Evaluation of loading strategies to improve tumor uptake of gemcitabine in a murine orthotopic bladder cancer model using ultrasound and microbubbles

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Abstract

In this study we compared three different microbubble-based approaches for the delivery of a widely used chemotherapy drug, gemcitabine: (i) co-administration of gemcitabine and microbubbles (Gem+MB), (ii) conjugates of microbubbles and gemcitabine-loaded liposomes (GemlipoMB), and (iii) microbubbles with gemcitabine directly bound to their surfaces (GembioMB). Both *in vitro* and *in vivo* investigations were carried out, respectively in the RT112 bladder cancer cell line and a murine orthotopic muscle-invasive bladder cancer model. The *in vitro* (*in vivo*) ultrasound exposure conditions were 1 (1.1) MHz centre frequency, 0.07 (1.0) MPa peak negative pressure, 3000 (20000) cycles and 100 (0.5) Hz pulse repetition frequency. Ultrasound exposure produced no significant increase in drug uptake either *in vitro* or *in vivo* compared with the drug-only control for co-administered gemcitabine and microbubbles. *In vivo,* GemlipoMB prolonged the plasma circulation time of gemcitabine, but only GembioMB produced a statistically significant increase in cleaved caspase 3 expression in the tumor, indicative of gemcitabine-induced apoptosis.

Keywords: gemcitabine, ultrasound-mediated drug delivery, bladder cancer, orthotopic model, liposome, microbubbles, nanoparticles, biotinylation.

Introduction

Gemcitabine is an antimetabolite that has been shown to be an effective chemotherapeutic agent both alone and in combination with other agents for treating pancreatic cancer (Burris and Storniolo 1997), non-small cell lung cancer (Copley-Merriman, et al. 1996), bladder cancer (Moore, et al. 1997, Schlack, et al. 2016), ovarian cancer (Shapiro, et al. 1996, Pignata, et al. 2017) and breast cancer (Seidman 2001). Its primary cytostatic effect arises from its structural resemblance to deoxycytidine (Ruiz Van Haperen and Peters 1994). The tri-phosphorylated form of gemcitabine can be incorporated into DNA during synthesis and allows only one additional nucleoside base to be added, resulting in 'masked chain termination' and subsequent apoptotic cell death (Huang and Plunkett 1995).

Although gemcitabine is effective in preventing tumor cell proliferation, its short plasma half-life limits its clinical efficacy. When used at standard clinical doses, gemcitabine generally has a half-life of < 10 minutes (Abbruzzese, et al. 1991). Once injected or infused intravenously, gemcitabine is rapidly inactivated to 2',2'-difluorodeoxyuridine (dFdU) by cytidine deaminase (CDA), either in the plasma or intracellularly. Similar to deoxycytidine, gemcitabine has a hydrophilic structure and nucleoside transporter proteins are required for its passage through cellular membranes. Unfortunately, since its clearance is fast and its cellular uptake is slow, high doses of gemcitabine are necessary to maintain therapeutic drug concentrations, inevitably resulting in significant side effects in normal tissues (Dasanu 2008, Haydock, et al. 2018). This is particularly problematic in patients suffering from pancreatic and muscle-invasive bladder cancers. For these patients, combination therapy with radiotherapy is often a primary

treatment option, but treatment tolerance is severely limited by acute intestinal toxicity (Nakamura, et al. 2012, Caffo, et al. 2016).

Consequently, there has long been an interest in improving the delivery of gemcitabine to address its limitations. Strategies that have been investigated include: (1) modifying the structure of gemcitabine by chemical methods and (2) incorporating it into drug-delivery vehicles. For example, gemcitabine has been structurally modified to include lipophilic groups to improve its intracellular transport (Castelli, et al. 2007) and protection groups to reduce its conversion to dFdU (Song, et al. 2005). It has also been encapsulated into liposomes and nanoparticles, resulting in enhanced cytotoxicity and prolonged circulation profiles (Yalcin, et al. 2018). In particular, encapsulating the drug within nanoparticles of < 200 nm diameter can facilitate uptake in the tumor through the compromised endothelium, a process known as the enhanced permeability and retention (EPR) effect. It is, however, difficult to achieve a therapeutically relevant drug dosage by this process alone (Wilhelm, et al. 2016, Sindhwani, et al. 2020).

Drug uptake at tumor sites can be improved non-invasively by applying ultrasound in conjunction with microbubble contrast agents (Lawrie, et al. 2000, Mullick Chowdhury, et al. 2017). These agents are used clinically for diagnostic ultrasound imaging but can also be used therapeutically to increase local permeability (Price, et al. 1998, Cosgrove 2006). They consist of suspensions of bubbles, 1- 10 μm in diameter, containing an inert high molecular weight gas and stabilized against dissolution by a shell, typically made of lipid, protein or polymer. The response of microbubbles to an ultrasound field can cause cell permeabilization, blood vessel

extravasation and deposition of drugs. This can be spatially confined to a target region by controlling the positioning and focus of the ultrasound beam.

Previous studies have shown that ultrasound-enhanced delivery of gemcitabine by co-administration with microbubbles is feasible both preclinically (Kotopoulis, et al. 2014) and clinically (Kotopoulis, et al. 2013, Dimcevski, et al. 2016) in the treatment of pancreatic cancer. Further improvements can be achieved by attachment of drug molecules and/or nanoparticle carriers to the microbubble shell to improve localization (Kheirolomoom, et al. 2007, Lentacker, et al. 2009). Hydrophobic drugs can be incorporated directly into the lipid layer of microbubbles (Unger, et al. 1998); whilst hydrophilic drugs, such as gemcitabine, require structural changes to enable their attachment onto microbubbles. Nesbitt *et al.* demonstrated loading of a biotinylated version of gemcitabine onto microbubbles using a biotin-avidin linkage (Nesbitt, et al. 2018). Nanoparticles such as liposomes can also be used to load hydrophilic drugs onto microbubbles. Kheirolomoon *et al.* showed that 1,000s-10,000s of liposomes with sizes of 100-200 nm could be attached to each microbubble (Kheirolomoom, et al. 2007). Lentacker *et al.* conjugated doxorubicin-containing liposomes to microbubbles and demonstrated their superior *in vitro* cytotoxicity over doxorubicin liposomes alone (Lentacker, et al. 2010). These techniques have been demonstrated to improve uptake and drug effectiveness in multiple applications (Stride and Coussios 2019).

The aim of this study was to compare these different approaches (Figure 1) for delivering gemcitabine with and without ultrasound (US) exposure, specifically:

1) Gem+MB: co-administration of gemcitabine (gem) and perfluorobutane (PFB) microbubbles (MB). Biotinylated FITC (FITCbio) was used as a drug surrogate *in vitro* to facilitate imaging.

2) GemlipoMB: gemcitabine was encapsulated into liposomal nanoparticles (gemlipo) that were then conjugated to PFB MB using an avidin-biotin linkage. Fluorescent liposomes (lipo) were used as gemlipo surrogates and fluorescent liposomes conjugated to PFB MB (lipoMB) as gemlipoMB surrogates *in vitro*. Fluorescent microbeads were used as gemlipo surrogates *in vivo*.

3) GembioMB: a biotinylated version of gemcitabine (gembio) was synthesised and directly conjugated to PFB MB using an avidin-biotin linkage. MB conjugated to biotinylated FITC (FITCbioMB) were used as gembioMB surrogates *in vitro*.

We initially compared the three approaches by studying cytotoxicity *in vitro* using a clonogenic assay, a standard method to measure the proliferative death of antimetabolites. We then investigated the stability of gemcitabine in each approach using enzymatic and plasma assays. To facilitate direct imaging, ultrasound-mediated drug delivery was then investigated using fluorescent surrogates both *in vitro* in a bladder cancer cell line and *in vivo* in a murine orthotopic bladder cancer model. Gemcitabine delivery *in vivo* was subsequently quantified by liquid chromatography-mass spectrometry (LCMS) one hour after treatment and tumor cell apoptosis was measured 6 hours after treatment using a human cleaved caspase 3 ELISA assay.

