A Single Microbubble Formulation Carrying 5-Fluorouridine, Irinotecan and Oxaliplatin To Enable FOLFIRINOX Treatment Of Pancreatic And Colon Cancer Using Ultrasound Targeted Microbubble Destruction.

Jinhui Gao[1], Keiran A. Logan[1], Heather Nesbitt[1], Bridgeen Callan[1], Thomas McKaig[1], Mark Taylor[2], Mark Love[3], Anthony P. McHale[1*], Darren M. Griffith[4,5*], John F. Callan[1*]

1. Biomedical Sciences Research Institute, University of Ulster, Coleraine, Northern Ireland, UK; 2. Department of HPB Surgery, Mater Hospital, Belfast, Northern Ireland, U.K. BT14 6AB. 3. Imaging Centre, The Royal Victoria Hospital, Grosvenor Road, Belfast, Northern Ireland, U.K. BT12 6BA. 4. Department of Chemistry, RCSI, 123 St Stephens Green, Dublin 2. Ireland.
5. SSPC, Synthesis and Solid State Pharmaceutical Centre, Ireland
* Address to whom correspondence should be addressed: j.callan@ulster.ac.uk; ap.mchale@ulster.ac.uk; dgriffith@rcsi.ie

Abstract: FOLFIRINOX and FOLFOXIRI are combination chemotherapy treatments that incorporate the same drug cocktail (folinic acid, 5-fluorouracil, oxaliplatin and irinotecan) but exploit an altered dosing regimen when used in the management of pancreatic and colorectal cancer, respectively. Both have proven effective in extending life when used to treat patients with metastatic disease but are accompanied by significant adverse effects. To facilitate improved tumour-targeting of this drug combination, an ultrasound responsive microbubble formulation loaded with 5-fluorouridine, irinotecan and oxaliplatin (FIRINOX MB) was developed and its efficacy tested, together with the non-toxic folinic acid, in preclinical murine models of pancreatic and colorectal cancer. A significant improvement in tumour growth delay was observed in both models following ultrasound targeted microbubble destruction (UTMD) mediated FIRINOX treatment with pancreatic tumours 189% and colorectal tumours 82% smaller at the conclusion of the study when compared to animals treated with a standard dose of FOLFIRINOX. Survival prospects were also improved for animals in the UTMD mediated FIRINOX treatment group with an average survival of 22.17 ± 12.19 days (pancreatic) and 44.40 ± 3.85 days (colorectal) compared to standard FOLFIRINOX treatment (15.83 ± 4.17

days(pancreatic) and 37.50 ± 7.72 days (colon)). Notably, this improved efficacy was achieved using FIRINOX MB that contained 5-fluorouricil, irinotecan and oxaliplatin loadings that were 13.44-fold, 9.19-fold and 1.53-fold lower than used for the standard FOLFIRINOX treatment. These results suggest that UTMD enhances delivery of FIRINOX chemotherapy, making it significantly more effective at a substantially lower dose. In addition, the reduced systemic levels of 5-fluorouracil, irinotecan and oxaliplatin should also make the treatment more tolerable and reduce the adverse effects often associated with this treatment.

Keywords: FOLFIRINOX, FOLFOXIRI, microbubbles, ultrasound, pancreatic cancer, colorectal cancer.

1.0 Introduction: The FOLFIRINOX chemotherapy cocktail combines cytotoxic 5-fluorouracil (5-FU), irinotecan (IRIN) and oxaliplatin (Ox) alongside the non-cytotoxic vitamin folinic acid (FOL). FOLFIRINOX is normally indicated for patients with advanced pancreatic cancer and in this setting provides a mean overall survival of 11.1 months, which while appearing modest, is significantly better than the 6.8 months offered by the most widely used treatment (Gemcitabine monotherapy) [1]. However, this survival benefit does come at the cost of significant off-target toxicity and FOLFIRINOX is normally only indicated for those patients with an Eastern Cooperative Oncology Group (ECOG) performance status ≤ 1 [2]. FOLFOXIRI, which is used in the management of colorectal cancer, combines the same drugs as FOLFIRINOX but with modified 5-FU and IRIN dosing in an attempt to control toxicity [3]. Therefore, methods that can deliver these cytotoxic drugs more accurately to pancreatic and colorectal tumours and reduce off-target toxicity would improve the tolerability of the treatment and potentially widen its applicability to patients with higher ECOG performance scores.

Ultrasound targeted microbubble destruction (UTMD) is an emerging method of drug delivery and involves the rupture of micron sized bubbles (1-2 μm) using an ultrasound (US) stimulus [4]. The microbubbles (MBs) are stabilised by lipid, protein or polymer-based shells

that encapsulate a hydrophobic gas such a perfluorobutane (PFB). MBs have been widely used as contrast agents in medical ultrasound imaging and have a proven safety record [5]. However, at higher US intensities than those used for diagnostic applications, but still within levels considered safe for use in humans, the MBs rupture (inertial cavitation) shedding their shell fragments at the site of destruction [6]. As it is possible to focus US accurately in 3-dimensions in human tissue, MB destruction can largely be confined to the point of interest (i.e. a tumour) [4]. If the MBs are loaded with chemotherapy drugs, then targeted drug release occurs. An added benefit of UTMD is that the physical processes that accompany MB cavitation (i.e. microstreaming & microjetting) help drive the shell fragments deeper into the tumour tissue [7-9]. We, and others, have demonstrated the utility of UTMD as a method of drug delivery in several pre-clinical models of cancer [10-12]. A clinical study has also demonstrated the benefit of MB cavitation in improving the efficacy of gemcitabine treatment in patients with pancreatic cancer [13]. However, to date, there have been no reports detailing the use of UTMD to facilitate targeted FOLFIRINOX chemotherapy.

When considering delivery of FOLFIRINOX, of primary concern is 5-FU, IRIN and Ox as these are the cytotoxic drugs responsible for anti-tumour efficacy. Of less concern is folinic acid which is a non-toxic vitamin used to improve the effectiveness of 5-FU. However, loading three drugs on a single MB is challenging from a formulation perspective. We have previously demonstrated the ability of IRIN to load effectively within the acyl layer of phospholipid stabilized MBs as its free base [10]. We have also demonstrated the effectiveness of using the biotin-avidin interaction to attach biotin functionalized payloads to the surface of avidin functionalized MBs [14-15]. The ability to functionalize phospholipids with drug payloads offers a third option for incorporating drugs within MBs during their assembly [16].

In this manuscript, we report the synthesis of a 5-Fluorouridine (5-FUR) functionalized phospholipid (DBP-5FUR) to facilitate 5-FUR incorporation within the MB shell. 5-FUR is the nucleoside analogue of 5-FU and undergoes phosphorylation followed by reduction to the active metabolite 5-fluorodeoxyuridine monophosphate (FdUMP). FdUMP inhibits thymidine synthesis by irreversibly binding to thymidylate synthase and thus prevents DNA replication

[17]. 5-FUR was chosen over 5-FU as the 5' hydroxyl group on the sugar moiety of 5-FUR enables functional derivatives of 5-FUR to be prepared [18]. A biotin functionalized oxaliplatin derivative (biotin-Ox) was also prepared to enable attachment of Ox to the MB shell using the biotin-avidin interaction. The cytotoxicity of Pt(II) compounds such as Ox is attributed to their ability to bind DNA, resulting in adducts that trigger DNA perturbation damage responses and ultimately apoptosis [19-20]. In an attempt to reduce the off-target toxicity and resistance issues associated with Pt(II) compounds, a range of bio-reductive Pt(IV) prodrugs have also been developed [21-22]. Such compounds are readily functionalised at the axial positions to enable attachment to targeting ligands or drug-delivery vehicles [23-25]. Herein, a Pt(IV) Ox prodrug bearing *trans* axial hydroxo ligands was reacted with the carboxylic acid of biotin to generate the mono-substituted biotin derivative (biotin-Ox) [26]. US responsive MBs were then formulated incorporating IRIN and 5-FUR in the shell and the biotin-Ox attached to the biontinylated MB surface via avidin. The effectiveness of UTMD using the resulting FIRINOX MB formulation, in combination with non-MB associated systemic folinic acid treatment, was assessed in pre-clinical models of pancreatic and colorectal cancer.

