Supplementary Material

METHODS

Materials

CA4, CA4P, irinotecan, tripropionin, tetradecafluorohexane (C_6F_{12}), bis-Benzimide-H 33342 trihydrochloride (Hoechst 33342), dimethyl sulfoxide (DMSO) and ethyl-^{10}-hydroxycamptothecin (SN38), were all purchased from Sigma (Sigma-Aldrich, UK). 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin (SN38-G) was from Santa Cruz Biotechnology Inc (USA). Lipids 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N[biotinyl(polyethyleneglycol-2000] (DSPE-BPEG$_{2000}$), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were all purchased from Avanti® Polar Lipids Inc. (Alabaster, Alabama, USA). CA4 for the multiple treatment schedule was dissolved in DMSO then diluted in peanut oil. The DMSO content was 10% (v/v) and the final concentration of CA4 was 0.72 mg/mL or 25 mg/mL. CA4P was dissolved in 0.9% saline at 15mg/mL. Irinotecan was dissolved in DMSO then diluted in PBS. The DMSO content was 1.5% (v/v) and the final concentration of irinotecan was 3mg/mL.

Cell lines

Human colorectal adenocarcinoma cells (SW480) were grown in RPMI-1640 with 10% (v/v) foetal calf serum (Sigma-Aldrich, UK). These were maintained at 37ºC in 5% CO_2. The cells were authenticated by single tandem repeat (STR) profiling and screened negative for mycoplasma.

CA4 LOND production and characterisation

The lipid monolayer shell of the LONDs was composed of 75:20:5 mol% DSPC, cholesterol and DSPE-BPEG$_{2000}$. The lipids were dried under nitrogen for 30 min. Following this, the lipids were resuspended in 0.7 mL of tripropionin with CA4 by vortexing. Briefly the first homogenisation step began with blending using the rotor-stator system Polytron PT13000 D (Kinematica AG, Switzerland).

This was performed at 12,500rpm for 10 min at 40ºC and atmospheric pressure. The second homogenisation step was performed in a high-pressure homogeniser Emulsiflex-C5 (Avestin Europe GmbH, Germany) for 20 min at 175 MPa. The diameters of the LONDs were measured by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZSP (Malvern Instruments, UK) and a NanoSight (Malvern Instruments, UK). The NanoSight was also used to determine the concentration of the LONDs in the sample.

CA4 LONDs-MBs microfluidic production and characterisation

CA4 LOND-MBs were produced in a microfluidic device using a two-step process in a microspray regime [30]. The microchip was fabricated by Epigem Ltd (Redcar, UK) in poly(methyl methacrylate) (PMMA) and SU-8. The design of the microchip had a flow-focusing region for MB production using a microspray regime, followed by a serpentine to allow for slower mixing of MBs with neutravidin functionalised CA4 LONDs (approximately 1.14 s [31]) **(Supplementary Figure S1)**. The microchip had an inlet channel for the gas phase and two inlet channels for the introduction of the aqueous phase. Prior to CA4 LONDs-MBs preparation, CA4 LONDs were incubated with 3 μM of neutravidin for approximately 15 min (Thermo Scientific, UK) to allow for neutravidin functionalisation. The lipid preparation for the MBs consisted of 1mg/mL of lipids: 95:5mol% 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and DSPE-BPEG$_{2000,}$ which were mixed and dried under nitrogen for 1 h. A film of lipids was formed around the walls of the vial. The dried lipid film was resuspended in 1mL of PBS by vortexing and placed in an unheated ultrasonic water bath for 1 h. Following this, 10μl/mL of C_6F_{14} (Sigma-Aldrich, UK) was added to the lipid solution to increase MB lifetime [62]. The lipid solution was then introduced into the microchip through the aqueous phase inlet at 20μl/min and the gas perfluorobutane (C_4F_{10}) was flowed through the central inlet at 15 psi to form the core of the MBs. The neutravidin functionalised CA4 LONDs were incorporated into the chip further downstream at a concentration of 10^{11} LONDs/mL at 20μl/min, mixing together with the MB solution through the serpentine and achieving LONDs-MB binding via the biotin-neutravidin link. MB-LONDs were characterised in terms of size and concentration. Based on this data, neutravidin VEGFR2 antibody

(eBiosciences, UK) was added to the MBs at 0.1µg per 10^7 MBs. This was incubated for 20 min prior to use *in vivo*.