Materials and Methods

Cell Culture and Chemicals

The human bladder cancer cell line, RT112, was purchased from DSMZ (Braunschweig, Germany) and cultured in RPMI-1640 medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% penicillin/streptavidin (Gibco, Life Technology ltd, Milton Keynes, UK). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG(2000)Biotin), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG(2000)) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Gemcitabine hydrochloride (50 mg, Y0000657), cholesterol (>99% from egg white, C8667, Chol), S-(4-nitrobenzyl)-6-thioinosine (NBMPR), tetrahydrouridine, dansyl chloride, and 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) were purchased from Sigma. Biotin-4-fluorescein (FITC) and Coomassie Brilliant Blue dye were purchased from Thermo Fisher Scientific (Abingdon, UK). Fluorescent microbeads (FluoSpheresTM Biotin-Labeled polystyrene spheres, 0.2 µm diameter, yellow-green fluorescent 505/515) were obtained from Thermo Fisher Scientific (Abingdon, UK). The synthesis of biotinylated gemcitabine is described in the Supplementary Information.

Liposome Preparation

Gemcitabine-carrying liposomes were prepared at 40 mg/mL lipid concentration using DPPC : Chol : DSPE-PEG(2000)Biotin = 8 : 3 : 1 using the small volume incubation method as reported previously (Xu, et al. 2014). Fluorescent liposomes were prepared with a

concentration of 0.5 mg DiO per mL of the above liposome suspension. Lipids (and DiO for fluorescent liposomes) were dissolved in chloroform, mixed and dried overnight at 50 ^{o}C on a hot plate. The lipid film was then rehydrated with 1 mL distilled water and underwent 10 freeze/thaw cycles using a 70 ^{o}C water bath and liquid nitrogen for 5 min per step. Samples were then extruded through a 200-nm filter extrusion (Mini-extruder, Avanti Polar Lipids, Alabaster, AL, USA). The suspension was then centrifuged at 60,000 x g for 1 hour at 4 ^{o}C and then the supernatant removed. Without resuspension, the liposomes were incubated with 30 µL of 2 M gemcitabine solution for 3 hours and purified with another centrifugation step at 60,000 x g for 1 hour at 4 ^{o}C using PBS. The liposomes were centrifuged again at 60,000 x g for 1 hour at 4 ^{o}C after gemcitabine cleaning and kept in pellet form after removal of the supernatant. The blank liposomes (blank lipo) were made by replacing the 2 M gemcitabine solution with PBS in the above procedure. The gemcitabine loading was quantified by LCMS as described below. The characterization of the liposomes is described in the Supplementary Information.

Microbubble Preparation

Microbubbles were prepared by mixing DSPC, DSPE-PEG(2000), and DSPE-PEG(2000)Biotin at a molar ratio of 82 : 9 : 9. Chemicals were dissolved in chloroform, mixed and dried overnight at 50 ^{o}C. The lipid films were re-suspended with PBS (20 mg/mL) at 100 ^{o}C and sonicated for 120 sec at 20 % amplitude using a 20 kHz ultrasonic cell disruptor (Probe sonicator, Q125 Sonicator, Qsonica, Newtown, CT, USA). The solution was further sonicated for 30 sec at 60 % amplitude with perfluorobutane (F2 Chemicals Ltd, Preston, UK) and then placed

on ice for 3 min. The microbubble suspension (concentration = (3.93 ± 0.99) x 10^{10} microbubbles/mL and average diameter = 1.20 ± 0.03 μm as measured from 7 batches by light microscopy and a custom MATLAB (The Mathworks Inc., Natick, MA, USA) script as previously described (Sennoga, et al. 2010)) was then mixed with avidin (1 mg/mL, dissolved in PBS, CAT# 189725 from EMD Millipore, Burlington, MA, USA) for 5 min, washed by centrifuging at 280 x g for 5 min at 4 ^{o}C to remove unbound avidin, and replenished with an equal volume of PBS. This method generated microbubbles at (1.01± 0.18) x 10^{10} microbubbles/mL concentration and 1.27 ± 0.04 μm in diameter (measured as above from 17 batches of microbubbles). Liposomes, biotinylated gemcitabine, or biotinylated microbeads were mixed with microbubbles for at least 3 min at room temperature before the experiments started. Attachment of biotinylated gemcitabine with microbubbles was confirmed by centrifuging the gemcitabine/microbubble or biotinylated gemcitabine/microbubble mixtures (initial gemcitabine or biotinylated gemcitabine concentration = 10 μM and 1 x 10^{9} microbubbles/mL) at 280 x g for 5 min at 4 ^{o}C and then the gemcitabine concentration was quantified using LCMS (protocol as described below). The characterization of the microbubbles using fluorescence and transmission electron microscopy is described in the Supplementary Information.

Cell survival and hENT1 inhibition assay

RT112 cells (5 × 10^{5} cells/well) were plated in a 6-well dish. To test the cytotoxicity of the microbubbles alone, cells were incubated with microbubbles without gemcitabine (concentration = 1.11x 10^{8}/mL) at microbubble:cell (MB:cell) ratios of 10 and 20. The microbubble concentration was chosen based on previous studies that indicated efficient

ultrasound mediated gene transfection or membrane disruption required ~10-20 microbubbles per cell (Kaddur, et al. 2007, Tran, et al. 2007). After 24 hour incubation, cells were then trypsinized to enable a clonogenic assay to be performed as described in (Franken, et al. 2006). Briefly, 700-1000 cells were seeded into one 10 cm dish and the cells were cultured for two weeks before fixation and stained with methanol containing 0.1% (w/v) Coomassie Blue dye. Colonies with more than 50 cells were counted. The dose survival curve for the drug/drug-loaded microbubbles was constructed by treating RT112 cells with free gemcitabine (gem), gembio, or gemlipo (matched gemcitabine concentration) at 0, 1, 12.5, 25, 50, 100, and 200 nM for 24 hours. An equal number of blank liposomes were also tested (blank lipo). Cells were then again subjected to clonogenic assay.

Gemcitabine typically enters cells via the human equilibrative nucleoside transporter 1 (hENT1), which can be inhibited by S-(4-nitrobenzyl)-6-thioinosine (NBMPR)(Hioki, et al. 2018). A further experiment was therefore performed in which RT112 cells were treated with 50 nM free gemcitabine, gembio, or gemlipo (matched gemcitabine concentration) with or without 1 μM NBMPR for 24 hours and then subjected to clonogenic assay.

Gemcitabine stability assay

Suspensions of gemcitabine co-administered with microbubbles (gem+MB, microbubble concentration = $1x10^9$/mL), microbubbles conjugated to gemcitabine-loaded liposomes (gemlipoMB, microbubble concentration = $1x10^9$/mL) and microbubbles carrying gemcitabine (gembioMB, microbubble concentration = $1x10^9$/mL) all having a gemcitabine concentration of 1 μM were incubated in PBS buffer in the presence of human cytidine deaminase (15 μg/mL,

SRP6372 from Sigma Aldrich, Gillingham, UK) at 37 °C with agitation. 50 µL of the samples were collected from the 1 mL solution at 0, 1, 5, 10, 15, 20, 30, 45, 60, 90, 120 min and mixed with 5 µL of glacial acetic acid (ARK2183 from Sigma Aldrich, Gillingham, UK) to quench the enzyme activity. No replacement was made after sample collection. The samples were precipitated with acetonitrile (34851 from Sigma Aldrich, Gillingham, UK) and centrifuged at 12,000 x g for 5 min to remove proteins. The supernatants were collected, evaporated overnight, reconstituted and analyzed by LCMS (protocol described below). 5-Fluoro-5'-deoxyuridine (dFdU, F8791 from Sigma Aldrich, Gillingham, UK) and gemcitabine hydrochloride were used as standards.