2. Experimental

2.1 Reagents and Equipment: All chemicals and solvents were used as received from commercial suppliers. *cis, trans, cis*-(*trans-R,R*-1,2-diaminocyclohexane)dihydroxooxalato-platinum(IV), *cis, trans, cis*-[Pt(DACH)$(OH)_2$(Ox)], was synthesized as previously reported [18]. 1,2-dibehenoyl-sn-glycero-3-phosphocholine (DBPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol) -2000] (DSPE-PEG(2000)) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG(2000)-biotin) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). PFB was purchased from Apollo Scientific Ltd. (Cheshire, UK). Phosphate buffered saline (PBS) and avidin from egg white was purchased from Thermo Fisher, (Waltham, MA, USA). 5-Fluorouracil, 5-Fluorouridine, Folinic acid, Biotin, Agarose, MTT assay kit, Glycerol and Propylene glycol were purchased from Sigma Aldrich (St. Louis,

MO, USA). Irinotecan hydrochloride and Oxaliplatin were purchased from Hefei Joye Import & Export Co Ltd, (Hefei, Anhui, China). Phospholipase D from *Streptomyces* sp. was purchased from Sekisui Diagnostics (Burlington, MA, USA). Panc-01 and HT-29 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The T110299 cell line, a gift from Prof. Jens Siveke, (Klinikum rechts der Isar, Technical University Munich, Munich, Germany), was isolated from primary pancreatic tumours in KPC mice (Ptfla-Cre; LSL-$Kras^{G12D}$; LSL-$Trp^{53fl/R172H}$) [27]. Matrigel was purchased from BD Biosciences (San Jose, CA, USA). C57BL/6J and SCID mice (C.B-17/IcrHanHsd-PrkdcSCID) were bred in house. MBs were formed using a Microson ultrasonic cell disruptor, 100 W, 22.5 kHz, from Misonix Inc. (Farmingdale, NY, USA). Optical microscope images were obtained using a Leica DM500 optical microscope and fluorescence images were obtained using a NIKON Eclipse E400 Phase contrast microscope (Wetzlar, Germany). Analysis of MB size and concentration was determined using a bespoke MATLAB algorithm (2010B, MathWorks, Natick, MA, USA). UV-Vis spectra were recorded with a Varian Cary spectrophotometer (Palo Alto, CA, USA), using quartz cells (path length = 1 cm). UV absorbance of MTT was analysed using a Fluostar Omega plate reader (BMG Labtech, Ortenberg, Germany). NMR spectra were obtained on Varian 500 MHz instrument at 25.0 ± 1 °C (Palo Alto, CA, USA) and processed using TopSpin software (Bruker, Billerica, MA, USA). ESI-MS were obtained using a Finnegan LCQ-MS instrument (San Jose, CA, USA). Preparative RP-HPLC was undertaken using a Shimadzu LC-8 system (Shimadzu Corp., Kyoto, Japan). Statistical error in data was expressed as percentage of standard error of the mean and statistical analysis was undertaken using either a t-test or a one-way ANOVA followed by Tukey's post hoc test using Prism.

2.2 Synthesis of [(2R)-2,3-bis(docosanoyloxy)propoxy]({[(2R,3S,4R,5R)-5-(5-fluoro-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-3,4-dihydroxyoxolan-2-yl]methoxy}) phosphinic acid (DBP-5FUR): A $CHCl_3$ solution (30 mL) of DBPC (0.5 g, 0.554 mmol) was added to a solution of Phospholipase D (PLD) (10 mg, 2650 units) and 5-FUR (0.720 g, 2.74 mmol) in sodium acetate buffer (200 mM, pH 5.7, 10 mL) containing $CaCl_2$ (250 mM). The mixture was stirred

at 45°C for 6 hours followed by the addition of 2N HCl (5 mL), MeOH (20 mL) and $CHCl_3$ (20 mL). The mixture was shaken and the separated organic layer washed with H_2O (2 x 10 mL), dried using anhydrous sodium sulfate and concentrated to dryness *in vacuo*. The residue was purified by flash chromatography ($CHCl_3$:MeOH 10:1 - 6:1 v/v) and fractions containing DBP-5FUR were combined and concentrated to dryness. ^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD (2:1 v/v) d (ppm) 8.01 (*d*, 1H, CONHCO), 5.91 (*br d*, 1H, 1'(CH)), 5.20 (*m*, 1H, glycerol CH), 3.72-4.20 (*m*, 9H, 3'(CH), 2'(CH) 4'(CH), 5'(CH_2) glycerol CH_2, glycerol CH_2OPO), 2.27 (*m*, 4H, 2x $COCH_2$), 1.57 (*m*, 4H, 2xCH_2), 1.23 (*m*, 72H, behenoyl CH_2), 0.83 (*t*, 6H, 2xCH_3). -ve mode MALDI-MS: Calculated MW for $C_{56}H_{102}O_{13}N_2P_1F_1$ = 1060.71 Da, observed = 1059.48 Da.

2.3 Synthesis of cis, trans, cis-[Pt(DACH)(Biotin)(OH)(Ox)] (biotin-Ox): (+)-Biotin *N*-hydroxysuccinimide ester (0.1 g, 0.293 mmol) in anhydrous DMSO (4 mL) was added to a suspension of *cis, trans, cis*-[Pt(DACH)$(OH)_2$(Ox)] (0.126 g, 0.305 mmol) in anhydrous DMSO (8 mL). The reaction was stirred at room temperature for 4 days under an argon atmosphere. A small amount of white solid was removed by filtration. The yellow filtrate was concentrated using a DMSO trap to yield a sticky yellow oil, to which acetone (40 mL) was added, to providea white precipitate. The suspension was stirred for 1 hr and subsequently the solid was filtered, washed with acetone, diethyl ether and dried. Yield: 0.122 g (0.186 mmol, 60%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.63 (s, 1H, DACH NH), 8.19 (s, 1H, DACH NH), 7.86 (s, 1H, DACH NH), 7.18 (s, 1H, DACH NH), 6.42 (s, 1H, CONH), 6.38 (s, 1H, CONH), 4.29 (t, 1H, ^{3}J = 8 Hz), 4.11 (m, 1H), 2.84 (dt, 1H, ^{3}J = 8 Hz & ^{4}J = 2 Hz), 2.79 (dd, 1H, ^{3}J = 8 Hz & ^{4}J = 4 Hz), 2.58 (d, 1H, ^{3}J = 4 Hz), 2.54 (s, 2H), 2.16 (t, 2H, ^{3}J = 8 Hz), 1.27 (m, 10H), 1.07 (m, 2H,) ppm. ^{195}Pt NMR (86 MHz, DMSO-d_6) δ: 1406.7 ppm. EA calc. % for $C_{18}H_{30}N_4O_8PtS.1.5\ H_2O$ requires C, 31.58; H, 4.86; N, 8.18; S 4.68, found C, 31.60; H, 4.66; N, 7.84; S, 5.00 %. ESI-MS: m/z ([M+H]+) 658.1 ([M+Na]+) 680.1.

2.4 Preparation of FIRINOX MBs: FIRINOX MBs were prepared by first dissolving DBP-5FUR (4.0 mg, 3.7 μmol), DSPE-PEG(2000) (1.15 mg, 0.41 μmol) and DSPE-PEG (2000)-biotin

(1.24 mg, 0.41 µmol) in $CHCl_3$ to achieve a molar ratio of 82:9:9. A 100 µL aliquot of IRIN free base (prepared from Irinotecan $HCl.3H_2O$ by standard acid-base extraction) (10 mg), dissolved in $CHCl_3$ was added to this solution. The solvent was removed under vacuum at room temperature yielding a translucent film. The film was then reconstituted in 2 mL of a solution containing PBS, glycerol and proplyene glycol (PGP) (8:1:1 v/v) and heated in a water bath at 80°C for 30 min. The suspension was sonicated using a Microson ultrasonic cell disrupter at an amplitude of 22% for 1 min. The headspace of the vial was then replaced with PFB followed by further sonication at an amplitude of 90% in a PFB atmosphere for 30 sec. The MBs were then cooled on ice for 10 min followed by centrifugation at 100 g for 3 min and the liquid laying below the surface of the MB cake (infranatant) was removed. The MB cake was then washed a further 2 times with PGP before mixing with a solution of avidin in PGP (10 mg/mL) for 5 min on ice using an orbital shaker (150 RPM). The MBs were then centrifuged (100 g) for 3 min, the infranatant removed and the MB cake washed with PGP (2 mL) which was removed following centrifugation. The MB cake was again reconstituted in PGP (2 mL), mixed for 5 min with PGP containing biotin-Ox (1 mL, 5 mg/mL) and centrifuged (100 g) for 3 min. Following removal of the infranatant, the MB cake was then washed with PGP (2 mL), centrifuged and the MB cake isolated. This washing/centrifugation procedure was repeated twice before the cake was finally reconstituted in PGP (2 mL). Scheme 3 provides an illustration of the FIRINOX-loaded MBs. The concentration of 5-FUR, and Ox present in the FIRINOX MBs were determined using RP-HPLC while IRIN was determined using UV-Vis spectroscopy.

