Supporting Information

Characterisation of an alginate encapsulated LS180 spheroid model for anti-colorectal cancer compound screening

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Experimental Procedures. *Materials.* Sodium alginate, Poly-L-Lysine, paclitaxel, Adenosine triphosphate (ATP) disodium salt hydrate standard and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (Johannesburg, South Africa), calcium chloride dihydrate ($CaCl_2.2H_2O$) and sodium chloride (NaCl) from UnivAR (SAARCHEM, Johannesburg, South Africa). 1X 0.1 µm sterile filtered phosphate buffered saline (PBS) was purchased from Separations (HyClone; Johannesburg, South Africa). The ToxiLight® BioAssay kit was purchased from Lonza (Whitehead Scientific (Pty) Ltd., Cape Town, South Africa). The Quick Start™ Bradford Protein assay and 2 mg/ml bovine serum albumin (BSA) standards were purchased from Bio-Rad (Lasec SA (Pty) Ltd., Midrand, South Africa). The lysis buffer was donated by Professor Krzysztof Wrzesinski from Celvivo® ApS (Odense, Denmark). The CellTiter-Glo® Luminescent Cell viability assay was supplied by Promega (Anatech Instruments (Pty) Ltd., Johannesburg, South Africa). Microgravity ProtoTissue™ bioreactors were purchased from Celvivo® ApS (Odense, Denmark).

For paclitaxel, a stock solution of 1.171 mM was prepared on a weekly basis by dissolving paclitaxel powder in dimethyl sulfoxide (DMSO), and storing it as four separate aliquots at -20°C. Prior to daily dosing, the stock aliquots were removed from the freezer and thawed in a block heater at 37°C. The stock solution was then diluted to an intermediate concentration of 1.5 µM using culture medium, followed by further dilution in the range of 1 nM – 1 µM. The final DMSO concentration exposed to the cells never exceeded 0.1%.

Culturing LS180 cells. The human colorectal adenocarcinoma cell line, LS180 (American Type Culture Collection cat. no. CL-187™, ATCC, Manassas, VA), was cultured using standard tissue culture conditions in low-glucose Dulbecco's Modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Johannesburg, South Africa). Medium was supplemented with 1% non-essential amino acids (Lonza, Whitehead Scientific, Cape Town, South Africa); 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Johannesburg, South Africa); 1% penicillin/streptomycin (Lonza, Whitehead Scientific, Cape Town, South Africa), 2 mM L-Glutamine (Lonza, Whitehead Scientific, Cape Town, South Africa) and 1% Amphotericin B (Biochrom, Randburg, South Africa). Cultures were incubated at 37°C, 5% CO_2 and 95% humidified air in a Thermo Scientific CO_2 incubator, with growth medium exchanged every second day. Upon reaching 60% confluence, the cells were sub-cultured by scraping the cells from culturing flasks. For seeding, cells were subjected to trypsinisation to obtain a single cell suspension.

Two-dimensional anticancer activity pre-screening. For the MTT assay, 8,000 cells per well was seeded per well in 96-well plates, and incubated at 37ºC, 5% CO_2 and 95% humidified air for 24 h to allow cells to adhere. Culture medium was removed and cells were treated with 200 µl culture medium containing paclitaxel at various concentrations (0 h). Medium containing treatment was replenished in 24 h intervals, until the assay was performed after 96 h exposure. Untreated wells (to indicate 100% cell viability), DMSO background control wells (cells treated with DMSO-medium to eliminate background interference due to the vehicle) and dead cells standard wells (cells treated with Triton X-100 to indicate 97 - 100% cell viability inhibition) received fresh supplemented medium during medium exchanges. After 96 h exposure, medium was removed from all the wells and the cells were washed twice with 100 µl PBS. The dead cell standard wells were treated with 200 µl of 0.2% Triton X-100 (dissolved in PBS) for 15 min, and subsequently carefully washed twice with 100 µl PBS. All wells (excluding the DMSO background standard) then received 180 µl non-additive medium. A volume of 20 µl MTT stock solution (5 mg/ml) was added to each well (excluding the DMSO background control wells) to achieve a final MTT solution concentration of 0.5 mg/ml [55]. The 96-well plate was covered with aluminum foil and placed on a compact rocker for 5 min, followed by further incubation at 37ºC for 4 h. The medium in each well was replaced with 200 µl DMSO. The plate was then shaken on the compact rocker for 1 h. The amount of dye accumulated by the cells in each well was quantified by measuring the absorbance at 560 nm, with a reference wavelength of 630 nm using a SpectraMax® plate reader (Paradigm® Multi-Mode Detection Platform; Molecular Devices®; Separations, Gauteng, South Africa).