In vitro ultrasound exposure

The ultrasound exposure apparatus consisted of a 1 MHz single element transducer (model 8233 A101, Imasonic, Voray sur l'Ognon, France) mounted in the base of a Perspex container filled with ~800 mL of degassed, deionized water. Images of the setup are shown in the Supplementary Information (Figure S2) and further details may be found in (Aron, et al. 2019). The container lid was covered with an acoustic absorber and had an attached holder capable of supporting an inverted cell culture imaging dish (35 mm iBidi µ-dish) as described in (Carugo, et al. 2015). The holder was situated above the transducer such that the cell layer sat ~32 mm from the transducer face so that the full 19 mm diameter cell growth area of the dish was exposed to ultrasound. The cell dish was sealed by a polydimethylsiloxane (PDMS) lid and inverted to allow microbubbles to rise into contact with the cell layer. Sinusoidal waveforms were generated in an Agilent 33220A function generator (Keysight Technologies, Santa Rosa, USA) and transmitted to the transducer via a 55-dB power amplifier (1140LA, E&I, Rochester,

NY, USA). Microbubble response was monitored acoustically using a 7.5 MHz single element transducer (Olympus, Waltham, USA) aligned with the center of the cell culture dish. Acoustic responses were passed through a 2 MHz high pass filter (Allen Avionics, River Grove, IL) to remove the fundamental 1 MHz frequency and recorded to computer by oscilloscope (Handyscope HS3, TiePie Engineering, Sneek, The Netherlands). Recorded waveforms were processed in MATLAB to determine the contribution of broadband and harmonic components of microbubble response over time. Transducer calibrations were performed *in situ* using a 0.2-mm-diameter needle hydrophone (HNC-0200, Onda Corporation, Sunnyvale, CA, USA). For *in vitro* cell treatment, a function generator was set to generate pulses of 3000 cycles, at 100 Hz pulse repetition frequency with a peak negative pressure of 0.07 MPa. These parameters were selected on the basis of preliminary optimization studies to show the maximum DiO-labeled liposome uptake with minimal cell detachment after sonication (data not shown). Cell dishes for sonication were prepared as described in (Carugo, et al. 2015). Medium without serum was used to avoid protein precipitates in the serum that might serve as additional cavitation nuclei. For the *in vitro* experiments with liposomes, the liposome concentration was 6×10^{11}/mL and the microbubble concentration was 4×10^{8}/mL in each dish. For the *in vitro* experiments with FITC-biotin, the microbubble concentration was adjusted to 5×10^{8}/mL in each dish. The cells were insonated for 3 min and washed with PBS before flow cytometry analysis.

Flow cytometry for in vitro ultrasound-mediated delivery

For analysis of *in vitro* delivery efficiency, cells were rinsed with PBS, incubated with trypsin for 5 minutes, and removed from the dishes immediately after treatment. Cells were

208 then re-suspended in PBS without fixation. A flow cytometer (FACSort from Becton Dickinson,
209 Swindon, UK) was used to analyze the samples. Samples were studied with an excitation
210 wavelength of 488 nm and an emission wavelength of 530 nm over a 30 nm bandwidth. Results
211 were analyzed using Flowing software (Cell Imaging Core, Turku Centre for Biotechnology,
212 Turku, Finland).

213 ***In vivo ultrasound treatment***

214 All animal work was done in accordance with UK Home Office Guidelines, following the
215 ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, and approved by the
216 University of Oxford Animal Welfare and Ethical Review Body (AWERB), under University of
217 Oxford project licenses P4B738A3B and P8484EDAE. Six to eight-week-old female CD1NUDE
218 mice (from Charles River Laboratories, Harlow, UK) were injected orthotopically with RT112
219 bladder cancer cells under ultrasound guidance following the technique described by Jäger *et al*
220 (Jager, et al. 2013). To characterize the orthotopic models, microbubbles (made as described in
221 the previous section, concentration = 1 x 10^8 /mL, injected volume 200 µL) were injected
222 through a tail vein catheter into 3 animals (not used for subsequent treatment) and the
223 orthotopic bladder tumor was imaged using the contrast mode from a Vevo 3100 using a
224 MX250 probe (frequency = 18 MHz, transmit power = 25 %, acquisition frame rate = 15 Hz,
225 dynamic range = 40 dB, FUJIFILM Visual Sonics, Toronto, Canada).

226 Mice were randomly subjected to different treatment groups before tumor inoculation.
227 Five to seven days after tumor inoculation (average tumor size = 55.35 ± 2.62 mm^3 from 57
228 mice), mice were treated using a customized image-guided ultrasound treatment system as

shown in the Supplementary Information Figure S3a. Mice were placed on a platform supported by an acoustically transparent mylar membrane and a thin layer of ultrasound gel was applied to provide acoustic coupling between the skin and the mylar membrane. In this system, an 8 MHz imaging transducer (L11.5v, Verasonics, Kirkland, WA) was placed at right angles to a 1.1 MHz therapeutic transducer (H102, Sonic Concepts, Bothell, WA) and at 45^{o} to the horizontal in the water tank, such that their beams intersected at the tumor site. A custom beam expanding lens was used on the therapeutic transducer to enable ultrasound exposure of the whole bladder. The imaging ultrasound probe was then used to align the therapeutic beam with the tumor and to enable treatment monitoring. Initially, B-mode imaging was used to locate the bladder and the centre of the tumor. After the tumor was located and the ultrasound treatment started, pulse inversion contrast imaging was used to monitor the signal of microbubbles within the tumor before and after treatment, whilst passive acoustic mapping (PAM) was used to monitor the bubble signal during therapeutic ultrasound exposure as described in (Choi, et al. 2014, Coviello, et al. 2015). The therapeutic ultrasound settings were 1.1 MHz centre frequency, 1 MPa peak negative pressure, 1% duty cycle and 0.5 Hz pulse repetition frequency. Transducer calibrations were performed in free field conditions in a water tank using a 0.2 mm diameter needle hydrophone (HNC-0200, Onda Corporation, Sunnyvale, CA, USA). The parameters were chosen based on pilot studies to maximise the number of fluorescent microbeads delivered whilst minimising skin damage. The differences compared with the *in vitro* exposure conditions were due to the need to allow for tissue attenuation and replenishment of the tumor vasculature with microbubbles, based on the perfusion measurements as described below.

Microbubble/drug mixtures were injected 8 times, using 25 µL of solution each time, via a syringe pump (flow rate = 0.5 mL/min, AL-1000, World Precision Instruments, Hitchin, UK), and 40 ultrasound pulses were applied between each injection (Supplementary Information Figure S3b). The time between injections was chosen to be 80 s to match the therapeutic protocol and the tumor perfusion rate. The length of the extension tube for the syringe was 13 cm and the dead volume was ~7 µL. The syringe was always primed before the treatment started. Bead-conjugated microbubbles were made by mixing the biotinylated yellow-green fluorescent microbeads (Ex/Em: 505/515, conc = 2.28 x 10^{13} microbeads/mL) with the microbubbles (1 x 10^{9}/mL). The microbubble concentration was diluted to 3×10^{8}/mL for injection. For all experiments involving gemcitabine, an equivalent dose of 10 mg/kg was used. Mice receiving microbubble/drug injections but without ultrasound treatment were used as the control. The mice were sacrificed to collect blood and bladder tissue at specific time points as described below. Blood was collected from the left ventricle into a heparin-coated tube with 10 mg/mL tetrahydrouridine, a common inhibitor of CDA, to prevent further conversion of gemcitabine to dFdU and centrifuged at 3,000 x g for 10 min at 4 ^{o}C to separate out the plasma. The plasma and bladder samples were frozen on dry ice and kept at -80 ^{o}C prior to further analysis.