Mouse models

5×10^6 SW480 cells were subcutaneously injected into the right hind flank of 5-6 week old male BALB/c and CD-1® nude mice to form xenografts.

Tumour volume measurements with high-frequency ultrasound imaging (HFUS) and mechanical callipers

Tumour xenografts were imaged using a VisualSonics Vevo 770 high-frequency ultrasound system (Fujifilm VisualSonics Inc., Ontario, Canada) equipped with 40 MHz (RM-704) and 25 MHz (RM-710B) transducers as previously described [32,33]. 3D images of tumours were reconstructed and tumour volumes were determined as previously described [32,33,63].

Tumours were also measured with mechanical callipers twice weekly once the tumour became palpable (approximately 7-10 days following injection). Tumour volumes were calculated as follows:

$$Tumour\ volume = (length\ \times\ width)^{\frac{3}{2}} \times \frac{\pi}{6}$$

For tumour growth rate, ratio to day 0 was calculated by diving each tumour volume by the starting volume on day 0.

Determination of vascular perfusion using the perfusion marker Hoechst 33342

Hoechst 33342 was freshly prepared in sterile water at 4.5 mg/mL and administered via tail vein injection at 15 mg/kg 1 minute prior to sacrifice. Tumours were embedded in OCT compound, frozen on dry ice and transferred to liquid nitrogen. Ten-micrometre tumour cryosections were fixed in 70% ethanol (v/v) and subsequently immunostained with a rat anti-mouse antibody against CD31 (BD Biosciences, UK) at 1:100 (v/v) in antibody diluent (Thermo Fisher Scientific, UK). After washing, a

goat anti-rat IgG secondary antibody with Alexa Fluor 568 (Thermo Fisher Scientific, UK) was used for visualisation. Tumour sections were imaged using a Zeiss Axioimager Z1 fluorescence microscope with AxioVision software (Carl Zeiss Microscopy, USA). Perfusion was scored using a semi-quantitative scoring system to define none (score 0), weak (score 1), moderate (score 2) and high (score 3) intensity of Hoechst 33342 fluorescent staining by two independent observers blinded to the treatment groups. Representative images for the perfusion scoring are shown in **Supplementary Figure S2** for each data set. Data set 1 was used to score images represented in Figure 4 and data set 2 was used to score images represented in Figure 5 of the results section.

Tissue processing and Immunohistochemistry

1, 24 and 72 h after the treatments, tumour, liver, kidney, colon, spleen, heart and lungs were harvested from animals in each group. Tissues were cut in half and either fixed in 4% (w/v) paraformaldehyde (PFA) for immunohistochemistry (IHC) or snap-frozen in liquid nitrogen for liquid chromatography-tandem mass spectrometry (LC-MS/MS). PFA fixed tissues were processed using a tissue processor and embedded in paraffin. 5 µm central tumour sections were cut and one section from each tumour was stained with haematoxylin and eosin. Slides were digitally scanned at x 20 magnification using an Aperio digital slide scanner (AT2 Leica Biosystems) with ImageScope software (Leica Biosystems, Germany). ImageScope was used to manually annotate the boundary of the tumour area and the areas with haemorrhage (mm^2) and necrosis (mm^2) per tumour section, in order to calculate %haemorrhage or necrosis/mm^2 of tumour. The number of mitoses was also counted and the number of mitoses/mm^2 was determined.

IHC was performed for the detection of CD31-positive vessels in the absence of Hoechst 33342. Tumour sections were deparaffinised followed by heat-mediated antigen retrieval in citrate buffer, pH 6.0 in a microwave for 10 min. The sections were then blocked for endogenous peroxidase, avidin and biotin (Vectorlabs,, Burlingame, USA). Sections were then incubated with a monoclonal rat anti-mouse CD31 antibody (Dianova, Germany) at 1:20 (v/v) in antibody diluent for 1 h at room temperature. After washing, the sections were incubated with a biotinylated rabbit anti-rat secondary

antibody (DAKO, UK) at 1:200 (v/v) in antibody diluent for 30 min at room temperature. ABC/HRP was then applied for 30 min at room temperature. Blood vessels were visualised by staining with DAB. Images were acquired using a Nikon Eclipse E1000 microscope (Nikon Instruments Inc., Japan). The quantitation of CD31 positive blood vessels or microvessel density (MVD) was performed by identifying areas with the highest CD31 positive MVD ("hot spots") within the entire tumour section. Then, individual CD31 positive vessels were counted from the images using Image J ($1mm^2$ per field using a x10 objective).