The relative percentage viable cells were calculated using Equation 1, whereby the absorbance values for each group were expressed as percentage cell viability relative to the untreated control.

$$\%\ \text{Cell viability} = \frac{\Delta\text{sample} - \Delta\ \text{blank}}{\Delta\text{untreated} - \Delta\ \text{blank}} \times 100 \quad \text{Eq. 1}$$

where Δ sample is the difference between the wavelength value 560 nm and that of the background wavelength 630 nm, measured for all the sample groups on the plate. Δ Blank is the difference

between the wavelength value 560 nm and that of the background wavelength 630 nm of the DMSO control wells. Δ Untreated is the difference between the wavelength value 560 nm and that of the background wavelength 630 nm measured for the untreated control.

Equation 2 was subsequently used to determine the relative percentage cell viability inhibition (IC).

$$\% \text{IC} = 100\% - \% \text{ cell viability} \qquad \text{Eq. 2}$$

All MTT experiments were performed in six-fold. SPSS statistical analysis software (IBM Analytics, Version 25), in conjunction with the Probit Analysis Method, were used to calculate IC_{50} values and 95% confidence limit ranges for paclitaxel, using the data from the MTT analyses.

The IC_{50} values were then divided by the measured soluble protein content (μg) of the 8,000 cells seeded per well. The concentration of the treatment was then divided by the protein content in μg to obtain a 2D IC_{50} value of the treatment per μg of soluble protein.

Bioreactor setup. Bioreactors were prepared by filling the water chambers (C in Figure 4) with distilled water and adding approximately 8 ml culture medium to the cell chamber (B in Figure 4). The bioreactors were placed onto a drive-unit (BAM v 4.6 CelVivo ApS, Odense, Denmark) in an incubator to rotate and equilibrate overnight at 37°C and 5% CO_2. Prior to the transfer of the spheroids to the cell chamber of the bioreactor, all growth medium was removed, and 10 ml fresh medium was added.

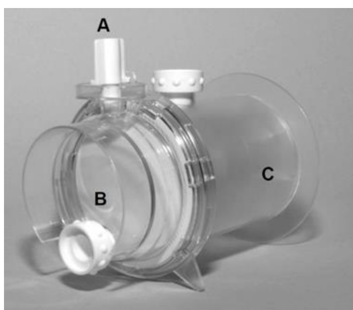

Figure 4. Photograph of a bioreactor used to grow the LS180 spheroids, showing the top opening (A), cell chamber (B) and water chamber (C).

Preparation of sodium alginate encapsulated spheroids. A sodium alginate solution of 2.5% *w/v* was prepared in PBS and autoclaved. The cross-linker solutions, which consisted of 50 mM $CaCl_2$ and 150 mM NaCl, was prepared in distilled water and filtered through a 0.8/0.2 μm Acrodisc® PF Supor® membrane syringe filter (PALL; Separations, Midrand, South Africa) to sterilize the solution.

A cell suspension was prepared from 80% confluent LS180 cells through trypsinisation. After diluting the cell suspension to 2,000 cells/μl the cell suspension was again centrifuged at 140 *x g* for 5 min, the supernatant was removed and the pellet gently re-suspended in the prepared sodium alginate solution at 37°C.