LCMS analysis of gembio and gemcitabine

Sample containing gemcitabine

To measure the gemcitabine concentration in each of the samples, gemcitabine was derivatized with dansyl chloride. For the liposome samples, 50 µL of the liposome suspension

(diluted to ~10^{13} liposomes per mL) was mixed with 50 µL water, 100 µL 0.1M sodium hydrogen carbonate (pH 11) and 100 µL 10 mg/mL dansyl chloride. The mixture was heated at 60°C for 5 min. The solution was then extracted with 1 mL methyl-tert-butyl ether and the solvent dried down in a centrifugal evaporator. The dried down sample was reconstituted in 25% acetonitrile before LCMS analysis (shown below). For the analysis of treated mice, gemcitabine or the relevant vehicle carrying an equivalent gemcitabine dose of 10 mg/kg was used for the *in vivo* ultrasound treatment as described above. Mice were sacrificed one hour after treatment to collect plasma and bladder samples. Bladder samples were homogenized on ice with PBS containing 10 mg/mL tetrahydrouridine. The sample lysates were stored at -80 °C before analysis. Samples were derivatized with dansyl chloride as described above.

The gemcitabine- dansyl chloride derivatized samples were analysed using a Waters Acquity H-class UPLC equipped with a mass spectrometer (Waters Acquity UPLC TQ detector, Waters UK, Elstree, UK). Separation utilized an ACE 3 µm, 2.1 x 100 mm column at 35 °C with eluents A: 10 mM formic acid and B: acetonitrile with a linear gradient of 30-100% B over 8 min returning to starting conditions in 0.1 min, run time 12 min, 0.25 mL/min. Gemcitabine derivative was detected at multiple reaction monitoring (MRM) 496.8>112 and dFdU derivative at MRM 497.8>113. Gemcitabine concentration was quantified using a standard curve prepared in water alone and treated as above.

Sample containing gembio

To measure the stability of gembio in plasma, mouse plasma (containing citrate as anticoagulant) was incubated with gembio (38 µg/ml) with and without the addition of avidin

(67 µg/ml) on ice or at 37 °C. Samples were removed every 4 min into perchloric acid to precipitate the protein, centrifuged and the supernatants measured for gembio stability.

Samples were analysed using a LCMS system (Waters e2695, Waters UK, Elstree, UK) equipped with a mass spectrometry detector (Waters QDa, Waters UK, Elstree, UK) operating in electrospray positive mode. Chromatography used a Waters Xbridge Ethylene Bridged Hybrid (BEH) C18 column (3.5 µm, 50 x 2.1 mm) maintained at 35 °C. Separation was achieved with eluent A: 0.1% formic acid; eluent B: acetonitrile; using a flow rate of 0.4 mL/min and a gradient from 2 % to 50 % B in 3 min with a 1 min hold, and then returning to starting conditions in 0.1 min. Data were acquired using Waters Empower 3 software (Waters UK, Elstree, UK). Gembio was measured at selected ion recording (SIR) 490 (M+H).

Microbead uptake by immunofluorescent staining and image analysis

For the fluorescent microbead experiments, beads labelled with a fluorophore having excitation and emission wavelengths of 505 and 515 nm respectively were used to enable them to be distinguished from other dyes used in the experiments. Excised bladders were snap frozen and cryo-sectioned to a thickness of 25 µm. The slides were then fixed with ice cold acetone for 10 min and stained with Hoechst 33342 for cell nuclei (1 µg/mL). Slides were scanned using an Aperio FL slide scanner (Leica Biosystems, Milton Keynes, UK) at 0.75 NA objective (Hoechst 33342: exposure time = 25 ms; Alexa 488 for microbeads: exposure time = 1 sec) and processed using Aperio ImageScope software (Leica Biosystems, Milton Keynes, UK). Quantification of the mean intensity of the fluorescent microbeads was conducted using the Positive Pixel Count FL Algorithm.

Tumor response by active human cleaved caspase 3 ELISA

The quantification of human cleaved caspase 3 was conducted according to the Human Cleaved Caspase-3 (Asp 175) SimpleStep ELISA Kit protocol (ab220655, Abcam, Cambridge, Uk). Briefly, bladder samples were homogenized using a 1 mL Dounce homogenizer (Sigma Aldrich, Gillingham, UK). The lysate was centrifuged at 18,000 x g for 20 min at 4 °C, and the supernatant quantified by BCA protein assay (Pierce™ BCA protein assay kit, Thermo Fisher Scientific, Abingdon, UK). The samples were then diluted to 3.5 mg/mL and subjected to ELISA analysis.

Statistical Analysis

Data were analyzed using Prism (GraphPad, San Diego, CA, USA) and presented as mean ± SEM. For the clonogenic assays, results were normalized to the sham control and then fitted using a relative weighted nonlinear regression applied to the dose-response model ($y = 100/(1 + (IC_{50}/x)^{Hill\ Slope})$). The IC_{10} and IC_{50} values were derived from this fit. Spearman 'r' correlation was used to study the relationship between two parameters with a two-tailed p value. A Student's t-test was used to compare two groups. Multiple group comparisons were made using one-way ANOVA followed by a *post hoc* Tukey's multiple comparison test.

Results

Characterization of microbubble constructs

Gemcitabine-encapsulated liposomes (gemlipo) and a biotinylated version of gemcitabine (gembio) were developed to facilitate the loading of gemcitabine onto microbubbles (MB). The gemlipo were characterised by dynamic light scattering for the diameter (size) and dispersion (size distribution). Freshly made gemlipo were 157.10 ± 1.22 nm in diameter ~~size~~ with a polydispersity index of 0.12 ± 0.01 (determined from 6 batches of liposomes, Figure 2a and 2b). The structure of gemlipo was found to be stable over time as the reconstitution of 3-month-old gemlipo from pellets showed a less than 10 % change in liposome diameter ~~size~~ (145.10 ± 0.40 nm with polydispersity index of 0.08 ± 0.01, from 3 batches of liposomes). The concentration of liposomes was (4.89 ± 0.76) x 10^{13} liposomes per mL, as measured by nanoparticle tracking analysis from 5 batches of liposomes. Gemcitabine concentration per mL of liposome suspension was 122.43 ± 18.52 nmoles (n=5).

The DSPC-based microbubble formulation generated microbubbles with a diameter range of 1-4 µm before liposomal conjugation, as measured from the microscopy images (Figure 2c and 2d). DSPE-PEG(2000)-biotin was used in the formulation as the linker for avidin and other biotinylated ligands. The attachment of avidin and gemlipo onto microbubbles was confirmed using FITC-labeled avidin and DiO-labeled liposomes, respectively (Supplementary Information Figure S4). The overlapping of the fluorescence signal with the microbubbles indicates the formation of avidin-biotin linkages (Supplementary Information Figure S4a) and similarly the successful conjugation of liposomes onto microbubbles (Supplementary

Information Figure S4b). Conjugation of gemlipo increased the microbubble size slightly, but most remained < 5 μm in diameter (Figure 2e and 2f). Attaching the liposomes did not seem to change the shape of the microbubbles as observed under TEM. The apparently non-spherical shape of the bubbles is likely due to the sample processing (Owen and Stride 2015). Attachment of gemcitabine onto microbubbles was verified using LCMS as described above. For gembioMB, 1.07 ± 0.04 μmoles of biotinylated gemcitabine was detected in suspensions containing 10^9 microbubbles per mL (n=3) after washing by centrifugation, while gemcitabine was not detected from the gem+MB mixture after washing.