Tissue processing for LC-MS/MS

Tissues were weighed and homogenised using a Bead Ruptor 24 Bead Homogenizer (OMNI International Inc.) for homogenisation in MeOH (1:4) (w/v). The instrument settings were: speed 5.6 m/s, number of cycles 2 x 45 s, pause in between cycles 30 s). After homogenisation, the samples were vortexed and centrifuged for 5 min at 10, 000 x g at 4ºC. The supernatant for CA4 detection was dried using a rotary evaporator (EZ2 plus rotary evaporator, Genevac Ltd, Suffolk, UK). The dried samples were reconstituted in MeOH at the original tissue mass. Plasma was mixed in MeOH (1:4), vortexed and centrifuged for 5 min at 10, 000 x g at 4ºC. The clear supernatant was dried as described above. The supernatant for irinotecan, SN38 and SN38G detection was diluted 1:20 in MeOH without drying.

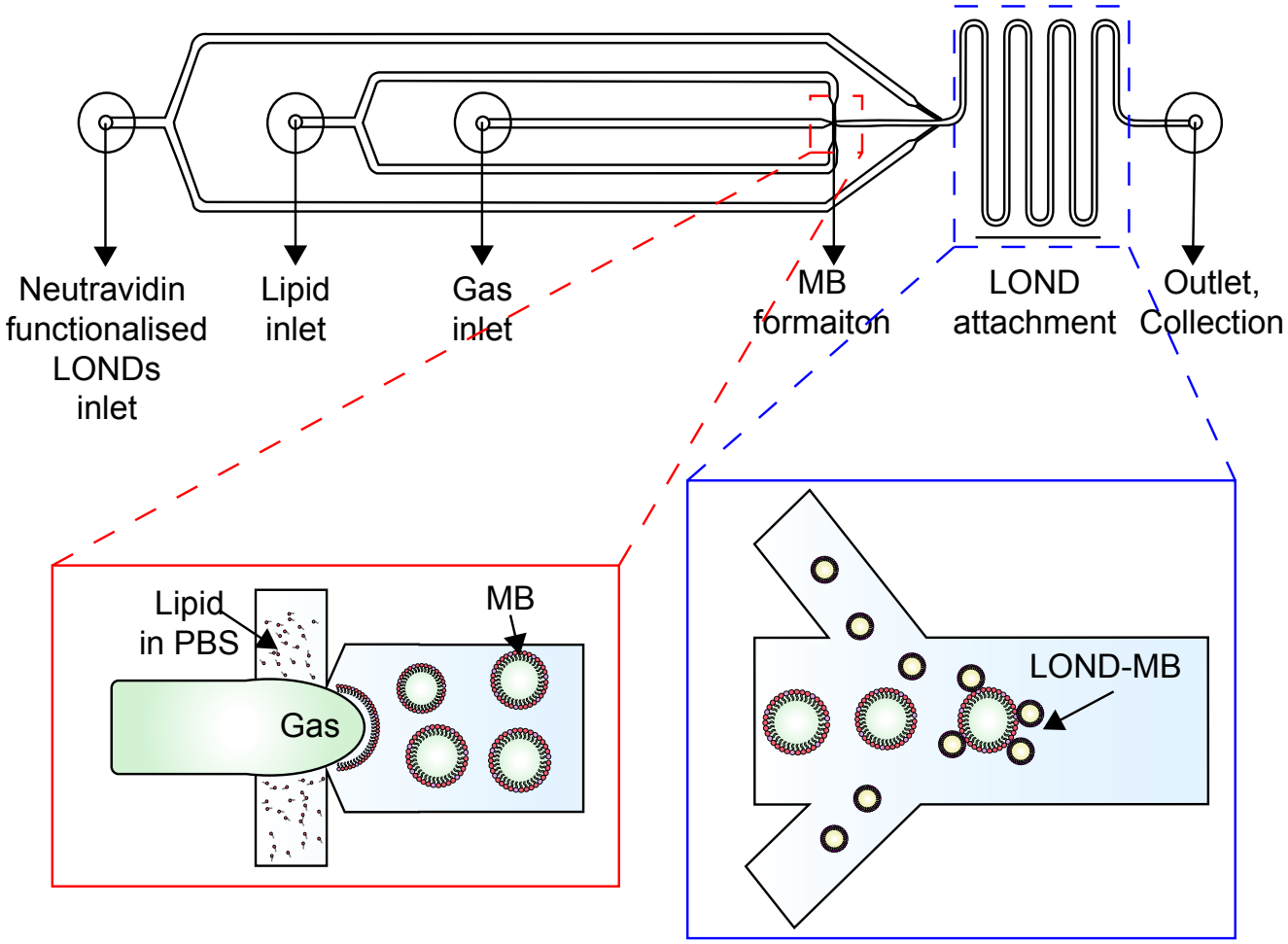
LC-MS/MS for CA4

CA4 was prepared in DMSO at a concentration of 10 µg/mL and a five point calibration curve using serial dilution of 1:1 in methanol (MeOH) was prepared. CA4 LONDs and CA4 LONDs-MBs were diluted accordingly within the range of the calibration curve in MeOH, vortexed and centrifuged at 10,000 x g for 4 min at 4ºC to remove debris and oil. The samples were analysed using a Waters Quattro Ultima triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA) with an electrospray ionisation (ESI) interface, in positive ionisation mode. Separation was obtained using an ACQUITY^{TM} UPLC BEH C18 column (1.7 µm, 2.1 x 100 mm) (Waters Corporation, Milford, MA, USA)