The LS180 cells, imbedded in the sodium alginate solution, were pipetted as 1 μl droplets onto square Perspex blocks covered with hydrophobic paraffin film. After the embedded cells were pipetted onto the blocks, a volume of 0.5 μl cross-linker solution was added to each droplet. Working quickly with an automatic pipette, it is possible to cast 300 spheroids in 6 min. The petri dish was then covered and incubated at room temperature for 5 min. Spheroids were collected into culture medium after incubation and transferred to the bioreactors. Each bioreactor used during characterization of the model contained 300 spheroids on day 0. The speed of each bioreactor was initially set at 16 rotations per minute (rpm).

Encapsulated LS180 cell spheroid maintenance. The day the spheroids were transferred to the bioreactors, was referred to as day 0. The culture medium was exchanged after three days of culturing during the first week, followed by medium exchange every second day thereafter. Rotation speed was adjusted daily to keep the spheroids suspended in essentially a 'stationary orbit'. Photomicrographs of the developing spheroids were taken daily using a Nikon Eclipse TS100 light microscope (Nikon Instruments, Tokyo, Japan) and a DFK 72AUC02 USB 2.0 industrial camera (The Imaging Source, Bremen, Germany).

Characterization of the sodium alginate encapsulated LS180 spheroid model. The optimized sodium alginate encapsulated LS180 spheroid model had to be fully characterized in terms of cell growth and cell viability. To establish the viability of the encapsulated cells, the model was characterized for a period of 20 days. The following parameters were measured: intracellular ATP, soluble protein content, extracellular AK and glucose consumption. During characterization, samples were collected three times per week.

For the first ten days, sampling in microcentrifuge tubes were as follows: five spheroids for the soluble protein assay and three for intracellular ATP assay. From day 11 and onwards, sampling in microcentrifuge tubes was as follows: one spheroid each for intracellular ATP and soluble protein assays. All experiments during this time consisted of two biological replicates, each with three technical replicates. For the duration of the characterization, 200 μl spent medium was sampled for every biological replicate.

After sampling of the spheroids, all culture medium was removed from the microcentrifuge tubes and the spheroids flash frozen with liquid nitrogen. The microcentrifuge tubes were then stored in a -150°C freezer until sample analysis.

The Bradford assay was used to determine the amount of soluble protein per spheroid. Samples were removed from the freezer and allowed to equilibrate to room temperature. Briefly, 450 μl water was added to each biological replicate and mixed vigorously to ensure spheroid lysis. In a clear bottom 96-well plate, 150 μl of each biological replicate was then loaded as three technical replicates, followed by the addition of 10 μl lysis buffer to each well. The protein assay dye reagent (40 μl) was then added to all wells and mixed vigorously. The plate was centrifuged at 1,218 *x g* for 2 min to remove all bubbles. The absorbance was measured with a Spectra-

max® Paradigm plate reader at 595 nm and all samples were quantified relative to a bovine serum albumin (BSA) standard.

The ATP assay was performed directly after sampling to ensure accurate results. An ATP standard concentration series was plated in triplicate (3 x 100 µl) and 100 µl CellTiter-Glo® luminescent lysis buffer was added in black, clear bottom 96-well plates. All culture medium was removed from the sampled spheroids in the microcentrifuge tubes. Following the addition of 300 µl PBS to each biological replicate, it was then mixed vigorously to ensure that spheroids were broken properly. Three technical replicates of each biological replicate were then plated in the 96-well plate. Finally, 100 µl of CellTiter-Glo® luminescent lysis buffer was added to all sample wells and mixed. The plate was covered and shaken in the dark for 40 min. The plate was centrifuged at 1,218 *x g* to remove all bubbles. The luminescence was measured with a Spectramax® Paradigm plate reader and all samples were quantified relative to the known ATP standard.