Cytotoxicity in vitro

Clonogenic assays were used to test the cytotoxicity of the various formulations on RT112 cells. Using 24 hours' incubation, we found no significant reduction in cell survival after treatment with microbubbles alone compared with untreated cells (Figure 3a). We also studied RT112 cells treated with gemcitabine (gem), gemcitabine-encapsulated liposomes (gemlipo), or biotinylated gemcitabine (gembio) using matched gemcitabine concentrations (Figure 3b). Using a relative weighted nonlinear regression on the dose-survival curves, the derived IC_{10}/IC_{50} doses were 13.84 ± 3.04 nM/55.20 ± 8.53 nM (gem, R^2 for goodness of fit = 0.92), 6.64 ± 3.98 nM/31.10 ± 1.07 nM (gemlipo, R^2 for goodness of fit = 0.998), and 20.35 ± 11.04 nM/61.4 ± 10.77 nM (gembio, R^2 for goodness of fit = 0.84). Blank liposomes produced no cytotoxicity and the curve was flat (Figure 3b). Gemlipo showed apparently higher cytotoxicity compared to gem and gembio as the dose-survival curve was shifted towards the left, but the difference was not statistically significant (Figure 3b). Gem and gembio showed comparable cytotoxicity.

NBMPR, an inhibitor of human equilibrative nucleoside transporters (hENTs) was used to inhibit the trans-membrane transport of gemcitabine by hENT1. NBMPR increased the survival of cells treated with 50 nM gem or 50 nM gembio, indicating gemcitabine resistance due to blockage of the major transporter (Figure 3c, gem *vs.* gem+NBMPR, $p < 0.05$; gembio *vs.* gembio+NBMPR, $p < 0.01$). However, no change in survival was found when the gemcitabine treatment was delivered by gemlipo in the presence of NBMPR (Figure 3c, gemlipo *vs.* gemlipo+NBMPR, p = NS), suggesting that an additional gemcitabine entry route may exist.

Stability of gemcitabine in vitro and in vivo

Under physiological conditions, gemcitabine can be converted into dFdU by CDA, primarily in the liver and plasma. An *in vitro* CDA assay was therefore used to test the relative stability of gemcitabine with the delivery vehicles. Gem+MB, gemlipoMB, and gembioMB all with an equivalent gemcitabine concentration of 1 μM were incubated with CDA. At 37^{o}C, the gemcitabine in the Gem+MB suspension was rapidly converted to dFdU within 10 min of incubation with CDA (Figure 4a). In contrast, in the gemlipoMB suspension, ~50% of the gemcitabine deaminated within the first minute and the remaining drug remained stable in the suspension (Figure 4a). The results for gembioMB were very different from those for gem+MB and gemlipoMB (Figure 4a). Less than 50% of gembio from gembioMB was deaminated within 10 min post incubation with CDA and the remaining gembio was not fully converted by CDA until 90 min. As the biotin group in the gembio was located on the 5' position of the deoxyribose group, which can be cleaved by esterases in the blood to become gem, we next assessed stability by incubating gembio with mouse plasma *in vitro*. Reduction of the gembio

394 signal was seen within 10 min (Figure 4b) even when the sample was placed on ice. However,
395 attachment of gembio to avidin improved its stability both on ice and at 37°C for at least 12 min
396 (Figure 4b). This suggests that conjugation to the microbubble may protect the gembio from
397 esterase activation into gemcitabine in circulation.

398 When gemcitabine vehicles (of 10 mg/kg of gemcitabine or equivalent dose) with
399 microbubbles were injected into mice, ~5 μg/mL of gemcitabine was still detected in plasma
400 one hour post-gemlipoMB injection, whereas the gemcitabine from the concentration-matched
401 gem+MB or gembioMB were mostly cleared by this time (Figure 4c). Six hours post-
402 administration of gem+MB or gembioMB, the gemcitabine level in the plasma was
403 undetectable by LCMS. In the gemlipoMB group, the plasma gemcitabine level reduced to 60-
404 70% of the 1-hour level and was still detectable 24 hours post- injection. As microbubbles
405 typically have a half-life of less than 10 minutes, this result may indicate that some liposomes
406 were separating from the microbubbles and still inhibiting gemcitabine deactivation.

407 ***Ultrasound enhanced delivery in vitro***

408 Since gemcitabine is not detectable by optical methods, fluorescent (DiO)-labelled
409 liposomes (lipo) and FITC-biotin (FITCbio) were used as the gemlipo and gem/gembio
410 surrogates respectively, to identify successful ultrasound-mediated delivery into individual cells.
411 Cells were treated with different drug or surrogate-microbubble combinations with or without
412 ultrasound. Cells subjected to microbubbles and ultrasound only were used as the control
413 group to gate the DiO or FITC positive cell population.

FACS analysis showed that conjugating liposomes to microbubbles produced a higher DiO-positive cell population than non-conjugated mixtures or liposomes alone following ultrasound exposure (Figure 5a, $p < 0.001$). A significant increase ($p<0.05$) in the fluorescent cell population was found between cells directly incubated with liposomes only (lipo) and those incubated with lipoMB+US (Figure 5c). No significant change in the fluorescent cell population, however, was found between cells incubated with lipoMB before and after ultrasound exposure. The apparent lack of a significant effect from ultrasound exposure could possibly be due to the high background noise in the fluorescence measurements, as $8.55 \pm 3.19\%$ of cells already showed fluorescence signals without ultrasound exposure.

Using FITC-biotin (FITCbio) as a drug surrogate, we found that FITCbio-microbubble conjugates produced more FITC-positive cells than the corresponding mixture or simply incubating FITC-biotin with cells (Figure 5b, $p < 0.01$). Simply incubating cells with FITCbio only resulted in 1.70 ± 0.17 % of cells showing fluorescence. A significant increase in the fluorescence signal was found before and after ultrasound exposure with FITCbioMB (Figure 5c, $p < 0.05$).

Effect of ultrasound on gem and gemlipo in vitro

We then studied whether ultrasound exposure could degrade gem or damage gemlipo by exposing gem+MB or gemlipoMB solutions to ultrasound for 3 min (Supplementary Information Figure S5). Two ultrasound pressures, 0.07 MPa and 0.7 MPa, were investigated (Supplementary Information Table S1) and representative area under the receiver operating characteristics curve (AUC) vs time plots for both ultrasound pressures obtained

435 (Supplementary Information Figure S5a). Solutions were sampled before and after ultrasound
436 exposure for LCMS analysis of gemcitabine concentration and the gemcitabine concentrations
437 compared using a paired t-test. No change in gemcitabine concentration was observed for
438 gemcitabine exposed to ultrasound with microbubbles at either pressure, indicating that
439 ultrasound exposure with these parameters does not cause gemcitabine degradation
440 (Supplementary Information Figure S5b). For the gemlipoMB study, CDA was added to the
441 insonated solution so that any gemcitabine leaking from the liposomes would be converted into
442 dFdU. Quantification of the remaining gemcitabine in the solution showed a trend of increased
443 leakage of gemcitabine from liposomes post-ultrasound exposure (p = NS, Supplementary
444 Information Figure S5c). The 0.07 MPa pressure caused ~15 % gemcitabine leakage from the
445 liposomes, while the 0.7 MPa pressure caused around 45 % gemcitabine leakage.

446 ***Ultrasound enhanced delivery in the orthotopic bladder cancer model***

447 A murine orthotopic muscle-invasive bladder cancer model was developed and used to
448 test the ultrasound-mediated drug delivery technique. We characterized the perfusion of the
449 tumor in this model by contrast imaging (Figure 6a, and Supplementary Information Video 1)
450 and showed the tumor to be well-vascularized, and thus suitable for microbubble-based drug
451 delivery. A burst-replenishment method was used to study how quickly the tumor could be re-
452 perfused with microbubbles (Figure 6b). Typically, microbubble signals were seen to reach their
453 peak within 2 s and, therefore, 2 s was chosen as the ultrasound pulse repetition period.

