly formed bubbles are formed in the immediate vicinity of pre-existing bubbles and coalesce with them (Figure 3B). Under 90-kHz ultrasound, the effect is more long-ranged (Figure 3C). The size of pre-existing bubbles almost triples during a 1-minute sonication by 90-kHz ultrasound.

The droplet-to-bubble transition and microbubble coalescence in tumor volume could be extremely beneficial for contrast-enhanced ultrasound imaging and drug delivery. Formation of large microbubbles in tumor tissue upon direct intratumoral injection of nanoemulsions was confirmed by ultrasound imaging [13,40]. As suggested by Figure 3, under hundred-kilohertz sonication, pre-generated large microbubbles may catalyze droplet-to-bubble transition in tumor-accumulated drug-loaded nanodroplets. Second tumor sonication by megahertz-range ultrasound would cavitate these small (micron to several microns) primary microbubbles and trigger drug release. These considerations can be used as a basis for an optimized, double-frequency drug delivery modality. Efficient ultrasound-mediated chemotherapy of a large breast cancer tumor was achieved using this approach (to be reported elsewhere).

3.4. Mechanism of the ultrasound-triggered drug release from nanodroplets/microbubbles

The experiments reported earlier [13,24] and in the next section reveal that the drug (doxorubicin or PTX) is tightly retained by the nanodroplets or microbubbles *in vivo* but is released under the action of ultrasound and effectively internalized by tumor cells.

The mechanism of the ultrasound-induced drug release from various nanoparticles (micelles, liposomes, emulsions) is not completely understood. For the PFP nanoemulsions, a possible drug release mechanism may involve droplet-to-bubble transition under the action of ultrasound. Due to a 125-fold density difference between PFP in liquid and gaseous phases, complete vaporization of the droplet results in a 125-fold increase of its volume accompanied by a 25-fold increase of surface area. This in turn results in a 25-fold decrease of the initial thickness of the bubble shell. This significantly increases surface area per copolymer molecule, which is expected to facilitate drug transfer from bubble to neighboring cell/cells, especially because the drug can be “ripped off” by ultrasound. This mechanism is schematically illustrated in Figure 4.

3.5. Therapeutic effects of nbGEN/ultrasound in the ovarian and breast cancer models

3.5.1. Systemic chemotherapy of a two tumor bearing mouse: effect of ultrasound—The results of chemotherapy of the mouse bearing two ovarian carcinoma

tumors inoculated in the right and left flank are presented in Figure 5. This mouse was treated by four systemic injections of the nanodroplet encapsulated PTX, nbGEN (20 mg/kg as PTX) given twice weekly; only one (the right) tumor was sonicated. The unsonicated left tumor grew with the same rate as control tumors (for which growth rates were measured separately). In contrast, the sonicated tumor appeared completely resolved after four treatments. These data indicate that without ultrasound, PTX is tightly retained by the nanodroplet carrier *in vivo*, which provides protection of healthy tissues. However, PTX is effectively released into the tumor volume under the action of ultrasound, which results in efficient tumor regression.

3.5.2. Pilot experiments with individual tumors—These experiments were performed using A2780 ovarian cancer tumors inoculated in the right flank of nu/nu mice. The goal of this pilot experiment was to monitor the degree of the therapeutic effect of the nanoemulsion encapsulated PTX combined with ultrasound in comparison with the therapeutic effect of empty (i.e. not drug loaded) PFP/PEG-PLLA nanoemulsion/ultrasound and that of Genexol PM without ultrasound (the latter was used as positive control). Two treatment rounds by the same regimen were given to the nbGEN/ultrasound group, with a two-week break between the treatments. The details of the experiment are described in Materials and Methods.

An effective tumor regression was observed in mice treated by nbGEN/ultrasound as presented in Figures 6 and 7A. For the mouse presented in Figure 6, the initial tumor volume of 1,650 mm³ dropped about an order of magnitude during the first treatment round. However, two weeks after the completion of the first treatment, the residual tumor visible in Figure 6C manifested signs of re-growth; a second treatment round using the same regimen decreased tumor growth rate to some extent but did not cause the same dramatic tumor regression that occurred during the first treatment round (data not shown). This indicated that residual tumor cells either acquired resistance to PTX or resistant cells were selected during the first treatment round. Normalized tumor growth/regression curve is presented in Figure 6B.