For the extracellular AK cell death assay, spent culture medium samples were collected in microcentrifuge tubes. The medium (3 biological replicates of 200 µl each) was centrifuged at 140 *x g* for 15 min, and 160 µl of the supernatant was then transferred to a new microcentrifuge tube. The transferred samples were once again centrifuged at 15,000 *x g* for 15 min and 140 µl of the supernatant transferred to a new tube. The samples were then flash frozen and stored at -150°C until further use.

All samples were removed from the freezer and equilibrated to room temperature. The three biological replicates were plated as three technical replicates each (20 µl per well) in black, clear bottom 96-well plates, and 100 µl of AK detection reagent was added. The plate was covered and placed on a compact rocker for 20 min, followed by centrifugation at 1,218 *x g* for 2 min to remove bubbles. The luminescence was measured with a Spectramax® Paradigm plate reader and all samples were quantified relative to a known dead cell standard to determine the number of dead cells per ml of culture medium.

The known dead cell standard was prepared by treating a known concentration of cells with Cyto-Tox Glo® digitonin lysis buffer (Promega; Anatech Instruments (Pty) Ltd., Johannesburg, South Africa). The cell concentration used was 1.117 x 106 cells/ml. The dead cell standard was diluted with heat-treated medium and exposed to the same conditions as the samples.

Following the second centrifugation step of the AK samples and the removal of 140 µl of the spent medium, the remaining medium was used to measure the glucose content of the spent medium.

The OneTouch® Select™ blood glucose monitoring system and OneTouch® Select™ test strips were used for the assay. 3 µl of the spent medium was used from each biological replicate, and loaded on the test strips by means of a pipette. The glucose concentrations were read and noted. Deducting the remaining glucose content from the glucose content in unspent medium, provides an approximation of glucose consumption by the cells in culture.

Data collected were analyzed to determine statistical significance. Data analysis was performed with Statistica® software (TIBCO Software Inc. (2017). Statistica® (data analysis software system), version 13. http://statistica.io.). One-way analysis of variance (ANOVA), followed by the Dunnett post-hoc test for comparison of multiple groups with a control group (time point 0), was used for analysis of model characterization data. Differences were considered statistically significant when $p < 0.05$.

Evaluation of the LS180 sodium alginate encapsulated spheroid model for anticancer treatment screening. The evaluation of the LS180 sodium alginate encapsulated spheroid model for chemotherapeutic treatment screening consisted of two separate experiments or biological replicates. During the first experiment, 150 spheroids were seeded per bioreactor, and then reduced to 50 spheroids per bioreactor when the spheroids were 10 days old. During the second experiment, only 100 spheroids were seeded per bioreactor, and was then reduced to 50 spheroids per bioreactor on day 10. The experiments commenced when the spheroids were 12 days of age. Sampling took place following 0 h, 24 h, 48 h, 72 h and 96 h of exposure to the model chemotherapeutic treatment.

During this study, different bioreactors were set up for each of the various treatment groups. All experimental procedures were the same as described for the characterization. Once the spheroids reached the age of 10 days, they were removed from the bioreactors and pooled together. Subsequently, 50 spheroids were sampled for each of the following treatment groups.

To ensure constant exposure of all spheroids to the same amount of paclitaxel, the total soluble protein content per bioreactor was determined before each new dosing. The initial dosages were based on the IC_{50} concentration for paclitaxel determined in 2D cultured LS180 cells. To establish the 2D IC_{50} concentration per protein, the determined 2D IC_{50} dose was divided by the measured soluble protein content per well containing 8,000 seeded cells. The soluble protein content was established as 2.606 µg soluble protein per well ($n = 6$; standard deviation = 0.233). These values were then multiplied by the amount of soluble protein per spheroid based on the number of spheroids left in the bioreactor for that specific day. This then gave the dosage per bioreactor per day.