454 To investigate ultrasound-mediated drug delivery, we used biotinylated fluorescent
455 microbeads conjugated to microbubbles, as surrogates for gemlipoMB to enable their location

to be detected *ex vivo*. Mice were treated with the microbubble-bead conjugates with or without ultrasound exposure. The overlay of the B-mode and passive acoustic mapping images shows that there was a strong microbubble signal from the bladder tumor area, indicating successful microbubble activation in the tumor (Figure 6c); and we observed minimal muscle bruising due to acoustic exposure (Figure 6d). Analysis of the fluorescent signal from microbeads in histological sections from the extracted bladder indicated that, when ultrasound was applied, the mean signal intensity from microbeads in the bladder increased by a factor of 1.7 (Figure 6e & 6f, control *vs.* US, $p < 0.01$), indicating that ultrasound mediated delivery was successful.

Gemcitabine uptake and tumor response after in vivo ultrasound-mediated delivery

Using the optimized exposure parameters, gemcitabine delivery was tested with the three different microbubble-drug combinations (gem+MB, gemlipoMB, and gembioMB). Passive acoustic mapping was used to monitor microbubble activity in the tumor area during the ultrasound exposure. Representative data from each group are shown in Figure 7a. Overall, we found no significant differences between the microbubble signals from the bladder tumor in the three treatment groups (Figure 7b). The gemcitabine content from the whole bladder was analysed one hour after treatment using LCMS. Although there was an increase in gemcitabine content in the bladder following ultrasound exposure, the difference compared to the no ultrasound control was not statistically significant for any of the delivery strategies (Figure 7c). Comparing the ultrasound-mediated approaches with administration of 10 mg/kg equivalent dose of gemcitabine, gem+MB+US had the highest gemcitabine uptake in the bladder (Figure

7c, gem+MB+US *v.s.* gemlipoMB+US, $p < 0.05$; gem+MB+US *v.s.* gembioMB+US, $p < 0.01$). On the other hand, a significantly higher concentration of gemcitabine could still be detected in the plasma of the mice treated with gemlipoMB+US (4.53± 1.58 μg/mL) compared to the other groups (Figure 7d, gemlipoMB+US *v.s.* gem+MB or gem+MB+US, $p < 0.01$; gemlipoMB+US *v.s.* gembioMB or gembioMB+US, $p < 0.001$).

As gemcitabine can be converted into other metabolites that were not studied using our current LCMS method, it is possible that the apparent lack of increase in gemcitabine concentration following ultrasound exposure was due to the conversion of gemcitabine into other metabolites *in vivo*. We therefore tested the cellular response in bladder tumors six hours after treatment using an ELISA analysis of an apoptosis protein, human active cleaved caspase 3 (Asp 175). We found an approximately 1.3-fold increase in the protein expression in tumors from the gembioMB+US group compared to the gembioMB group, indicating increased tumor apoptosis following ultrasound exposure, whilst no difference was found between gem+MB *v.s.* gem+MB+US and gemlipoMB *v.s.* gemlipoMB+US (Figure 7e, $p < 0.05$).

Comparison of the three delivery approaches

The findings for the three gemcitabine delivery approaches in terms of cytotoxicity, the stability of gemcitabine with CDA/plasma, and the feasibility of ultrasound-mediated drug delivery *in vitro* and *in vivo* are summarized in Table 1.

Discussion

Cytotoxicity of individual components

The cytotoxicity, as measured by the proliferative cell death from the clonogenic assays, observed in our three approaches was primarily due to gemcitabine, because we found no significant cell killing effect of microbubbles or blank liposomes (Figure 3a and 3b). Despite modification in its chemical structure, gembio demonstrated similar cytotoxicity to gem but gemlipo was slightly more toxic than gem or gembio (Figure 3b). While the cytotoxicity of both gem and gembio was diminished after hENT1 inhibition, gemlipo demonstrated similar cytotoxicity before and after hENT1 inhibition (Figure 3c). This suggests that gemlipo does not require hENT1 for gemcitabine entry into cells. The fusion of liposomes with cell membranes has been proposed as one of the routes for drug uptake and therefore might cause gemcitabine entry into cells without the need for the hENT1 transporter (Mamot, et al. 2003).

Effect of ultrasound on gemcitabine and gemcitabine loaded liposomes

No degradation of gemcitabine was seen after exposure to ultrasound at peak negative pressures of 0.07 MPa or 0.7 MPa (Supplementary Information Figure S5b). Gemcitabine has been shown to be very stable in aqueous solution and insensitive to light damage (Xu, et al. 1999) and thus is also insensitive to sonoluminescence, which has been shown to occur at low US pressures (Beguin, et al. 2019). In contrast, when the same pressures were used on gemcitabine-encapsulated liposomes, we found that both ultrasound pressures increased the leakage from liposomes (Supplementary Information Figure S5c), suggesting that the lipid bilayers of liposomes may be damaged by cavitation. This damage may or may not be desirable

depending on where it occurs. If the liposomes are damaged away from the tumor, delivery will be reduced as the released gemcitabine will be converted into dFdU by cytidine deaminase. On the other hand, if the liposome damage occurs within the tumor vasculature, it may promote drug delivery by facilitating entry into the tumor cells.

Comparison of the three delivery approaches

The advantage of the gem+MB approach is that it is straightforward to implement clinically. However, under the conditions used in this study, gemcitabine was shown to be readily deactivated both in the *in vitro* CDA assay and *in vivo,* as shown by the murine plasma data (Figure 4). Following ultrasound exposure, gem+MB produced the highest gemcitabine uptake one hour after treatment but the difference was not significant compared with gem+MB without ultrasound, nor was there a significant increase in tumor apoptotic response 6 hours post-treatment (Figure 7c and Figure 7e). Encapsulating gemcitabine into liposomes (gemlipoMB) made the gemcitabine more resistant to CDA deactivation (Figure 4a) and prolonged the half-life of gemcitabine (Figure 4c & 4d). The deamination of gembioMB was slower than gem+MB in the *in vitro* CDA assay (Figure 4a). Since gembio can be cleaved by esterases to become gem, we also incubated gembio with mouse plasma to study the gembio deactivation. When gembio was incubated in murine plasma, the deactivation was reduced on additional avidin binding (Figure 4b). It is possible that the presence of the avidin could hinder the hydrolysis of the ester bonding by making it less accessible to esterase, a key enzyme in the blood and tissue activating gembio for downstream metabolism. As the gembio is conjugated to microbubbles through avidin linkage, we expected gembioMB would protect gembio from rapid

deactivation by CDA *in vivo*. However, in our *in vivo* data, in contrast to gemlipoMB we were unable to detect gemcitabine in the serum six hours after administration (Figure 4c).

For gembioMB+US, we found that ultrasound exposure increased expression of human cleaved caspase 3 in the bladder tumors, which is indictive of enhanced gemcitabine delivery (Figure 7e). This suggests that some of the gemcitabine might have been converted into further cytotoxic metabolites by the time of tissue collection. Unfortunately, we were not able to obtain information regarding other gemcitabine metabolites in our pharmacokinetic analyses using LCMS. Intracellularly, gemcitabine can also be converted into dFdCMP, dFdCDP, and dFdCTP, and the latter two metabolites contribute to gemcitabine cytotoxicity (Kroep, et al. 2002). Previous studies using patient-derived peripheral blood mononuclear cells showed that the intracellular concentration of gemcitabine metabolites increased during drug infusion but still reached high levels within the first two hours (Derissen, et al. 2018). However, no information has been reported for bladder tumors. As caspase 3 is an important protein in cellular apoptosis, has been shown to predict response to chemotherapy (Liu, et al. 2018) and is required for gemcitabine-induced apoptosis on multiple myeloma cells (Nabhan, et al. 2002) and pancreatic cancer cells (Chandler, et al. 2004), our finding of increased human cleaved caspase 3 expression implies that gembioMB with ultrasound exposure may improve bladder tumor cell death and hence tumor control.