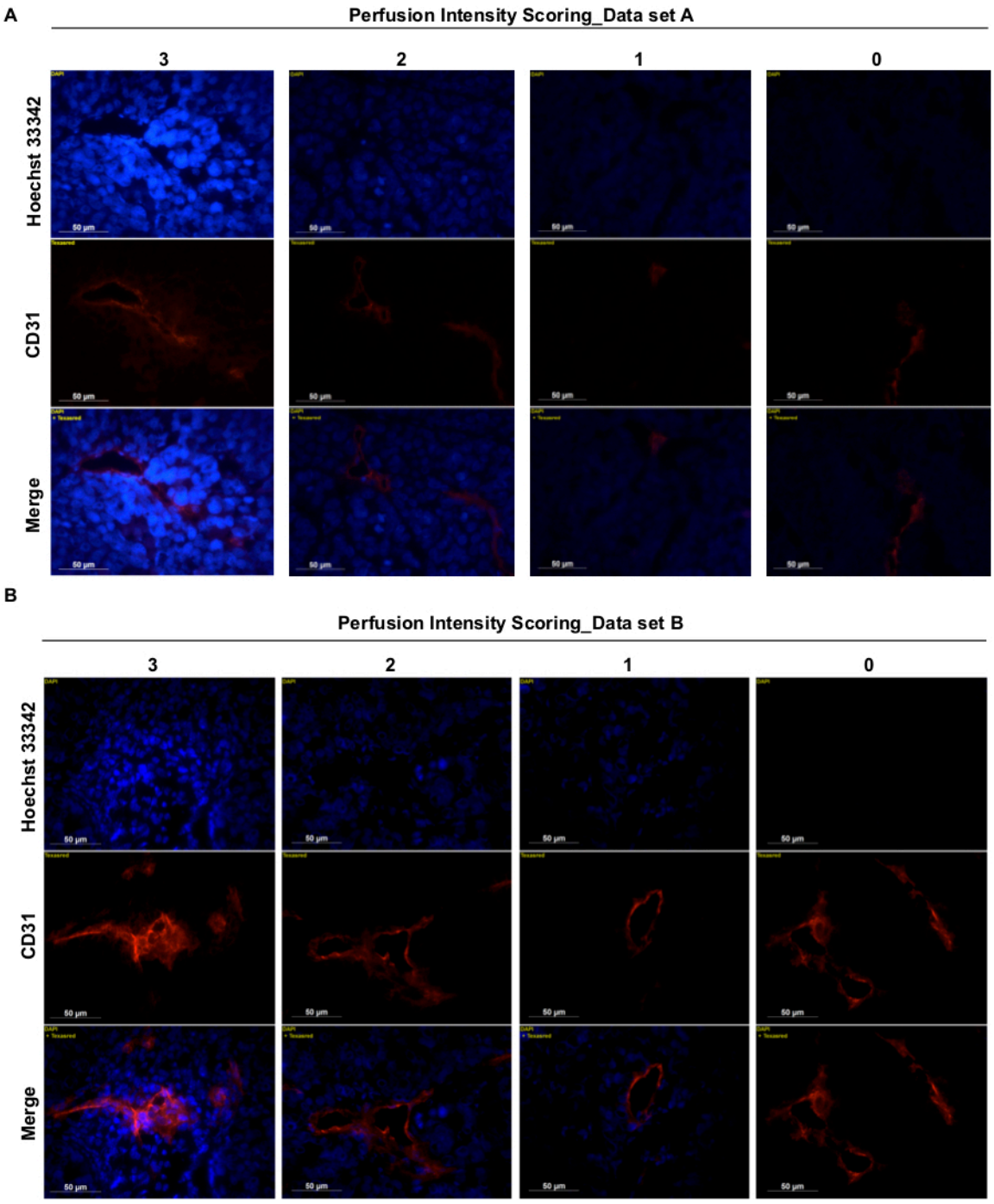
that was maintained at 40ºC. The autosampler was maintained at 8ºC. The following instrument parameters were in place: ion source temperature 120ºC; desolvation temperature 300ºC; Capillary voltage 3kV; Cone energy 20 V; Gas flow desolvation 650 L/hr; Cone 71 L/hr. The samples were eluted using a stepwise gradient in mobile phase A (90:10:0.1% (v/v) MeOH:H_2O:formic acid) and mobile phase B (90:10:0.1% (v/v) H_2O:MeOH:formic acid). The flow rate was 0.25 mL/min and 10 µl was injected for each analysis. The initial gradient of 70% A: 30% B was gradually increased over 18 min to 80% B and 20% A, held for 2 min then returned to the initial gradient over 1 min and held for 4 min, with a total run time of 25 min. The analysis was performed in a multiple reaction monitoring (MRM) mode following optimisation to obtain the product ions with the highest signal. The full MRM settings are shown in **Supplementary Table S1**. Mass Lynx software (Waters Corporation, Milford, MA, USA) was used to calculate the peak area (PA) under the curve and this was compared to a calibration curve to determine the concentration of CA4 in the samples.