2.5 In vitro cytotoxicity of 5-FUR and DBP-5FUR in Panc-01 2D cells: Panc-01 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 1 mg/mL glucose supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin,10% fetal bovine serum (FBS) and grown in a humidified 5% CO_2 atmosphere at 37 °C. Panc-01 cells were seeded in a 96-well plate at 5×10^3 cells per well and incubated for 24 hours. The medium was then removed from each well and replaced with 100 µL of treatment suspension and 100 µL of

fresh medium. 48 hours after treatment an MTT assay was carried out. Absorbance was recorded at 570 nm using the FLUOstar Omega microplate reader. Data are expressed as % cell viability by comparison with untreated controls.

2.6 In vivo cytotoxicity of biotin-Ox and Ox in C57BL/6 mice bearing KPC-derived T110299 xenograft tumours: All animals employed in the *in vivo* experiments were treated humanely and in accordance with the licensed procedures under the UK Animals (Scientific Procedures) Act 1986. T110299 mouse pancreatic cancer cells were maintained in DMEM medium supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 10% fetal bovine serum (FBS), 1% Non-essential amino acids and 1% L-Glutamine in a humidified 5% CO_2 atmosphere at 37 ºC. Cells (5 ×10^5) were suspended in 100 µL of PBS and implanted in the dorsum of 6-week old C57BL/6 mice. Tumour volume was calculated using the equation: tumour volume = (length × width × height) / 2. Once tumours reached an average volume of approximately 100 mm^3, they were randomly allocated into 3 groups: untreated group (n=4, received no treatment), Ox treated group (n=4, received I.P injection [Ox] = 5 mg/kg) and biotin-Ox treated group (n=4, I.P injection, [active Ox] = 5 mg/kg). Animals were treated on days 0, 5, 12, 19 with the tumour volume and body weight measurements recorded at the indicated times.

2.7 Treatment of Panc-1 3D Spheroids using FIRINOX MBs: 96 well plates were coated with agarose solution (15 mg/ml in DMEM - low glucose, 60 µL/well) and air-dried in a laminar-flow hood for 30 min. Panc-01 cells were maintained in DMEM containing high glucose (4.5 g/L) supplemented with 10% (v/v) foetal bovine serum in a humidified 5% CO_2 atmosphere at 37°C. 6×10^3 cells were seeded in each well of the agarose coated plate and placed in an incubator (37°C, 5% CO_2) for 96 hours to generate spheroids. The spheroids were incubated in a PBS: medium (1:1 v/v) solution containing the FIRINOX MBs ([IRIN] = 50 µM; [Ox] = 48 µM; + [5-FUR] = 90 µM) and selected wells treated with US using a Sonidel SP100 sonoporator (1 MHz,

30 sec, 3 W/cm^2, duty cycle = 50%, and PRF = 100 Hz, PNP = 0.45 MPa) for 30 sec from underneath the plate using US gel to mediate contact. Untreated spheroids and spheroids treated with US only were used for comparative purposes. Following treatment, the spheroids were incubated for a further 48 hours, after which the medium was removed and spheroids washed 3 times with PBS. The spheroids were then treated with a solution of propidium iodide (P.I.) in PBS (100 µg/ml) and incubated for 30 min after which time the P.I. solution was removed, and the spheroids washed 3 times with PBS. Microscopy images were recorded using a NIKON Eclipse E400 phase contrast microscope in bright field and fluorescence modes (540 nm band pass excitation and 590 nm long pass emission filters). Image J software was used to quantify P.I. fluorescence and the latter was expressed as a % of P.I. fluorescence intensity/µm^2, i.e. the P.I. fluorescence was normalized according to the area of the spheroid.

2.8 UTMD mediated treatment of mice bearing subcutaneous KPC pancreatic tumours using FIRINOX MBs and folinic acid: T110299 cells were maintained as described in section 2.6. 5×10^5 cells were re-suspended in PBS and implanted into the rear dorsum of 6-week old female C57BL/6 mice. Tumour formation occurred approximately 2 weeks after implantation and once tumours became palpable, dimensions were measured using Vernier callipers. Once tumours reached approximately 100 mm^3, animals were randomly allocated into the following groups: Group 1 received no treatment. Group 2 received an I.V. injection of the FOLFIRINOX free drug cocktail (i.e. non-MB bound) with Ox (2.5 mg/kg) administered first and immediately followed with folinic acid (IP, 50 mg/kg). After 30 minutes, IRIN (25 mg/kg) was administered followed immediately with 5-FU (25 mg/kg). Group 3 received folinic acid (IP, 50 mg/kg) followed by FIRINOX MBs ([IRIN] = 7.3 ± 1.5 mg/kg, [Ox] = 1.55 ± 0.17 mg/kg, [5-FUR] = 2.09 ± 0.19 mg/kg) administer via tail vein injectionwith US applied to the tumour during and after injection for a total of 3.5 min. US was administered using a Sonidel SP100 sonoporator (3.5 W/cm^2, 1 MHz, 30% duty cycle, and PRF = 100 Hz; PNP = 0.48 MPa; M.I. = 0.48) and US gel used to mediate contact. Group 4 received the same treatment as for Group 3 but without US.

Animals were treated on days 0, 3, 6, 8 and both the tumour volume and body weight measurements recorded at the times indicated. For the Kaplan Meier plot, as all the tumours were not the same volume at the start of the experiment, a value of 7 times the starting tumour volume was chosen to illustrate the time at which the animals were removed from the study.

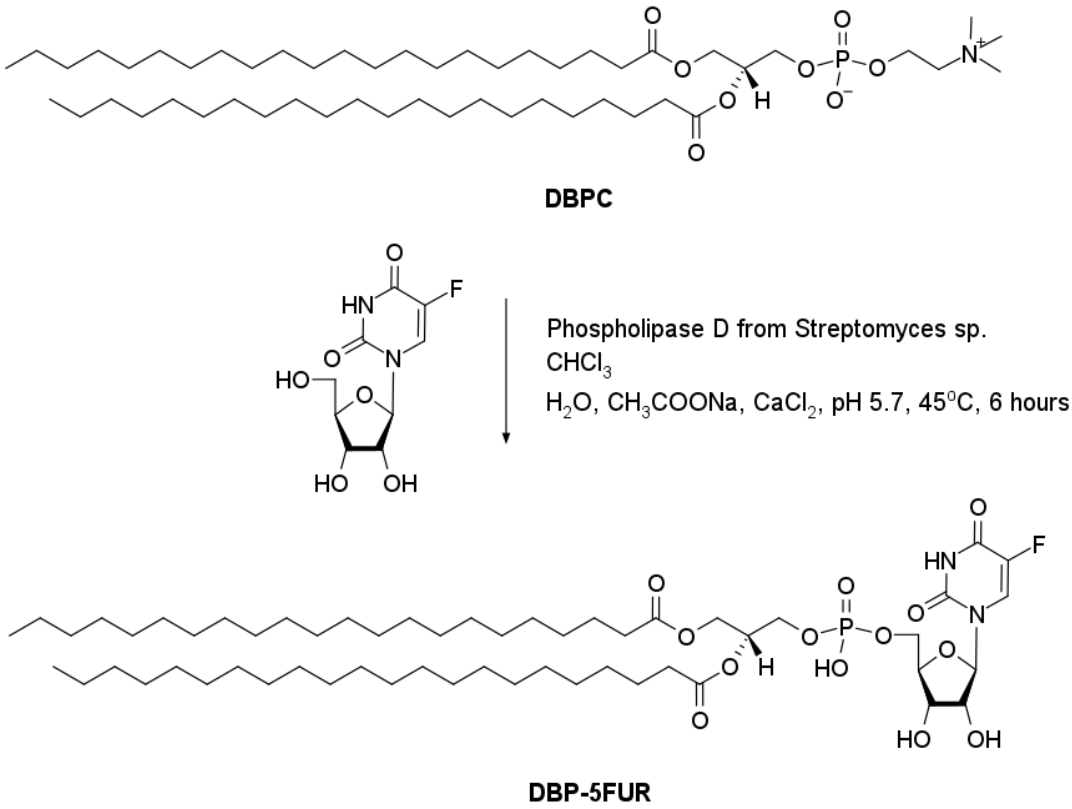
2.9 UTMD mediated treatment of mice bearing subcutaneous HT-29 colon tumours using FIRINOX MBs and folinic acid: HT-29 cells were maintained in DMEM medium supplemented with 10% foetal calf serum. Cells (1 x10^6) were re-suspended in 100 µL of Matrigel® and implanted into the rear dorsum of 6-week old female SCID (C.B-17/IcrHan®Hsd-Prkdc^{scid}) mice. Tumour formation occurred approximately 4 weeks after implantation and once tumours became palpable, dimensions were measured using Vernier callipers. Tumour volume was calculated using the equation as described in section 2.6. Once tumours reached an average volume of approximately 100 mm^3, animals were randomly allocated to groups essentially as described in section2.8. In this experiment the standard FOLFIRINOX treatment was as described in section 2.8. However, here the FIRINOX MB drug concentrations used were: [IRIN] = 2.72 ± 1.64 mg/kg, [Ox] = 1.63 ± 0.19 mg/kg and [5-FUR] = 1.86 ± 0.14 mg/kg. Again, the MB formulation was injected intravenously with US applied to the tumour during and after injection for a total of 3.5 min followed by an IP injection of folinic acid at a dose of 50 mg/kg. Animals were treated on days 0, 3, 7, 13, 17 and both the tumour volume and body weight measurements recorded at the times indicated in Figure 5. For the Kaplan Meier plot, as all the tumours were not the same volume at the start of the experiment, a value of 7 times the starting tumour volume was chosen to illustrate the time at which the animals were removed from the study. Where appropriate, the US conditions were as per 2.8.