Figure 7A shows growth curves of the control and treated ovarian carcinoma tumors. Growth curve for the nbGEN/ultrasound treatment was located below the curve for the Genexol PM treatment indicating that drug was effectively released from the nanoemulsion under ultrasound action. Similar results were obtained in the pilot experiments with breast cancer (N=3) (Figure 7B) and full-scale experiments involving pancreatic tumor-bearing mice (N=6), where the advantage of the PTX/nanoemulsion/ultrasound (nbGEN/ultrasound) treatment over micellar PTX (Genexol PM) treatment with or without ultrasound was clearly observed (see below).

A thin line with open triangles in Figure 7A suggests the absence of therapeutic effect of empty (i.e. not drug-loaded) nanodroplets combined with ultrasound. Similar results were obtained earlier for empty micelle/ultrasound treatment using a larger group of animals (N = 13) [9]. The absence of therapeutic effect of empty droplets combined with ultrasound strongly indicates that the therapeutic effect of the drug-loaded droplets is caused by the cytotoxic action of the chemotherapeutic drug rather than cancer cell killing by ultrasound. The ultrasound role consists of effective release of the drug from carrier in tumor interstitium and perturbation of cell membranes, which results in enhanced internalization of the released drug [9,10,12,13, 24,52]. Ultrasound can also increase the inter-endothelial gaps thus enhancing carrier extravasation [46–51]. It should be noted that excessive pressure and/or shear stresses associated with circulating bubbles may cause collateral endothelial lining damage in normal tissues; preventing of these unwanted effects requires simulation of bubble/vessel properties [53,54].

3.6. Therapeutic effects of nbGEN/ultrasound in pancreatic cancer model

Pancreatic cancer (pancreatic ductal adenocarcinoma, PDA) is the fourth most common cause of cancer death in the United States. Most of the PDA tumors are inoperable at the time of

diagnosis due to the extended state of tumor progression, regional proliferation, poor general health of patients, and multiple aggressive micrometastases that are resistant to chemotherapy and radiation treatment. Patients with PDA have a dismal prognosis.

The only FDA-approved chemotherapeutic agent for pancreatic cancer is gemcitabine (GEM), but the response rate to chemotherapy is less than 25% [55]. An attempt was made to treat pancreatic cancer by extracorporeal high intensity focused ultrasound (HIFU), with a marginal success [56]. New approaches to treatment of PDA are urgently needed. In this context, ultrasound-mediated chemotherapy by nanodroplet-encapsulated drugs may offer an innovative approach to a PDA therapy. Here we describe the first results of the experiments on using nanodroplet plus ultrasound modality to treatment of gemcitabine-resistant MiaPaCa-2 xenografts in nu/nu mice. Two treatment rounds (twice weekly for two weeks) with a two-week break between the treatment rounds were given. Six animals per experimental group were used. It was shown earlier by others that MiaPaCa-2 cells were sensitive to treatment by micellar-encapsulated PTX Genexol PM [57]. This was also observed in the present study. The introduction of a micellar encapsulated PTX, Genexol PM (GEN) into the drug formulation dramatically sensitized the pancreatic tumor to the chemotherapeutic action of the drug (Figure 8A). A very significant tumor regression was observed in all groups that comprised Genexol PM. We have found that treatment by drug combination Genexol PM +GEM appeared somewhat superior to that by Genexol PM alone. Note that gemcitabine has very high aqueous solubility and therefore does not encapsulate either in micelles or in droplets but circulate independently of Genexol PM or nbGEN. Note also that in a nanoemulsion formulation nbGEN, PTX is exclusively encapsulated in nanodroplets (Table 1 above and Figure 1 of Supplementary Material) which, if not irradiated, retain the drug very tightly (See Figure 5), as was also reported in refs.[13,24].