Characterising ultrasound-mediated drug delivery using drug surrogates

As gemcitabine is hard to detect visually *in vitro*, fluorescent drug surrogates were used to measure the efficiency of ultrasound-mediated drug delivery. DiO labelled liposomes and

biotinylated FITC were used as surrogates for gemlipo and gembio in an *in vitro* acoustic setup. In both cases, we found conjugating the surrogate drug to microbubbles significantly increased delivery compared with co-administration, as measured by the percentage of fluorescent labeled cells by flow cytometry (Figure 5a & 5b).

A limitation of the *in vitro* acoustic setup is the absence of flow, without which microbubbles will not encounter the same kind of shear stress as in the blood vessels and the drug will not be cleared by the circulation. Liposomes in this system may have a high chance of fusing with the cell membrane and hence release drugs into cells, which could account for the high uptake signal seen when incubating cells with liposomes or liposome-carried microbubbles without ultrasound exposure (Figure 5c).

For the *in vivo* study, fluorescent microbeads were used as a surrogate for gemlipo. Such beads are commonly used to simulate and observe ultrasound-mediated delivery (Mannaris, et al. 2019) and the density of the microbeads (1.05 g/mL) is very close to that of liposomes (~1 g/mL). The microbeads were also conjugated to microbubbles via biotin-avidin linkage before administration *in vivo*. We observed a significant enhancement of fluorescent microbead delivery in the tumor following ultrasound exposure (Figure 6e & 6f). However, as noted above, increased drug uptake was not observed when gemlipo were used. This may call into question the relevance of polymeric beads as drug surrogates for *in vivo* studies and further research is needed.

Limitations and clinical relevance

As mentioned in the introduction, improving the delivery of chemotherapeutics such as gemcitabine is highly desirable for reducing off-target toxicity in combination therapies. In a parallel study, we investigated the response of the same orthotopic MIBC model to radiotherapy following exposure to either Gem+MB or GembioMB and ultrasound (Ruan 2021). Tumor volume was measured over a period of 7 weeks post treatment and acute tissue toxicity was assessed using an intestinal crypt assay in mice culled 3.75 days post treatment. There was no statistically significant difference in tumor growth delay between the positive control treatment (conventional chemoradiation regime) and either of the microbubble-based treatments. However, in contrast to conventional chemoradiation, neither of the microbubble-treatments resulted in acute intestinal toxicity. Consistent with the results of the present study, GembioMB produced a greater therapeutic effect than Gem+MB. Whilst the results of both studies are very encouraging for the clinical application of microbubbles and ultrasound for drug delivery, there are multiple areas in which further investigation is needed.

We found that gem+MB+US produced a higher gemcitabine uptake in the bladder compared to gemlipoMB+US and gembioMB+US *in vivo* (Figure 7c). It is possible that bioconjugation of gemcitabine to microbubbles through liposomes or avidin-biotin linkage may change the biodistribution of gemcitabine. Previous studies have shown that microbubbles primarily accumulate in lung and liver as early as 10 min post injection (Barrefelt, et al. 2013), while the accumulation of liposomes occurs primarily in the liver, kidney and spleen, within 1 hour (Emerson, et al. 2000). Thus, further analysis from those tissues would be required to obtain the biodistribution of gemlipoMB and gembioMB. We also found a high concentration of gemcitabine was still present in plasma from gemlipoMB+US group, indicating that further

optimization of the ultrasound treatment procedure (e.g., prolonged treatment time) could be exploited to increase the gemcitabine uptake with this approach (Figure 7d).

Ultrasound exposure only caused a slight increase in gemcitabine concentration in the bladder as detected by LCMS in all three approaches, and the effect was not statistically significant (Figure 7c). Although a few previous studies have shown improved drug uptake in tumors using LCMS (Yan, et al. 2013, Huang, et al. 2018), the drugs in these studies, such as doxorubicin or paclitaxel, are relatively stable *in vivo* compared to gemcitabine and cellular uptake is mediated by passive diffusion. On the other hand, a recent study on pancreatic cancer cell lines showed that ultrasound-mediated drug delivery could only lead to moderate (non-significant) increases in gemcitabine uptake as the status of nucleoside transporters in the cells are the major determinants of gemcitabine uptake (Bjånes, et al. 2020). As the study also showed that significant increases in gemcitabine can be found when the nucleoside transporters are inhibited, it is possible the sensitivity of our study could be improved by using tumors from hENT1 knockdown cell lines. Similarly, it may be desirable in future work to analyze the excised tissue before homogenization to confirm the ratio of tumorous to healthy bladder tissue and if possible, remove the latter.

Whilst only gembioMB+US produced a significant increase in tumor apoptosis six hours post treatment (Figure 7e), it should be noted that one sample from gemlipoMB+US also showed strong cleaved caspase 3 expression. A potential explanation is the variability of the tumor microenvironment in the *in vivo* models. Tumors with leakier or denser vessels may

respond better to ultrasound-mediated drug delivery as more microbubbles can accumulate in tumor and trigger much stronger tumor effect after ultrasound exposure.

A potential limitation of gembioMB for clinical translation is the immunogenicity of avidin, which could pose a safety concern. However, several recent studies have, shown the feasibility and safety of using avidin-biotin systems clinically (De Santis, et al. 2003, Paganelli, et al. 2007, Veyrat-Follet, et al. 2009, Petronzelli, et al. 2010). Alternatively, use of other non-avidin-based linkages can also be considered. For example, Geer *et al.* conjugated doxorubicin-loaded liposomes onto microbubbles by a maleimide-thiol system (Geers, et al. 2011). Use of non-avidin-based approaches has also been used to increase the loading capacity of microbubbles (Kitano, et al. 1991). It has previously been reported that the use of neutravidin, a non-glycosylated avidin with lower isoelectric point, increased liposomal conjugation to microbubbles more than 3-fold (Kheirolomoom, et al. 2007). Additional studies are needed to explore whether these conjugation strategies can also improve the conjugation and the efficacy of ultrasound-mediated drug delivery.

Conclusions

In this study we investigated three approaches for improving gemcitabine delivery using ultrasound and microbubble-mediated drug delivery. In the first approach, we investigated whether ultrasound exposure following co-administration of microbubbles and gemcitabine could improve the uptake of gemcitabine. In the second approach, we encapsulated gemcitabine within liposomes and then conjugated the liposomes to microbubbles using an avidin-biotin

linkage. In the third approach, gemcitabine was biotinylated and then conjugated to the microbubble surface again using an avidin-biotin linkage. We found that co-injection of microbubbles and gemcitabine did not produce a statistically significant increase in drug uptake (as measured by LCMS) or therapeutic effect (as measured by cell apoptosis) either *in vitro* or *in vivo* following ultrasound exposure compared with the drug-only control. The microbubble-liposome conjugates increased the plasma half-life of gemcitabine and drug uptake *in vitro* but did not increase either drug uptake or cell apoptosis *in vivo*. Interestingly, this was despite an increase in fluorescence intensity being observed when a fluorescent particle was used as a drug surrogate. The ultrasound mediated delivery of the direct microbubble-drug conjugates, however, did produce an increase in tumor cell apoptosis six hours post-treatment in the *in vivo* model. The results confirm that microbubbles and ultrasound provide a means of enhancing drug delivery to tumors but indicate that the design of the microbubble-drug conjugate should be modified according to the drug or drugs being delivered. Fluorescent drug/carrier surrogates should be used with caution as their distribution may not be reflective of that of the drug/carrier even when matched in terms of physical characteristics. With drugs such as gemcitabine that are rapidly degraded, tissue analysis should focus on the relevant metabolites rather than the drug itself to quantify uptake.