The treatment groups and their IC_{50} dosages per protein were established as:

- untreated control,
- paclitaxel [IC_{50}] (concentration paclitaxel per µg protein, based on the IC_{50} values obtained in the 2D cultures following the MTT assay) = 0.006 mg/ml/µg protein,
- paclitaxel 2[IC_{50}] (two times the concentration paclitaxel per µg protein, based on the IC_{50} values obtained in the 2D cultures following the MTT assay) = 0.012 mg/ml/µg protein.

Each treatment groups' spheroids were placed in their respective bioreactors, and the bioreactors were then filled with preheated culture medium. Bioreactors were returned to the drive unit, and speed was adjusted until they reached the age of 12 days.

The total soluble protein content of each bioreactor needed to be determined daily, prior to the drug dosing. This quantification then enabled daily dosing of the spheroids per protein content of each bioreactor. The soluble protein content was determined following sampling of one spheroid from each treatment group, as previously described, with the exception that the assay was conducted immediately, and not following storage at -80°C. Each spheroid sampled per treatment group were lysed and divided into three technical replicates.

The measured soluble protein content was used to determine the dose per protein for the various treatment groups. The soluble protein content (µg) per spheroid was multiplied by the number of spheroids in each bioreactor per day to obtain a total protein mass (µg) for the bioreactor, and the doses were adapted accordingly. This adjustment ensured that the spheroids were constantly exposed to a constant amount of drug for the duration of the experiment.

Following sampling for the soluble protein content, intracellular ATP, extracellular AK, as well as glucose content, the bioreactors were filled with the prepared treatments of the previous day. At time point 0 h, the bioreactors were filled with unspent medium. Once the soluble protein content for each treatment group was determined and the new dose per bioreactor calculated, the new culture medium containing the treatments was prepared and the medium of each bioreactor changed.

For paclitaxel, a stock solution was prepared and the necessary dilutions were made in preheated culture medium.

qRT-PCR was performed. After sampling of the spheroids, all culture medium was removed from the microcentrifuge tubes and 200 µl RNAlater (Whitehead Scientific (Pty) Ltd., Cape Town, South Africa) solution was added to each sample tube. The microcentrifuge tubes were then stored in a -80°C freezer until further use.

Before ribonucleic acid (RNA) extraction, samples were thawed at room temperature followed by centrifugation (100 *x g* for 10 min). Total RNA was extracted using the PureLink™ RNA Mini Kit according to the manufacturer's guidelines, followed by RNA quantification using a NanoDrop™ One/OneC UV-Vis spectrophotometer (Thermo Fisher, Wilmington, DE, USA). Complementary deoxyribonucleic acid (cDNA) was synthesized using 2 µg of total RNA and the High-Capacity cDNA reverse transcription kit, followed by real-time PCR using TaqMan™ Fast advanced Master Mix (Thermo Fisher Scientific, Johannesburg, South Africa), according to the manufacturer's guidelines, on the C1000Touch™ Thermal Cycler with 96-Well Fast Reaction Module (Bio-Rad, Singapore). FAM-labelled TaqMan™ Gene Expression Assays were used for the following genes: P-glycoprotein (P-gp) (ABCB1-Hs00184500_m1), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) and TATA-box binding protein (TBP) (Hs00427620_m1). GAPDH and TBP were used as housekeeping genes to normalize the data, and to guarantee the comparability of the calculated mRNA (messenger RNA) expression in all samples analyzed. All PCR analyses were performed in biological triplicates. Threshold cycle (Ct) values and data were further analyzed with Bio-Rad CFX Maestro Software v1.1 (Bio-Rad CFX Maestro. Ink). Relative gene expression was calculated using the 2-ΔΔCt method [56]. Real-time PCR data were analyzed by means of one-way ANOVA with Bonferroni post-hoc test using Bio-Rad CFX Maestro 1.1 (4.1.2433.1219) software. Differences were considered statistically significant when $p < 0.05$.