Statistics

All statistical analyses were performed using GraphPad Prism version 7 software (GraphPad Software Inc., La Jolla, California, USA). The statistical tests used for each experiment are described in the text and figure legends.



Supplementary Figure S1: LOND-MB production. The illustration shows the microchip design. Liquid is pumped through opposite inlets while the gas is flown through the centre into a flow-focusing region for MB production. Further downstream post-MB production, neutravidin functionalised LONDs are introduced, these go through a serpentine channel for slow mixing of MBs with LONDs. The serpentine channel allows for extra time (approximately 1.14 s) for neutravidin functionalised LONDs to bind onto the surface of the MBs by increasing their contact time before collection through the outlet.

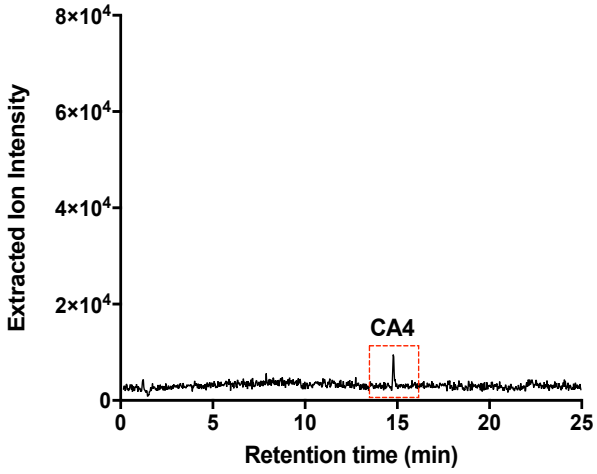


Supplementary Figure S2: Representative images showing perfusion scoring scale used. Blue is DAPI, nuclei while red is CD31 blood vessels. Two blinded reviewers used these representative images to score images from each treatment group and the median score was used.

