Supplementary Materials

MR-labelled liposomes and focused ultrasound for spatiotemporally controlled drug release in triple negative breast cancers

Maral Amrahli, Miguel Centelles, Paul Cressey, Martynas Prusevicius, Wladyslaw Gedroyc, Xiao Yun Xu, Po-Wah So, Michael Wright, and Maya Thanou

Scheme S1. Synthesis of Gd.DOTA.DSA. Reagents and conditions: (i) dioctadecylamine, HBTU, DMAP, OH.Gly.NH.Boc, dry CHCl₃, RT; 1 = 90 %; (ii) TFA: DCM (3:7), RT; 2 = 95 %; (iii) NHS-DOTA, TEA, dry CH₂Cl₂, 35 °C; 3 = 76 %; (iv) GdCl₃·6H₂O, H₂O, slow reflux; 4 = 82 %. 2. DSA. ¹H (400 MHz; CDCl₃; 296 K) δ 3.84 (s, 2H, OCC₂H₂NH₂), 3.29 (t, J = 8.0 Hz, 2H, OCNCH₂), 3.11 (t, J = 7.8 Hz, 2H, OCNCH₂), 1.50 (m, 4H, OCNCH₂C₂H₂), 1.25 (s, 60H, alky chain CH₂), 0.88 (t, J = 6.3 Hz, 6H, CH₃); ¹³C (100 MHz; CD₂Cl₂; 296 K) δ 166.6 (OCN), 48.8 & 48.1 (OCN C₂H₂), 41.6 (OCNCH₂NH₂), 41.1 (CH₃C₂H₅CH₂), 30.9-30.8 (alkyl chain CH₂), 29.9 (OCNCH₂C₂H₅CH₂CH₂), 28.8-28.3 (OCNCH₂C₂H₅CH₂), 24.2 (CH₂C₂H₅), 15.4 (CH₃). TLC (15% MeOH in CH₂Cl₂ with 0.5 % NH₃) gave Rf 0.55 with the DSA spot showing red after sequential vanillin and ninhydrin stains. ESI-MS calcd. for C₃₈H₇₈N₂O [M+H]⁺: 579.8 a.m.u. Found [M+H]⁺ 579.7 a.m.u. 3. DOTA.DSA. ¹H (400 MHz; CDCl₃/CD₂OD) δ 3.45 (br, 2H, NCCH₂CONH), 3.10 (br, 6H, NCH₂CONH), 3.00 (br, 2H, OCNCH₂), 2.80 (br, 16H, NCH₂CONH), 2.28 (br, 2H, OCNCH₂), 2.16 (br, 2H, OCNCH₂NH), 1.44 (m, 4H, OCNCH₂CH₂), 1.18 (s, 60H, alky chain CH₃), 0.80 (t, J = 6.6 Hz, 6H, CH₃). ¹³C (100 MHz; CD₂Cl₂/CD₂OD; 296 K) δ 47.0 (OCNCH₂), 41.5-38.5 (NCH₂NH₂ & NCH₂COOH), 32.3 (CH₂C₂H₅CH₂), 31.5-28.5 (alkyl chain CH₂), 22.9 (CH₃CH₂), 14.1 (CH₃); others could not be distinguished. ESI-MS calcd. for C₅₄H₁₀₄N₆O₈ [M+H]⁺: 965.8 a.m.u. Found [M+H]⁺ 965.7 a.m.u with major fragments seen at 579.6, 522.3, 444.1 and 387.1 a.m.u. corresponding to 2, (C₃₈)₂NH, DOTA-glycine and DOTA respectively. 4. Gd.DOTA.DSA. The presence of Gd disrupted the ¹H and ¹³C NMR spectra. ESI-MS calcd. for C₅₄H₁₀₄N₆GdO₈ [M+H]⁺: 1121.7 a.m.u. Found [M+H]⁺ 1120.6 a.m.u. with major fragments seen at 1076.5, 1032.5, 1005.7, and 988.8 a.m.u. (all + p ESI) corresponding to loss of COO, 2x COO, 2x CH₂COO, and 3x COO respectively.
Figure S1. Assessing doxorubicin release under FUS, measured by intrinsic doxorubicin fluorescence; (A) schematic showing a polyacrylamide gel embedded flow-tube, light source and camera, around a FUS system; 1. camera, lens, and filter; 2. transducer; 3. gel block; 4. flow tube; 5. focus; 6. acoustic foam; 7. water bath; 8. LED lights; (B) photographs of the setup around the transducer; (C) close up of the gel block and flow tube; (D) view showing the flow-tube and an indication of the FUS focus with the fine-wire thermocouple visible in reflection. Pulsed FUS insonation of a flowing iTSL stream then causes synchronised fluorescence intensity increases, indicating boluses of released doxorubicin; (E) Three representative frames showing (left to right) FUS-off, start of FUS and fluorescence increase, and FUS-off again and wash out of the release doxorubicin bolus.
Figure S2. Supporting data for the assessment of FUS-induced doxorubicin release: (A, B) this prototype used a 3.4 MHz FUS transducer (Precision Acoustics, UK) focused on iTSL-DOX / agar phantoms embedded in a larger polyacrylamide gel block. Low gel-point agar allowed the iTSL-DOX to be immobilised without heating above the T_m, while the polyacrylamide allow for placement and retention without interfering with FUS transmission. Focus temperature was measured with a fine-wire thermocouple and doxorubicin release by intrinsic fluorescence using a monochromatic LED (460 nm) source, combined with a domestic camera with glass photographic (‘G’) filters and video collection settings. This approach was sufficient for a proof-of-principle but had limitations of poor spectral specificity and low frame rates (6-8 fps). Example frames showing (C) embedded iTSL-DOX/agar as a pale pink cylinder under white light. The encasing polyacrylamide is optically clear, while the supporting acrylic cylinder can be seen, along with the transducer face on the right; (D) Under blue light but no FUS the encapsulated doxorubicin shows deep-red fluorescence; (E) This significantly increases in brightness once the FUS is turned on (160 mV input to amplifier; 100 % duty cycle). This change is irreversible and demonstrates the fluorescence de-quenching seen from iTSL-released doxorubicin.