3. Results and Discussion

3.1. Synthesis of DBP-5FUR and biotin-Ox.

The 5-FUR modified DBPC conjugate (DBP-5FUR) was prepared using the enzyme phospholipase D from *Streptomyces* sp. to catalyse a transphosphatidylation reaction

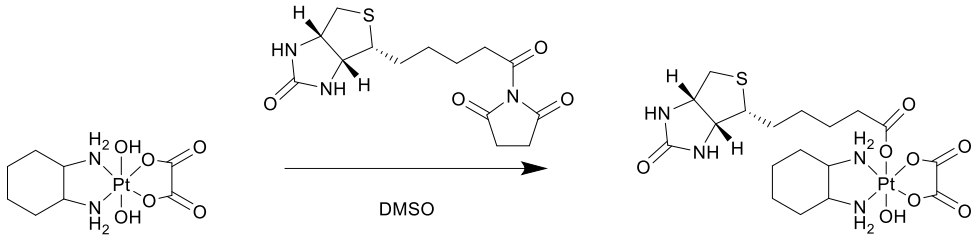
between the phospholipid and the 5’ hydroxyl group of 5-FUR (Scheme 1) [18]. This resulted in the formation of a phosphoester bond between the 5’ sugar hydroxyl of 5-FUR and the phosphate group of DBPC with the choline unit of the phospholipid eliminated.



Scheme 1. Synthesis of DBP-5FUR.

Following purification using column chromatography, DBP-5FUR was characterized using ^{1}H NMR and MALDI-TOF mass spectrometry. The stacked ^{1}H NMR spectra for 5-FUR, DBPC and DBP-5FUR are shown in Figure S1 (ESI), and clearly reveal signals from both the 5-FUR and DBP components but importantly reveal that protons associated with choline methyl (3.15 ppm) and methylene groups (3.60 and 4.15 ppm) which were present in the spectrum of DBPC were absent in the spectrum of DBP-5FUR. In addition, the resonance corresponding to the methylene protons adjacent to the 5’-hydroxyl group centered at 3.75 ppm in 5-FUR, shifted downfield to 3.95 ppm in DBP-5FUR, reflecting their new chemical environment adjacent to the phosphodiester group. Other important signals include the methine ring proton of the 5-FUR component observed at 8.00 ppm, the furanose sugar

methine proton at 5.85 ppm and the chiral methine proton adjacent to the ester of the aliphatic lipid chain at 5.20 ppm. The methyl protons of the lipid chains were observed at 0.80 ppm while the majority of methylene protons of the lipid chains were observed on a large resonance at 1.20 ppm with the exception of those alpha and beta to the ester carbonyl at 2.25 and 1.60 ppm respectively. The remaining protons were assigned as described in Figure S2 (ESI). The MALDI-TOF mass spectrum of DBP-5FUR, (Figure S3, ESI) in the negative mode reveals the parent ion with m/z = 1059.7 Da, which is consistent with the expected mass of 1060.7 Da.



Scheme 2. Synthesis of biotin-Ox.

The biotin functionalised oxaliplatin derivative, biotin-Ox, was prepared by reaction of *cis, trans, cis*-[Pt(DACH)(OH)$_2$(Ox)] with the activated NHS ester of biotin ((+)-Biotin *N*-hydroxysuccinimide) in anhydrous DMSO (Scheme 2) [26]. The structure of biotin-Ox was confirmed using ^{1}H-NMR, ^{195}Pt-NMR, ESI-MS and elemental analysis (Figure S4, S5 and S6, ESI). The ^{1}H NMR spectrum (DMSO-d6) has four broad signals between 7.18 and 8.63 ppm associated with the four protons of the DACH amino groups in the unsymmetrical Pt(IV) complex. The characteristic carbamide protons of biotin feature as two sharp doublets at 6.38 and 6.42 ppm. The chiral methine protons on the bridging carbons of the biotin ring were observed as resonances at 4.11 and 4.29 ppm while the remaining methine and methylene protons of the biotin ring adjacent to the thioether sulphur atom were observed at 2.84 and 2.54 ppm respectively. The methylene protons adjacent to the ester group of the linker were observed as a triplet at 2.16 ppm while the methine protons of the DACH ring were found at

2.79 and 2.58 ppm. The remaining 14 methylene protons associated with DACH (8) and the biotin carboxylate ligand (6) are accounted for as outlined in the experimental. The ^{195}Pt NMR signal at 1406.7 ppm is consistent with previously reported values for similar oxaliplatin-based Pt(IV) complexes [23]. ESI-MS in positive mode featured the required molecular ion for biotin-Ox and its Na^+ adduct at 658.1 and 680.1 Da respectively and also featured the expected Pt isotopic pattern. Elemental analysis was consistent with one DACH ligand, one oxalato, one hydroxo ligand and one biotin carboxylato ligand per Pt centre.

3.2. Efficacy of DBP-5FUR and biotin-Ox

In advance of preparing MB formulations using DBP-5FUR and biotin-Ox, it was first necessary to ensure both retained their efficacy following chemical modification. A cell viability experiment was undertaken in which human pancreatic Panc-01 cells were treated with DBP-5FUR and 5-FUR using the MTT assay to determine the percentage of viable cells 48 hours following treatment. The results are shown in Figure 1a and reveal DBP5-FUR to exhibit less toxicity than 5-FUR at 0.05 and 0.5 μM but greater cytotoxicity at the higher concentrations of 2.5 and 5.0 μM, although the differences were not statistically significant. Based on these results the DBP-5FUR was considered sufficiently active for use in the FIRINOX MBs.