Compound	Precursor/product ion (m/z)	Dwell (sec)	Cone voltage (V)	Collision energy (eV)
Combretastatin A4	317.5 > 286.2	0.20	15	15
Combretastatin A4	317.5 > 271	0.20	15	15
Combretastatin A4 G	493 > 302.21	0.20	15	25
Combretastatin A4 G	493 > 317.31	0.20	15	25
Irinotecan	587.3 > 124.0	0.15	25	45
Irinotecan	587.3 > 167.0	0.15	25	45
SN38	393.2 > 264.2	0.15	35	30
SN38	393.2 > 293.0	0.15	35	30
SN38	393.2 > 349.1	0.15	35	30
SN38G	569.8 > 393.8	0.20	35	30
SN38G	569.8 > 349.9	0.20	35	30

Supplementary Table S1: Multiple reaction monitoring (MRM) settings for CA4, CA4G, irinotecan, SN38 and SN38G detection.

A



B

Extraction efficiency			
Sample	**CA4 (PA)[a]**	**MeOH (PA)[b]**	**Extraction (%)**
Tumour	30043	30368	98.9
Liver	32438	30294	107.1
Spleen	29029	28805	100.8
Kidney	30928	28818	107.3
Colon	31800	30091	105.7
Plasma	29772	29376	101.3

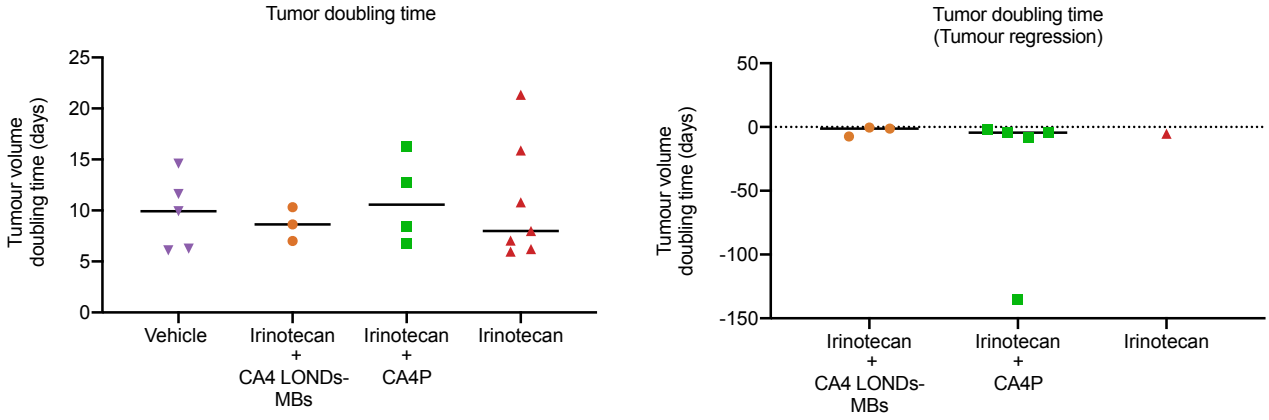
Supplementary Figure S3: (A) CA4 Limit of detection (LOD) at 10ng/mL. Retention time of CA4 at approximately 14.8 min. Total run time 25 min. (B) Tissue homogenates (tumour, liver, spleen, kidney, colon and plasma) were spiked with 1µg/mL of CA4 and compared with 100% methanol spiked in the same way. % extraction efficiency was expressed as the ratio of the peak area (PA) of CA4 spiked into the relevant tissue (a) by the PA of CA4 spiked in methanol (MeOH) (b) (a/b*100). The high extraction efficacy for all homogenates indicates good MeOH extraction of the poorly water soluble CA4 can be obtained.