At the start of the treatment, all formulations comprising Genexol PM were very effective in inducing dramatic tumor regression (Figure 8A). The differences between formulations and treatment protocols appeared at later stages. In this report, we compare the effect of ultrasound on the tumor treatment by micellar and nanodroplet PTX formulations. As shown in Figure 8B, application of ultrasound with a micellar PTX formulation accelerated and enhanced tumor regression during the first treatment round but the differences were quickly eliminated during the treatment break and did not reappear in the second treatment round (Figure 8B, dashed curves). Tumor re-growth that had started during the treatment break continued during the second treatment round.

The effect of ultrasound on the nanodroplet encapsulated PTX was more pronounced. In Figure 8B, tumor growth curves are compared for the treatment by a combination drug Genexol PM +GEM (solid curves) that was delivered either as a micellar formulation (GEN+GEM) or nanodroplet formulation plus ultrasound (nbGEN+GEM+US). Starting with the thirds week of treatment, statistically significant differences in a two-tail paired T-test were observed between the ultrasound-treated and untreated group ($p = 0.02$). The differences were preserved throughout the experiment. Even more pronounced differences ($p = 0.0004$) were observed between the nbGEN+GEM+Ultrasound group and Genexol PM group (i.e. our positive control) thus underscoring the effect of ultrasound on the drug release from phase-shift nanoemulsions. Tumor accumulation of nanodroplets after systemic injections of nanoemulsions was clearly manifested by increased echogenicity of tumor ultrasound images (Figure 9).

It is noteworthy that only the nanodroplet+ultrasound formulation induced some tumor regression during the second treatment round. Even more importantly, the number of metastatic sites was substantially lower in the ultrasound-treated groups than in all other groups indicating that in our experiments, ultrasound treatment suppressed tumor metastasis. As an example, the

mean number of metastatic sites was 1.2 ± 0.7 in the nbGEN+GEM+Ultrasound treated group, 2.2 ± 1.5 in the Genexol PM+GEM group without ultrasound, and 3.8 ± 0.4 in the GEM alone group. The suppression of metastasis by ultrasound treatment was unexpected and its mechanism is presently unclear. This is a very important observation because a possible role of ultrasound in promoting metastasis has been discussed in the literature [58–60]. Our data show that at least at ultrasound intensities used in this study, ultrasound combined with chemotherapeutic agent does not promote but actually reduces metastasis.

In conclusion, combination tumor treatment with a phase-shift nanoemulsion formulation of PTX and ultrasound appears superior to treatment by micellar formulations of PTX with or without ultrasound. We consider these data very encouraging and deserving translational research. Note however that for all studies tumor models, tumor re-growth started after the completion of the first treatment round. Similar results were observed earlier with breast cancer tumors [13,24]. A second treatment round with the same regimen was less effective than the first one; tumor growth was delayed but some tumor regression was observed only in the nanodroplet/ultrasound treatment group. This indicates that resistant cells were either developed or selected during the first treatment round.

4. Conclusions

A novel ultrasound-mediated chemotherapeutic modality is based on systemic injections of drug-loaded nanoemulsions that convert into microbubbles *in situ* under the action of therapeutic ultrasound. This modality proved very effective in inducing dramatic tumor regression in ovarian and pancreatic cancer models. However recurrence of more resistant tumors was observed some time after the completion of the treatment. Current efforts are directed toward suppressing development of drug resistance in the course of chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Droplet vaporization temperature as a function of droplet size for the surface tension values of 30 mN/m and 50 mN/m.

Fig. 2.
Fig. 2A. PEG-PCL nanodroplets inserted in PBS through a 18 G needle (left) or 26 G needle (right). Bubbles formed when nanoemulsion is injected through a thin needle are visualized as

bright spots (indicated by arrows in the right panel); bubbles rise to the sample surface while droplets precipitate to the bottom of a test tube.