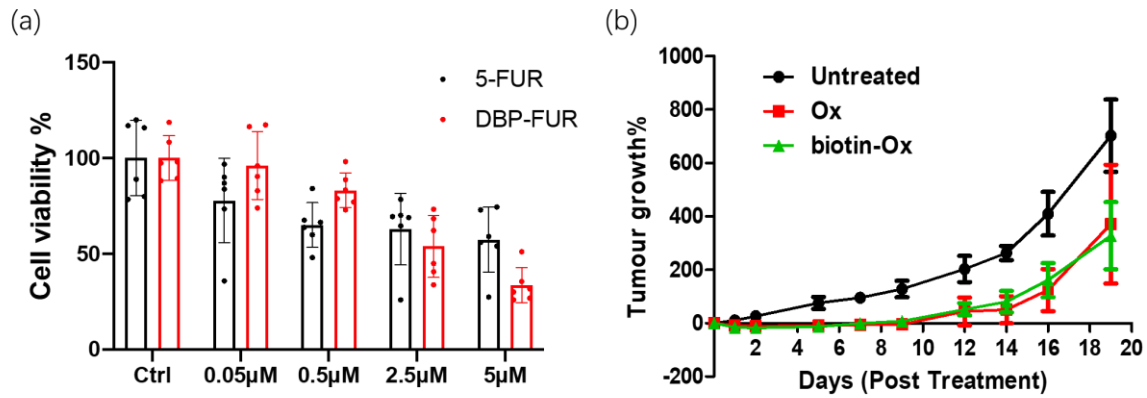
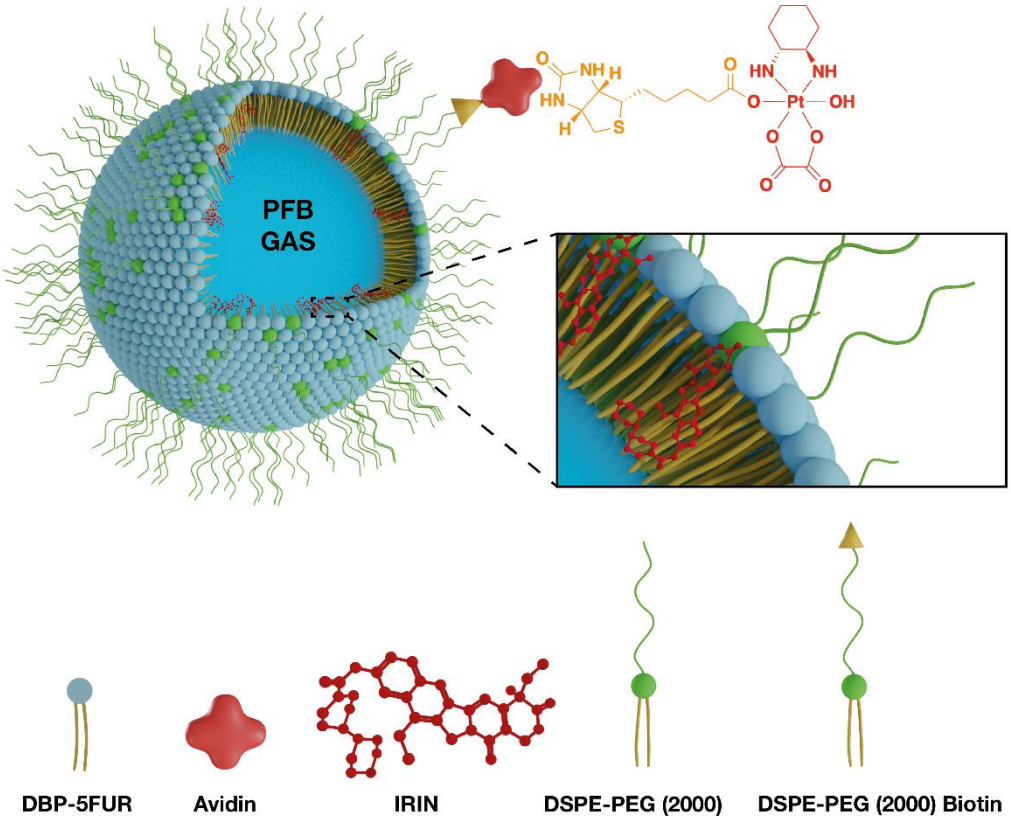


Figure 1 (a) Plot of cell viability against concentration for 5-FUR and DBP-5FUR in Panc-01 cells. Error bars represent ± SEM where n = 6 (b) Plot of % tumour growth against time for

mice bearing subcutaneous KPC tumours and treated with Ox or biotin-Ox. Error bars represent ± SEM where n = 4.

The efficacy of biotin-Ox was tested in a murine model of pancreatic cancer bearing syngeneic KPC-derived T110299 pancreatic tumours. Pt(IV) complexes such as biotin-Ox are kinetically more inert than their corresponding Pt(II) complexes and the Pt(IV) to Pt(II) conversion is facilitated by endogenous bio-reductive processes [24-25]. Most solid tumours have hypoxic fractions ranging between 10 and 30% and thus provide an ideal environment for the activation of bio-reductive prodrugs [28]. This is particularly true for pancreatic tumours where the pO_2 can be as low as 5.3 mmHg [29]. When the animals were treated with matched doses of biotin-Ox or Ox, a similar growth delay profile was observed for both compounds suggesting that the biotin-Ox was effectively activated in the tumour microenvironment. In addition, the biotin-Ox treatment was also well tolerated with no evidence of acute weight loss throughout the experiment (Figure S7, ESI).



Scheme 3. Schematic representation of the FIRINOX MB. Avidin functionalised IRIN loaded MB were first prepared using DBP-5FUR as a constituent lipid and subsequently bound with biotin-Ox.

3.3. Preparation of FIRINOX MBs

Confident that both DBP-5FUR and biotin-Ox demonstrated satisfactory efficacy, MBs were then prepared comprising both cytotoxic agents in addition to IR. A chloroform solution of IR free base was added to a chloroform solution containing DBP-5FUR, DSPE-PEG(2000) and DSPE-PEG (2000)-biotin in molar ratio of 82:9:9. The chloroform was then evaporated, the resulting film hydrated and heated above the lipid phase transition temperature. The solution was then cooled and sonicated under a headspace of PFB to generate the 5-FUR and IRIN loaded MBs which were purified using low-speed centrifugation. The MBs were coated with avidin and mixed with biotin-Ox to generate the final FIRINOX MB suspension (Scheme 3). A representative optical micrograph image of the MB suspension is shown in Figure 2a and reveal spherical particles with a mean diameter of 1.10 ± 0.76 µm and yield of 6.47×10^9 MB / mL (Figure 2b). The loading of each of the drugs on the FIRINOX MBs was determined using UV-Vis spectroscopy for IRIN (68 ± 11 µg/10^8MB) and RP-HPLC for Ox (73 ± 13 µg/10^8 MB) and 5-FUR (52 ± 10 µg/10^8 MB).

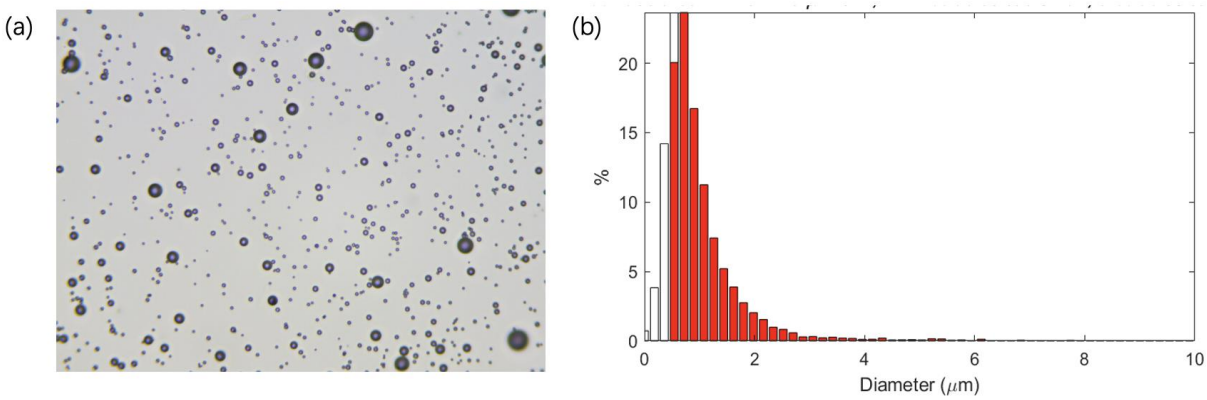


Figure 2 (a) and (b) representative optical micrograph of FIRINOX MB (n = 20) and representative size distribution analysis of FIRINOX MB (n = 20) respectively.

3.4. UTMD mediated efficacy of FIRINOX MBs in Panc-01 3D spheroids

The efficacy of UTMD using the FIRINOX MB formulation was first determined in a Panc-01 3D spheroid model of pancreatic cancer. Panc-01 3D spheroids were treated with FIRINOX MBs with (+US) or without (-US) US treatment and in the absence or presence of FIRINOX MBs. Untreated spheroids and those treated with ultrasound alone were used as controls. 48 hours later the cell viability of the spheroids was determined using P.I. staining. P.I. is a fluorescent DNA intercalating agent, which is excluded from crossing into cells with viable intact plasma membranes [30]. In turn, fluorescence intensity inversely correlates with cell viability. The results (Figure 3) show a 64.77% increase in P.I. fluorescence for spheroids treated with FIRINOX MB +US compared to the 31.13% increase associated with FIRINOX MB - US. In addition, the FIRINOX MB +US treated spheroids were notably smaller than the FIRINOX MB - US treated spheroids, most likely as a result of cell division being inhibited due to ultrasound enhanced delivery into the spheroid.