Mouse no.	Group	Tumour (PA)	Liver (PA)	Plasma (PA)
1	CA4 LONDs	-	-	-
2		-	178	-
3		-	12591	-
1	Free CA4 in	148	4153	435
2	DMSO/peanut oil	-	2079	-
3		103	2140	48

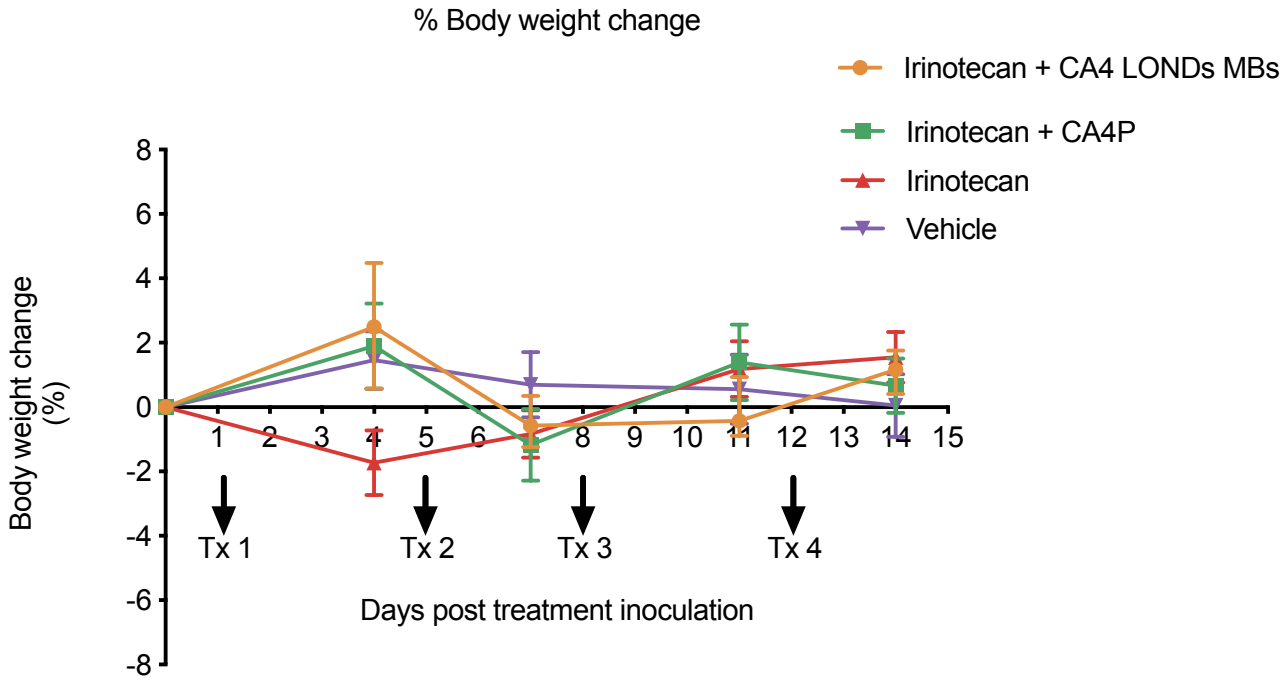
Supplementary Table S2: Peak area (Peak area) of CA4G detected in tissues treated with CA4 LONDs (12.8mg/kg) and free CA4 in DMSO/peanut oil (50mg/kg)

CA4 LONDs-MBs Prep.	CA4 LONDs-MB concentration x10^7/mL	Size µm ± SD	CA4 mg/mL	Dose mg/kg
1	9	4 ± 2.6	0.0002	0.001
2	5	4.4 ± 2.6	0.0001	0.001
3	2	3.7 ± 2.6	0.0001	0.001
4	0.7	4.2 ± 3.1	0.0002	0.001
5	1	3.6 ± 2.5	0.00006	0.0003
Mean	3.5	4	0.0001	0.001
SD	3.5	0.3	0.00006	0.0003