Fig. 2B. PEG-PCL nanodroplets injected in the agarose gel through a 18 G (left) or 26 G (right) needle. Injection through the thin needle results in immediate formation of very bright bubbles whose size and brightness increase with time; the brightness of the droplets also increases with time suggesting a post-injection droplet-to-bubble transition.

Fig. 3. PFP/PEG-PLLA microbubbles in a plasma clot before (A) and after sonication for 1 min by 1-MHz, 3.4 W/cm² (B) and 90-kHz, 2.8 W/cm² ultrasound (C) at room temperature.

Fig. 4.
Schematic illustration of drug transfer from nanodroplets through microbubbles into cells under the action of ultrasound.

Fig. 5.

Photographs of a mouse bearing two ovarian carcinoma tumors (A) - immediately before and (B) - three weeks after the treatment; a mouse was treated by four systemic injections of nanodroplet-encapsulated PTX, nbGEN (20 mg/kg as PTX) given twice weekly; the right tumor was sonicated by 1-MHz CW ultrasound (nominal output power density 3.4 W/cm^2 , exposure duration 1 min) delivered 4 hours after the injection of the drug formulation. Ultrasound was delivered through a water bag coupled to a transducer and mouse skin by Aquasonic coupling gel.

Fig. 6.

Fig. 6A. Effective regression of the ovarian carcinoma tumor treated by nbGEN/ultrasound combination as described in Materials and Methods. The first photograph was taken before the start of the treatment, the second – two weeks later, i.e. immediately after the last treatment of the first treatment round. The third photograph was taken one week after the completion of the first treatment round.

Fig. 6B. Normalized tumor growth/regression curve for the mouse presented in Figure 6A.

Fig. 7.

Fig. 7A. Ovarian tumor growth curves for control tumors (open circles), and tumors treated by micellar PTX formulation Genexol PM (GEN, filled triangles), nanodroplet PTX formulation combined with ultrasound (nbGEN+ultrasound, filled circles), and empty nanodroplets combined with ultrasound (open diamonds). Mean values plus/minus standard errors are presented for control and nbGEN+ultrasound (N = 3). Arrows indicate days of treatment. Fig. 7B. Breast tumor growth curve for control tumor (open circles), and tumors treated with a micellar PTX formulation Genexol PM (GEN, filled triangles), and nanodroplet PTX formulation combined with ultrasound (nbGEN+ultrasound, filled circles). Mean values plus/minus standard error are presented (N = 3). Arrows indicate days of treatment.

Fig. 8.

Fig. 8A. Pancreatic tumor growth curves for control mice (open squares) and mice treated by Gemcitabine (GEM, filled squares), Genexol PM (GEN, open circles), and nanodroplet/ultrasound formulation nbGEN+GEM+ultrasound (filled triangles). Ultrasound parameters: frequency 1 MHz, nominal power density 3.4 W/cm², duration 30 s. Mean values plus/minus standard errors are presented (N = 6). Arrows indicate days of treatment.

Fig. 8B. Pancreatic tumor growth curves for mice treated by Genexol PM (GEN, open circles, dashed line), Genexol PM/ultrasound (GEN+US, filled circles, dashed line), Genexol PM + GEM (open triangles, solid line), and nbGEN+GEM+ultrasound (filled triangles, solid line). Ultrasound parameters as in 8A. Mean values plus/minus standard errors are presented (N = 6).

Fig. 9.
Ultrasound images of a pancreatic tumor before (left) and 5.5 h after systemic injection of nbGEN (right).

Table 1
Size distribution parameters of micellar and nanoemulsion systems at room temperature.

Samples	Peak 1		Peak 2		Peak 3	
	Radius, nm	Volume fraction	Radius, nm	Volume fraction	Radius, nm	Volume fraction
0.25% PEG-PLLA micellar solution	22.2	15%	114.7	85%	-	-
GEN in 0.25% PEG- PLLA micellar solution	29.3	69%	188.9	31%	-	-
1% PFP/0.25% PEG- PLLA emulsion	-	-	117.5	27%	592.6	73%
GEN in 1% PFP/0.25% PEG-PLLA emulsion	19.3	7%	122.9	31%	718.4	62%