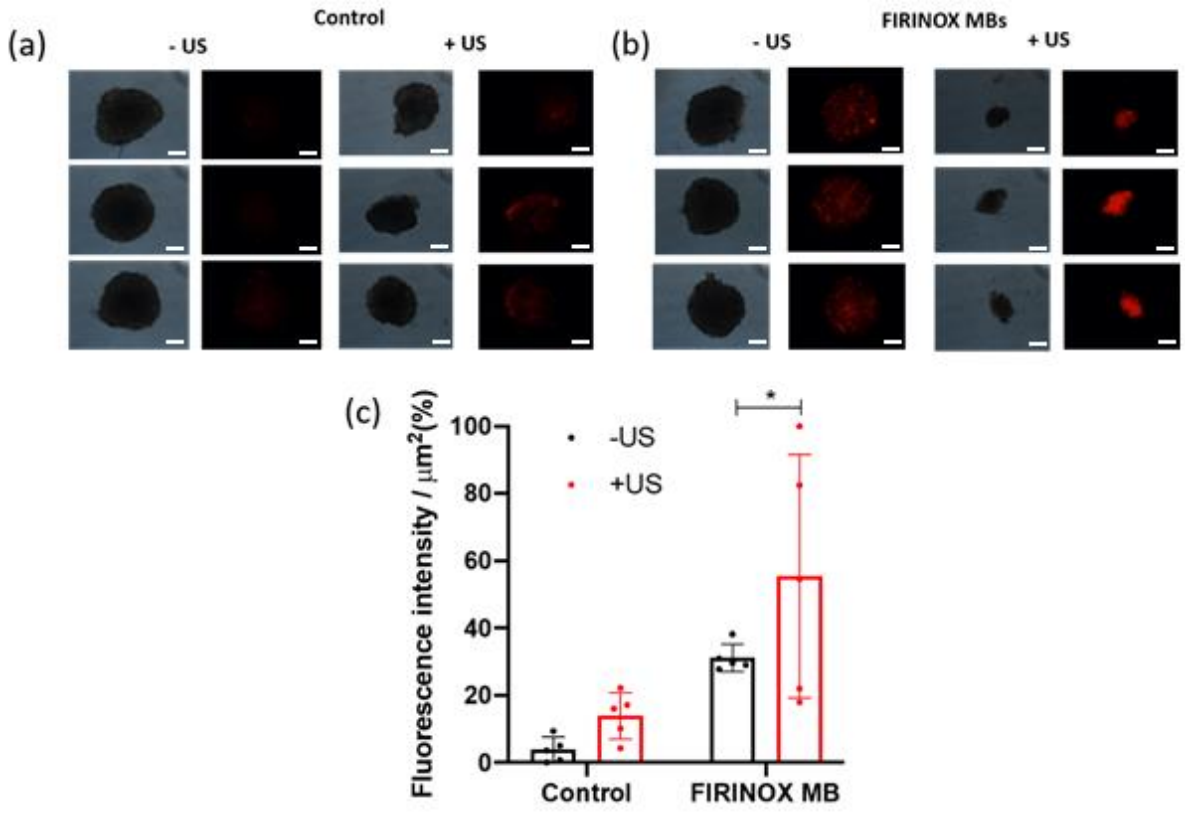


Figure 3 (a) Optical (grey panels) and (b) fluorescence (black panels) microscope images of Panc-01 3D spheroids treated with (+US) or without (-US) ultrasound treatment in the absence (a) or presence (b) of FIRINOX MBs. FIRINOX MBs contained 50 µM IRIN, 90 µM DBP-5FUR and 48 µM biotin-Ox. Scale bar = 200 µM. Error bars represent ± SEM where n = 5, *p ≤ 0.05.

3.5. UTMD mediated efficacy of FIRINOX MBs in mice bearing T110299 (KPC) pancreatic tumours.

Encouraged by the spheroid study results, the effectiveness of UTMD mediated FIRINOX treatment was then assessed *in vivo* using the T110299 murine pancreatic tumour model. Tumour bearing animals were randomly allocated into 4 groups that received either FIRINOX MB + folinic acid, FIRINOX MB + folinic acid +US, free (i.e. non-MB bound) FOLFIRINOX or no treatment. For the non-MB bound FOLFIRINOX treatment, the dose was chosen to represent a standard mouse-based treatment and used 5-FU (25 mg/kg), IRIN (25 mg/kg) and Ox at (2.5 mg/kg) together with folinic acid (50 mg/kg) [31]. By comparison, the chemotherapy dose administered using the FIRINOX MBs was 5-FUR (2.09 ± 0.19 mg/kg), IRIN (7.25 ± 1.47 mg/kg) and Ox (1.55 ± 0.16 mg/kg) alongside folinic acid (50 mg/kg) (Figure 4a). The FIRINOX MBs were delivered intravenously via the tail vein with US applied to the tumour for 3.5 min during and following injection in order to ensure rupture of the MBs in the tumour vasculature. The effects of each treatment on tumour growth are shown in Figure 4b and reveal a minor, non-statistically significant reduction in tumour volume of 88.32% 11 days after the initial treatment for the FIRINOX MB - US group compared to untreated animals. This was not surprising since a lower dose of the chemotherapy drugs was used in this formulation. However, tumours in animals treated with FIRINOX MB +US group were significantly smaller than untreated animals (477.79%, **p ≤ 0.01) and animals treated with the free drug combination (189.22%, *p ≤ 0.05) at the same time point. This significant improvement in efficacy can be attributed to UTMD providing enhanced delivery of the chemotherapy combination that, in turn, enables a significant improvement in efficacy at a substantially reduced dose.

A Kaplan Meier plot (Figure 4c) was also constructed to determine the effect of the treatment on survival. While no significant improvement in overall median survival was observed for the FIRINOX MB +US group, the two longest surviving animals in this group were removed from the study on days 27 and 45 compared to days 16 and 24 for the group treated with free FOLFIRINOX. In addition, the average lifetime of animals (Figure 4d) in the FIRINOX MB +US group was 22.17 ± 12.19 days which was significantly longer than animals in the untreated group (12.67 ± 2.25 days, $p \leq 0.05$) and considerably longer than animals in the FOLFIRINOX (15.83 ± 4.17 days) or, FIRINOX MB - US groups (12.67 ± 2.33 days). Importantly, the FIRINOX MB +US treatment was also well tolerated with animals displaying no acute weight loss throughout the experiment (Figure S8, ESI).