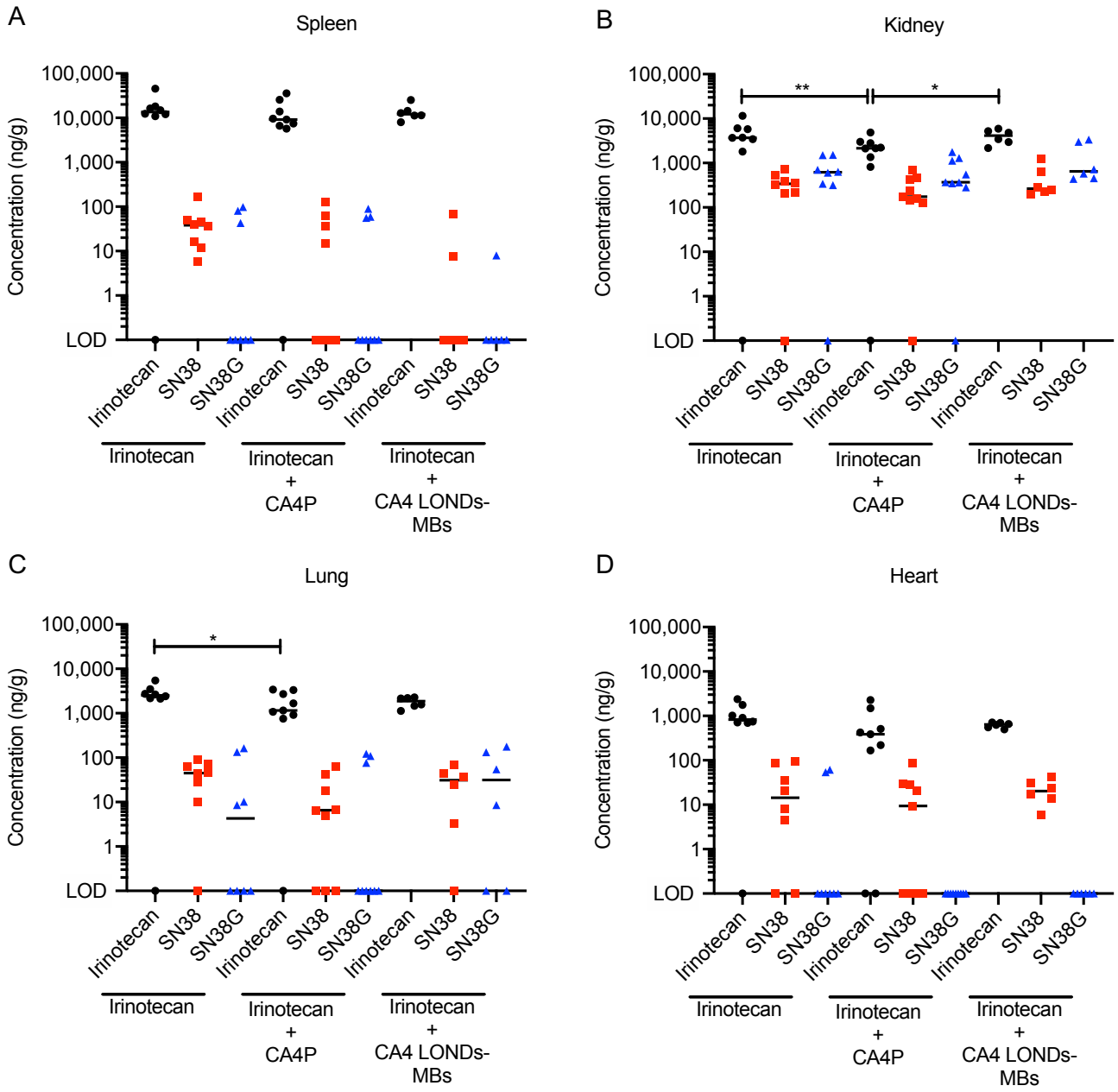
Supplementary Table S3: Characterisation of CA4 LONDs-MBs. A total of five preparations of CA4 LONDs-MBs were prepared for the treatments. The mean ± SD concentration, diameter and CA4 loading measured by LC-MS/MS for the five treatments was $3.5 \times 10^7 \pm 3.5 \times 10^7$ MBs/mL, 4 ± 0.3 µm and 0.0001 ± 0.00006 mg/mL respectively.



Supplementary Figure S4: Tumour doubling time in days, divided in two graphs for simplicity. Negative values indicate tumour regression. Horizontal lines (-) represent the median.



Supplementary Figure S5: Percentage body weight change. Data represent the mean and error bars the SEM for each treatment group. 10% body weight reduction is considered significant and 20% is where the animal would need to be euthanised for health reasons.



Supplementary Figure S6: Drug biodistribution by LC-MS/MS in spleen, kidney, lung and heart following multiple treatments with irinotecan and/or CA4P or CA4 LONDs MBs. (A) Spleen; (B) Kidney, ** p = 0.004, * p = 0.02; (C) Lung, * p = 0.02; (D) Heart.

References

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