SUPPLEMENTARY INFORMATION

Aromatic carbohydrate amphiphile disrupts cancer spheroids and prevents relapse

Alexandra Brito,[a-d] Patrícia M. R. Pereira,[c] Rui L. Reis,[a,b] Rein V. Ulijn,[d-f] Jason S. Lewis,[c,g-j] Ricardo A. Pires,*[a,b] and Iva Pashkuleva*[a,b]

[a]3B's Research Group, I3Bs - Research Institute on Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, Parque de Ciência e Tecnologia, Zona Industrial da Gandra, 4805-017 Barco, Guimarães, Portugal; [b]ICVS/3Bs - PT Government Associate Laboratory, Braga/Guimarães, Portugal; [c]Department of Radiology, Memorial Sloan Kettering Cancer Center; [d]Advanced Science Research Center (ASRC) at the Graduate Center, City University of New York (CUNY), 85 St Nicholas Terrace, New York, New York 10031, USA; [e]Department of Chemistry, Hunter College, City University of New York, 695 Park Avenue, New York 10065, USA; [f]PhD programs in Biochemistry and Chemistry, The Graduate Center of the City University of New York, New York 10016, USA; [g]Department of Radiology, Weill Cornell Medical College, New York, NY 10065, USA; [h]Molecular Pharmacology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA; [i]Department of Pharmacology, Weill Cornell Medical College, New York, NY 10065, USA; [j]Radiochemistry and Molecular Imaging Probes Core, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

Materials and Methods

*Synthesis and purification of N-fluorenylmethyloxycarbonyl-glucosamine-6-phosphate (**1**).*

The compound **1** was obtained from the respective glucosamine-6-phosphate using our previously described one-step procedure.[1-3] Briefly, the sodium salt of the glucosamine-6-phosphate was dissolved in water (30 g/L) in the presence of 2 eq sodium hydrogen carbonate (Riedel-de Haen, Germany) and reacted with 1.5-2 eq 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl, Sigma, Germany) dissolved in dioxane until complete consumption of the carbohydrate (detected by TLC). The product was purified by column chromatography (230-400 mesh silica gel) to remove the non-reacted Fmoc (eluent EtOAc:MeOH:H_2O, 5:3:2). The purity of the compound was confirmed by HPLC, MS, and NMR.

Monolayer (2D) cell cultures

SaOs2 osteosarcoma cells, MCF-7 and HS578T breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). SaOs2 and HS578T cells were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM, Sigma or MSKCC media preparation core). MCF-7 cells were cultured in Eagle´s Minimum Essential Medium (EMEM; Corning, NY, USA or MSKCC media preparation core) with 1.5 g/L sodium bicarbonate, non-essential amino acids, L-glutamine and sodium pyruvate. Both culture media were supplemented with 10% (v/v) of fetal bovine serum (Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B (Sigma). The cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Spheroids formation and characterization

Spheroids were generated by growing cancer cell suspensions in agarose-coated 96 well plates.[4] The agarose prevented the cells from attaching to the bottom of the wells. Briefly, 150 µl/well of 1.5% (w/v in PBS) agarose solution (MP Biomedicals, Santa Ana, CA, USA) was added to the wells of a 96-well microplate. Cells (SaOs2, MCF-7 or HS578T with densities ranging from 2,500 to 20,000 cells per well) were then added and the microplate was hand-spun gently. Clusters of cancer cells were observed after 24 h of seeding, except for SaOs2 cells. Within the following 24 h the clusters formed spheroids for the HS578T cells, *i.e.* stable round aggregates which are not dislodged by pipetting. Images of clusters/aggregates/spheroids were obtained at 24, 48 and 72 h after cell culturing using the image analysis system consisting of Nikon Eclipse Ti fluorescent microscope and an Andor iXon EMCCD camera. The size of at least 6 spheroids was calculated for each cell line by measuring two orthogonal diameters (d1 and d2) using the line morphometry function. Volumes were calculated using equation 1:

$$\text{Volume} = \frac{4}{3}\pi r^3 \qquad \text{equation 1}$$

where $r = \frac{1}{2} \times (d1 + d2)$ is the geometric mean radius. Average cell number per spheroid was also determined at 24, 48 and 72 h after cell culturing by trypsinizing six different spheroids, mixing the cell suspension with Trypan blue (Sigma) and counting the number of total and viable cells, using a Vi-CELL XR, Cell Counter - Beckman Coulter.