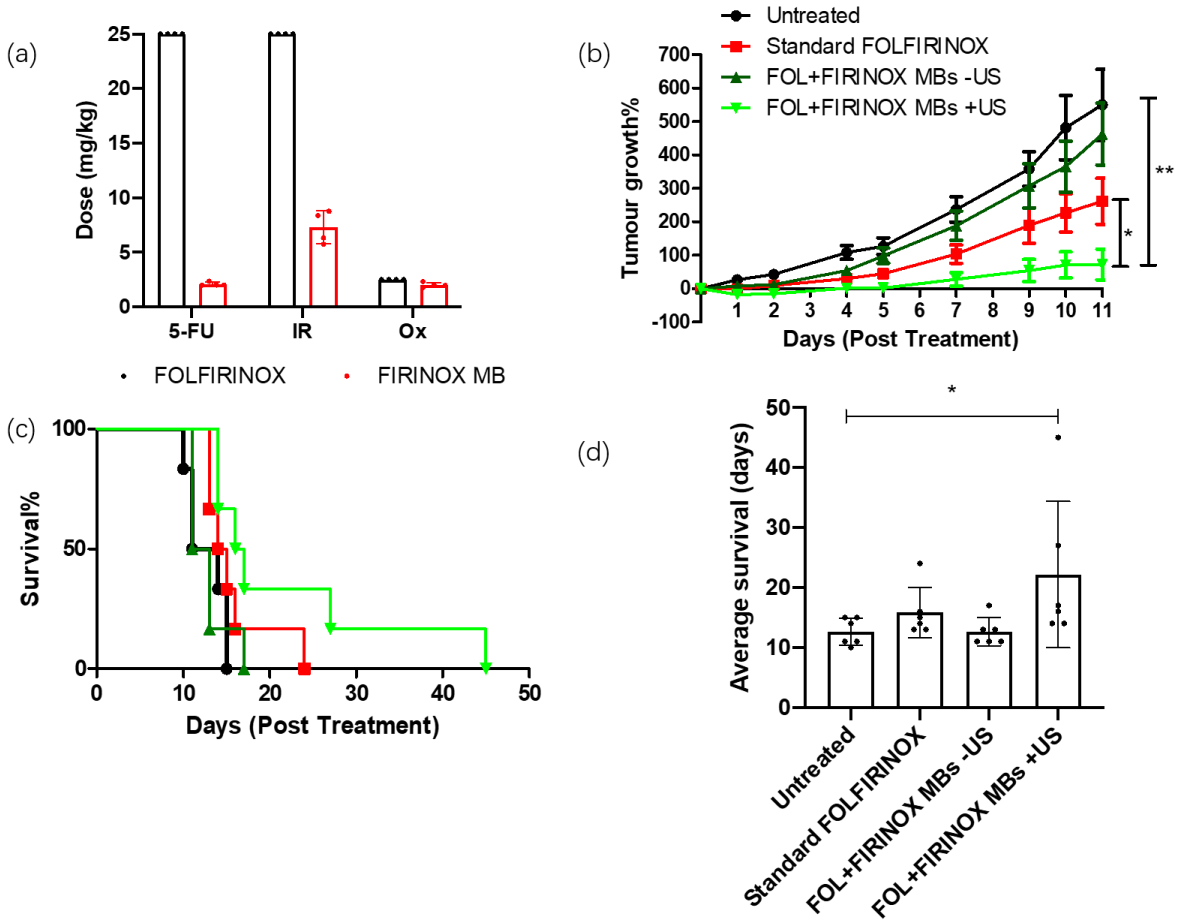


Figure 4 (a) Plot of dose in mg/kg for 5-FU / 5-FUR, IRIN and Ox used in the FIRINOX MB and FOLFIRINOX treatments. (b) Tumour growth, (c) Kaplan-Meier and (d) average survival plots for untreated animals and animals treated with FIRINOX MBs ± US or FOLFIRINOX. Error bars represent ± SEM where n = 6. *p ≤ 0.05, **p ≤ 0.01.

3.6. UTMD mediated efficacy of FIRINOX MBs in mice bearing HT-29 colorectal tumours.

Having demonstrated the effectiveness of UTMD mediated FIRINOX MB treatment in a murine model of pancreatic cancer, we were also interested to determine its potential as a treatment for colorectal cancer, as the same chemotherapy drugs form part of the FOLFOXIRI regimen used in the management of this disease [32]. Human HT-29 colorectal tumours were established in SCID (C.B-17/IcrHan®Hsd-Prkdc^{SCID}) mice and animals were randomly allocated into the same treatment groups used in treating the pancreatic cancer described above. The same free (i.e. non-MB bound) FOLFIRINOX drug concentrations were used in this study but since different batches of FIRINOX MBs were used, the administered dose of each drug was slightly different at 5-FUR (1.86 ± 0.14 mg/kg), IRIN (2.72 ± 1.64 mg/kg) and Ox (1.63 ± 0.19 mg/kg) (Figure 5a). Again, a significant improvement in tumour growth delay was observed for UTMD FIRINOX MB +US group with tumours 195% smaller than untreated tumours 16 days following the initial treatment while animals treated in the standard FOLFIRINOX were 113% smaller at the same time point. The Kaplan-Meier curve (Figure 5c) also showed that animals treated with FIRINOX MBs +US lived longer with an average survival of 44.40 ± 3.85 days compared to FOLFIRINOX (37.50 ± 7.72 days), FIRINOX MB - US groups (36.00 ± 6.78 days) or the untreated group (34.00 ± 8.37 days) (Figure 5d). In accordance with the pancreatic cancer *in vivo* experiment, the FIRINOX MB treatment was well tolerated but a significant drop (*p ≤ 0.05) in average weight was observed for animals treated with FOLFIRINOX 4 days after the initial treatment (Figure S7, ESI). Combined, these results suggest UTMD FIRINOX treatment is well tolerated and has the ability to effectively control tumour growth in preclinical models of both pancreatic and colorectal cancer.

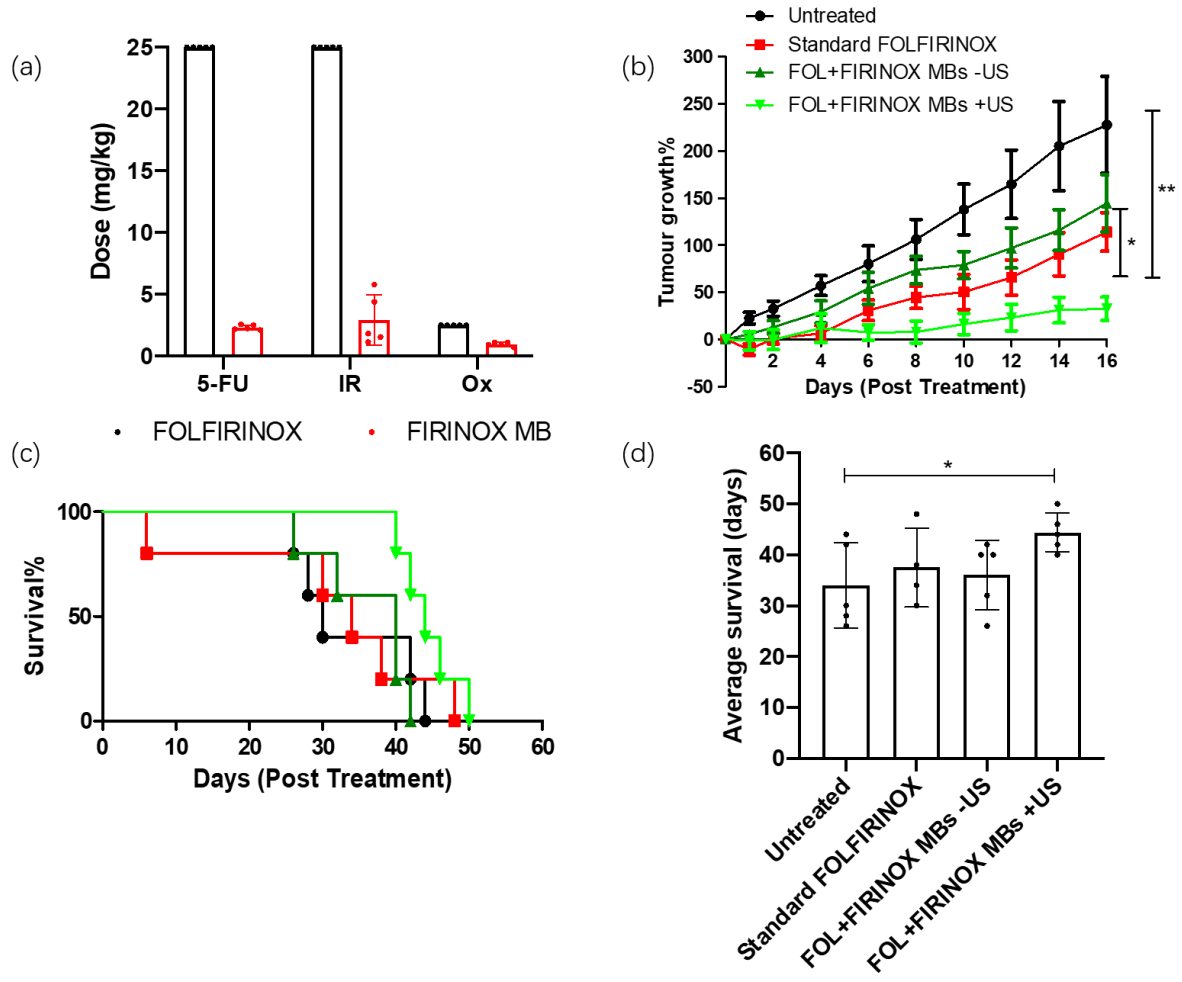


Figure 5 (a) Plot of dose in mg/kg for 5-FU / 5-FUR, IRIN and Ox used in the FIRINOX MB and FOLFIRINOX treatments used in the colorectal cancer study. (b) Tumour growth, (c) Kaplan-Meier and (d) average survival plots for untreated animals and animals treated with FIRINOX MBs ± US or FOLFIRINOX with untreated animals as comparison, Error bars represent ± SEM where n = 5. *p ≤ 0.05, **p ≤ 0.01.

4. Conclusion

Novel DBP-5FUR and biotin-Ox conjugates were successfully prepared and used to formulate a single microbubble formulation carrying FIRINOX chemotherapy. UTMD treatment using the FIRINOX MBs was successful in treating Panc-01 3D spheroids and controlling the growth of tumours in murine models of pancreatic and colorectal cancer. The FOLFIRINOX and FOLFOXFIRI regimens are widely used in the management of pancreatic and colorectal

cancer but are accompanied by significant off-target toxicity. The use of UTMD to more effectively deliver the chemotherapy components of this toxic cocktail to solid tumours not only enhances efficacy but also significantly reduces the overall chemotherapy burden. For pancreatic cancer in particular, survival prospects have remained relatively unchanged over the past 5 decades and new approaches are urgently required to enable more effective management of this disease. UTMD mediated FIRINOX treatment could find a role in both a neo-adjuvant setting to enable tumour debulking in advance of surgery or as a more tolerable treatment to prolong life in patients with metastatic disease.