See also: http://youtu.be/N6N6GgY49CA
Figure S3. Preclinical FUS studies; Study outline - once tumours were ~ 5 mm each animal received: (i) a leading FUS treatment (42-43 °C; 3 min); (ii) injection of iTSL-DOX at t = 0; (iii) a second FUS treatment (42 °C for 3 min) applied once imaging observed iTSL-DOX had accumulated in the tumour; (iv) monitoring by whole body NIRF, tumour sizing, and weight measurement until the end of the study.
Figure S4. Effect of serum on doxorubicin release from iTSL-DOX samples incubated at 37 °C for up to 60 min in buffer (50 mM HEPES with 5 w% glucose; pH 7.4), compared with buffer containing 50 v% foetal bovine serum (FBS) as a blood analogue. Release was monitored by the increase of intrinsic doxorubicin fluorescence (Ex$_{480}$/ Em$_{590}$ nm) as it leaves the self-quenched encapsulated state. N = 3, values are mean ± SD.
Figure S5. Storage stability of iTSL-DOX; Doxorubicin release was studied by incubating samples for 3 min at 32-46 °C. The graphs show %release for stocks either left at (A) room temperature for 10 min, 3 h, or 24 h (n = 3; mean ± SD) or in; (B) cold storage at ~5 °C (stacked curves; n = 3; mean ± SD). Little or no change is seen in the thermal release profiles as the liposomes ages; (C) representative average particle diameter and PDI data also shows no significant changes on storage for 2 months.
Figure S6. Gadolinium leakage analysis using dialysis membranes and total reflection X-ray fluorescence (TXRF). The potential for loss of the metal from Gd.DOTA.DSA was established by assaying the amount of Gd$^{3+}$ able to escape through a dialysis membrane from an inner chamber containing either 0.2 mg/mL gadolinium standard or iTSL (equivalent to 0.38 mg/mL Gd) and into a cuvette containing reverse osmosis (RO) grade water at RT or 50 % (v/v) fetal bovine serum at 4 °C (to avoid serum degradation). The cuvettes were placed on a magnetic stirrer and 10 μL samples were taken at 1-48 h time points. These were analysed to determine the concentration of gadolinium (n = 3; mean ± SD). A scaled baseline is also given for n = 11 samples of RO water.
Figure S7. Collated pixel intensities from matched ROIs in all $T_1$ map slices underwent frequency distribution analysis in Prism (Graphpad Software, San Diego CA, USA) with bin-width 50 over 800-3000 units. The resulting histograms were then non-linear regression fit to Gaussian curves and the resulting best-fit value means and S.D.s (equivalent to the distribution breadth) cross-compared for each animal ($n = 3$), time-point, and ROI. Significance markers refer to ANOVA 1-way analyses on the collated raw data using default settings: *** $P < 0.0002$, **** $P < 0.0001$. Little or no difference is seen from neither the Gadovist reference nor the muscle tissue controls. Significant mean reduction is seen in the majority of tumours immediately post-injection. There is often an increase in the distribution SD, signifying significant heterogeneity. This likely relates to the increased tumour vascularity and/or the presence of a low-infusion core.
Figure S8. Double-tumour mouse studies with only the right-side tumour treated by FUS. The groups were: nil-drug ± FUS (n = 3) and iTSL-DOX ± FUS (n = 10); (A) average tumour sizes (mean ± SEM). Mice were injected (i. v. tail) to 6 mg/kg equivalent doxorubicin on day 0 and FUS treatment was applied pre/post injection; (B) average body weights and; (C) Kaplan-Meier plots showing survival. Weights are given as mean ± SEM.

For these double-tumour studies, mouse survival is limited by the growth of the non-FUS tumour, which receives only a reduced dosage of iTSL-DOX. The approach allows for more direct comparison of the effects of FUS across the two tumours of the same animal but reduces overall survival improvements compared to the single-tumour studies.
| Formulation                        | $T_{on}$ | $T_m$ | $T_{cl}$ |
|-----------------------------------|----------|-------|---------|
| 100 mol% DPPC                     | 39.3     | 41.7  | 42.8    |
| 30 mol% Gd.DOTA.DSA, 70 mol% DPPC | 39.4     | 41.6  | 42.4    |
| Reference LTSL                    | 40.2     | 42.1  | 43.7    |
| iTSL                              | 41.2     | 43.3  | 45.8    |

**Table S1.** Measurements carried out on a TA Instruments Nano DSC in HEPES/glucose buffer, against a reference of the same. Values are indicative examples with estimated error $\pm 0.2$ °C. $T_{on/\text{cl}}$ are calculated as the first and last temperatures at which the thermal power is 5% of $T_m$.