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Figure Captions

Figure 1. Schematics of the three gemcitabine delivery approaches investigated in the study.

Figure 2. Characterization of liposomes and microbubbles. a) Representative image from dynamic light scattering measurements of the size distribution of liposomes after extrusion. b) Electron micrograph of gemcitabine loaded liposomes. c) Representative size distribution of microbubbles as measured by optical microscopy (the white columns indicate microbubbles with diameters < 0.5 μm i.e. smaller than the optical resolution limit). d) Representative phase contrast image of microbubbles. e) Representative size distribution of liposome-conjugated microbubbles. Conjugation of DPPC-based liposomes increased the size of microbubbles from 1.37 ± 0.88 μm as shown in (c) to 2.83 ± 1.51 μm. f) Transmission electron micrograph of liposome-conjugated microbubbles.

Figure 3. Relative cytoxicities of the delivery vehicles. a) Cytotoxicity of microbubbles aloen. RT112 cells were incubated with microbubbles (conc = 1.11×10^8/mL) for 24 hours and survival was estimated by clonogenic assay. No cytotoxicity was observed (n = 3). b) Dose-survival curves for cells treated with gem, gemlipo, gembio, and equivalent blank liposome (n=3 for gem, gemlipo, and blank lipo, n=6 for gembio). c) Inhibition of hENT1 by NBMPR diminished the cytotoxicity of gem and gembio but not gemlipo. RT112 cells were treated with 50 nM gemcitabine, gembio, or gemlipo (containing 50 nM gemcitabine) for 24 hours and survival was estimated by a clonogenic assay (n=3 for each group, *p < 0.05, **p < 0.01).

Figure 4. Stability of gemcitabine in the delivery vehicles. a) Gem+MB, gemlipoMB, and gembioMB were incubated with CDA at 37^{o}C. The representative time course profile of gemcitabine showed gemlipoMB were able to retain gemcitabine and protect it from deactivation while gembioMB produced different enzymatic kinetics compared to gem. b) Stability of the gembio in murine plasma with or without avidin under different incubation conditions (n = 2 for each group). The binding of gembio to avidin inhibited the inactivation of gembio into biotinylated dFdU. c) Time course analysis of gemcitabine concentration in mouse plasma after administration of gem+MB, gemlipoMB, and gembioMB (n = 2 for each group).

Figure 5. *In vitro* demonstration of ultrasound-mediated gemcitabine delivery using drug surrogates on RT112 cells. The uptake of the drug surrogates by cells was analyzed by flow cytometry. a) DiO-labelled liposomes (lipo) were used as gemcitabine-encapsulated liposome surrogates. Conjugating of liposomes to microbubbles increased liposomes uptake as shown by a higher DiO positive cell population (***$p < 0.001$, n = 3 for each group). b) Biotinylated FITC (FITCbio) was used as biotinylated gemcitabine surrogate. Conjugating FITCbio to microbubbles improved the FITCbio uptake by cells as shown by the higher FITC positive cell population (**$p < 0.01$, n = 3 for FITCbio and FITCbio+MB+US, n=4 for FITCbioMB+US). c) Ultrasound exposure improved the uptake of drug surrogates (*$p<0.05$, n=4 for FITCbioMB and FITCbioMB+US, n=3 for all other groups).

Figure 6. Ultrasound-mediated drug delivery *in vivo*. a) B-mode and contrast images of an orthotopic bladder tumor after microbubble injection. The tumor area is circled in green. b)

Representative destruction-reperfusion plot using high intensity ultrasound pulses (indicated by red vertical lines) during contrast imaging. The perfusion curve from the tumor region (as highlighted in green from panel a) shows the signal reached the peak within 3.2 seconds. Once the high intensity pulse was applied, microbubbles were destroyed, and it took 0.6~0.8 seconds for reperfusion to occur. c) B-mode and passive acoustic mapping overlay. The color bar shows the source power signal from the bladder area normalised to its maximum value. The solid red line indicates the tumor outline as determined from the B-mode images. d) Image of mouse indicating minimal muscle damage from the current ultrasound treatment regimen. The area exposed to the ultrasound beam is indicated by black arrows. e) Fluorescence signals from microbeads after ultrasound exposure - in the orthotopic model. Two tumor sections were obtained from either the control or the ultrasound-treated group and the fluorescence signal of the microbeads were quantified. (**$p < 0.01$). f) Representative fluorescence images from control and ultrasound-treated tissue sections. Cell nuclei were stained with Hoechst 33342 (grey) and microbeads are showed in red. The region of intense Hoechst 33342 staining corresponds to the tumor which occupies most of the bladder. The scale bar represents 800 μm.

Figure 7. *In vivo* ultrasound mediated gemcitabine delivery.

a) Example of the passive acoustic mapping source power signal measured during ultrasound treatment. The timing of the signal level spikes corresponds to the injection of the drug-microbubble solution after every 40 bursts. b) Passive acoustic mapping source power for each approach (n=12 for gem+MB+US, n=11 for gemlipoMB+US, n=14 for gembioMB+US). c) LCMS

915 analysis of murine bladder subjected to ultrasound and gem+MB, gemlipoMB, and gembioMB
916 treatment. For each approach, ultrasound exposure did not significantly increase gemcitabine
917 uptake in the bladder. d) LCMS analysis of murine plasma from c) (n = 5 for gem and gem+MB, n
918 = 7 for gemlipoMB, n = 8 for gemlipoMB+US and gembioMB, n = 11 for gembioMB+US. *p <
919 0.05, ** p < 0.01, *** p < 0.01). e) ELISA quantification of human cleaved caspase 3
920 demonstrated that tumors exposed to ultrasound following gembioMB administration had
921 increased cleaved caspase 3 expression, indicative of enhanced gemcitabine delivery (n = 4 for
922 gembioMB+US, n=3 for the rest. *p < 0.05).

923 **Table 1.** Comparison of the three approaches from this study

Type	**Cell survival from clonogenic assays**	**Stability under CDA and plasma**	**Drug uptake by US (drug surrogates)**	***In vivo* UMD: Gemcitabine uptake by LCMS**	***In vivo* UMD: Tumor apoptosis by cleaved caspase ELISA**
gem+MB	IC_{10} /IC_{50} = 13.84 ± 3.04 nM/61.4 ± 10.77 nM	No gem detectable after 10 min incubation in CDA and 6 hours in plasma	No effect in enhancing the uptake *in vitro* (FITCbio+MB)	Produced the highest gemcitabine uptake of the three approaches but US had no statistically significant effect	No enhancement after US
gemlipoMB	IC_{10} /IC_{50} = 6.64 ± 3.98 nM/31.10 ± 1.07 nM Shown to overcome the hENT1 deficiency-based resistance	25% gem still detectable after 10 min in CDA and 24 hours in plasma	Showed ~1.5 fold increased uptake *in vitro* (DiO-labeled liposomes, lipoMB) Showed 1.7-fold increased uptake *in vivo* (fluorescent microbeads)	No enhancement after US	No enhancement after US
gembioMB	IC_{10} /IC_{50} = 20.35 ± 11.04 nM/55.20 ± 8.53 nM	Around 75% gembio detectable after 10 min in CDA but no gem detectable 6 hours in plasma	Showed ~7.5-fold increased uptake *in vitro* (FITCbioMB)	No enhancement after US	Enhanced cleaved caspase 3 expression after US

924 CDA: cytidine deaminase; UMD: ultrasound-mediated delivery; US: ultrasound exposure