Acknowledgements

JFC thanks Norbrook Laboratories Ltd for an endowed chair and Mrs Margaret McGowan for a charitable donation toward this research. DMG gratefully acknowledges funding received from the Synthesis and Solid State Pharmaceutical Centre (SSPC), financed by a research grant from Science Foundation Ireland (SFI) and co-funded under the European Regional Development Fund under Grant Number 12/RC/2275_P2. DMG thanks Dr. Diego Montagner (Maynooth University) for kindly running the ^{195}Pt NMR sample.

References

1. FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer. N Engl J Med 2011; 364:1817-1825 DOI: 10.1056/NEJMoa1011923

2. Conroy T, Gavoille C, Samalin E, Ychou M, Ducreux M. The Role of the FOLFIRINOX Regimen for Advanced Pancreatic Cancer. Current Oncology Reports. 2013;15(2):182-189.

3. Masi G, Allegrini G, Cupini S, Marcucci L, Cerri E, Brunetti I et al. First-line treatment of metastatic colorectal cancer with irinotecan, oxaliplatin and 5-fluorouracil/leucovorin (FOLFOXIRI): results of a phase II study with a simplified biweekly schedule. Annals of Oncology. 2004;15(12):1766-1772.

4. Chen H, Hwang J. Ultrasound-targeted microbubble destruction for chemotherapeutic drug delivery to solid tumors. Journal of Therapeutic Ultrasound. 2013;1(1):10.

5. Moran C. Ultrasonic contrast agents. Clinical Ultrasound. 2011;:77-89.

6. Ammi A, Cleveland R, Mamou J, Wang G, Bridal S, O'Brien W. Ultrasonic contrast agent shell rupture detected by inertial cavitation and rebound signals. IEEE Transactions on Ultrasonics, Ferroelectrics and Frequency Control. 2006;53(1):126-136.

7. Tranquart F, Bettinger T, Hyvelin J. Ultrasound and microbubbles for treatment purposes: mechanisms and results. Clinical and Translational Imaging. 2014;2(1):89-97.

8. Fan Z, Kumon R, Deng C. Mechanisms of microbubble-facilitated sonoporation for drug and gene delivery. Therapeutic Delivery. 2014;5(4):467-486.

9. Prentice P, Cuschieri A, Dholakia K, Prausnitz M, Campbell P. Membrane disruption by optically controlled microbubble cavitation. Nature Physics. 2005;1(2):107-110.

10. Gao J, Nesbitt H, Logan K, Burnett K, White B, Jack I et al. An ultrasound responsive microbubble-liposome conjugate for targeted irinotecan-oxaliplatin treatment of pancreatic cancer. European Journal of Pharmaceutics and Biopharmaceutics. 2020;157:233-240.

11. Logan K, Foglietta F, Nesbitt H, Sheng Y, McKaig T, Kamila S et al. Targeted chemo-sonodynamic therapy treatment of breast tumours using ultrasound responsive microbubbles loaded with paclitaxel, doxorubicin and Rose Bengal. European Journal of Pharmaceutics and Biopharmaceutics. 2019;139:224-231.

12. Nesbitt H, Sheng Y, Kamila S, Logan K, Thomas K, Callan B et al. Gemcitabine loaded microbubbles for targeted chemo-sonodynamic therapy of pancreatic cancer. Journal of Controlled Release. 2018;279:8-16.

13. Dimcevski G, Kotopoulis S, Bjånes T, Hoem D, Schjøtt J, Gjertsen B et al. A human clinical trial using ultrasound and microbubbles to enhance gemcitabine treatment of inoperable pancreatic cancer. Journal of Controlled Release. 2016;243:172-181.

14. McEwan C, Kamila S, Owen J, Nesbitt H, Callan B, Borden M et al. Combined sonodynamic and antimetabolite therapy for the improved treatment of pancreatic cancer using oxygen loaded microbubbles as a delivery vehicle. Biomaterials. 2016;80:20-32.

15. McEwan C, Owen J, Stride E, Fowley C, Nesbitt H, Cochrane D et al. Oxygen carrying microbubbles for enhanced sonodynamic therapy of hypoxic tumours. Journal of Controlled Release. 2015;203:51-56.

16. Márquez M, Dotson R, Pias S, Frolova L, Tartis M. Phospholipid prodrug conjugates of insoluble chemotherapeutic agents for ultrasound targeted drug delivery. Nanotheranostics. 2020;4(1):40-56.

17. Ward T, Hartzer M, Blumenkranz M, Lin L. A comparison of 5-Fluorouridine and 5-Fluorouracil in an experimental model for the treatment of vitreoretinal scarring. Current Eye Research. 1993;12(5):397-401.

18. Shuto S, Ueda S, Imamura S, Fukukawa K, Matsuda A, Ueda T. A facile one-step synthesis of 5′-phosphatidylnucleosides by an enzymatic two-phase reaction. Tetrahedron Letters. 1987;28(2):199-202.

19. Kelland L. The resurgence of platinum-based cancer chemotherapy. Nature Reviews Cancer. 2007;7(8):573-584.

20. Wheate N, Walker S, Craig G, Oun R. The status of platinum anticancer drugs in the clinic and in clinical trials. Dalton Transactions. 2010;39(35):8113.

21. Gibson D. Multi-action Pt(IV) anticancer agents; do we understand how they work?. Journal of Inorganic Biochemistry. 2019;191:77-84.

22. Gabano E, Ravera M, Osella D. Pros and cons of bifunctional platinum(iv) antitumor prodrugs: two are (not always) better than one. Dalton Transactions. 2014;43(26):9813.

23. Shi Y, Liu S, Kerwood D, Goodisman J, Dabrowiak J. Pt(IV) complexes as prodrugs for cisplatin. Journal of Inorganic Biochemistry. 2012;107(1):6-14.

24. Wexselblatt E, Gibson D. What do we know about the reduction of Pt(IV) pro-drugs?. Journal of Inorganic Biochemistry. 2012;117:220-229.

25. Johnstone T, Suntharalingam K, Lippard S. The Next Generation of Platinum Drugs: Targeted Pt(II) Agents, Nanoparticle Delivery, and Pt(IV) Prodrugs. Chemical Reviews. 2016;116(5):3436-3486.

26. Wilson J, Lippard S. Synthetic Methods for the Preparation of Platinum Anticancer Complexes. Chemical Reviews. 2013;114(8):4470-4495.

27. Duewell P, Beller E, Kirchleitner S, Adunka T, Bourhis H, Siveke J et al. Targeted activation of melanoma differentiation-associated protein 5 (MDA5) for immunotherapy of pancreatic carcinoma. OncoImmunology. 2015;4(10):e1029698.

28. Moulder J, Rockwell S. Hypoxic fractions of solid tumors: Experimental techniques, methods of analysis, and a survey of existing data. International Journal of Radiation Oncology*Biology*Physics. 1984;10(5):695-712.

29. Matsumoto S, Kishimoto S, Saito K, Takakusagi Y, Munasinghe J, Devasahayam N et al. Metabolic and Physiologic Imaging Biomarkers of the Tumor Microenvironment Predict Treatment Outcome with Radiation or a Hypoxia-Activated Prodrug in Mice. Cancer Research. 2018;78(14):3783-3792.

30. Crowley L, Scott A, Marfell B, Boughaba J, Chojnowski G, Waterhouse N. Measuring Cell Death by Propidium Iodide Uptake and Flow Cytometry. Cold Spring Harbor Protocols. 2016;2016(7):pdb.prot087163.

31. Erstad D, Sojoodi M, Taylor M, Ghoshal S, Razavi A, Graham-O'Regan K et al. Orthotopic and heterotopic murine models of pancreatic cancer and their different responses to FOLFIRINOX chemotherapy. Disease Models & Mechanisms. 2018;11(7):dmm034793.

32. Masi G, Allegrini G, Cupini S, Marcucci L, Cerri E, Brunetti I et al. First-line treatment of metastatic colorectal cancer with irinotecan, oxaliplatin and 5-fluorouracil/leucovorin (FOLFOXIRI): results of a phase II study with a simplified biweekly schedule. Annals of Oncology. 2004;15(12):1766-1772.