*Treatment of spheroids and 2D flat cultures with **1***

Monolayers and spheroids cultures were supplemented with 0.5 mM or 1 mM of compound **1**, dissolved in media specifc for each cell line. The supplemented cultures were maintained for 24, 48 or 72 h and then analyzed as described below.

Lactate dehydrogenase (LDH) assays

The cytotoxicity of **1** was assessed using the CytoTox 96H Non-Radioactive cytotoxicity assay kit (Promega, USA) according to the manufacturer's instructions. Unlike other studies reporting the use of MTT for quantification spheroid viability, we were not able to apply this assay because the results were neither reproducible nor consistent (data not shown). Therefore, we used CytoTox 96 Assay to quantify the LDH released from the spheroids as an indicator of cytotoxicity. Briefly, 50 µL of culture medium was collected from the well (96-well plate) containing cell spheroids and mixed with 50 µL of the CytoTox 96 Reagent in dark for 30 min at room temperature. Then 50 µL of stop solution was added to each well and the absorbance was recorded at 490 nm using a microplate reader (PowerWave HT Microplate Spectrophotometer). The average values of the culture medium background were subtracted from all values of experimental wells. The protein concentration was determined by Pierce BCA Protein Assay Kit after scrapping spheroid cultures in 1% (m/v) SDS solution in PBS (pH 7.0), and LDH activity was normalized to the protein concentration. The results were normalized to the maximal LDH release, which was determined by treating the control wells for 60 min with 1% Triton X-100 to lyse all cells.

Transfection assay

GLUT1 was depleted using a pool of three target-specific 19–25 nt siRNA and CAV1 with a pool of 20–25 nt siRNA (Santa Cruz Biotechnology). [4] Cancer cells were transfected with either SLC2A1-siRNA, siRNA-CAV1 or scrambled (scr) siRNA (controls). Each transfection was performed for 5 h with 2.4 µM of siRNA in transfection medium (Santa Cruz) containing 0.5 µL/cm^2 of transfection reagent (Santa Cruz). After incubation with siRNA , complete media was added and the cells were incubated for 48 h. CAV1 and GLUT1 downregulation was assessed at 24, 48 and 72 h post-transfection by Western Blotting.

ALP inhibition

For alkaline phosphatase inhibition assays, we used the Pierce phosphatase inhibitor (Thermo Scientific) according to the manufacturer instruction (1 tablet per 10 mL of culture medium with FBS).

Preparation of cell extracts and Western Blot

Scraped monolayer cells and spheroids cultured for 48 h were collected at 1,500 g for 5 min, washed twice with ice-cold PBS and whole protein lysates were extracted using RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM ethylene glycol tetra-acetic acid, 1% Triton X- 100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethanesulfonyl, 2 mM iodoaceta- mide, and 1x protease inhibitor cocktail (Roche, Indianapolis, USA)). Cell extracts were centrifuged at 16,000 g for 10 min at 4°C. After centrifugation, supernatants were used for protein quantification using the Pierce BCA Protein Assay Kit, followed by denaturation of the sample with Laemmli buffer. For the Western Blot analysis, 40 µg proteins were loaded per lane on sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE). Following electrophoresis and transfer to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA), the blots were incubated in 5% (w/v) BSA in TBS-T (20 mM Tris, 150 mM NaCl, Tween 0.2%, pH 7.6). Membranes were then incubated with rabbit anti-GLUT1 1:1,000 (Thermo Fisher Scientific), rabbit anti- CAV1 1:1,000 (Thermo Fisher Scientific), rabbit anti-ALP 1:1,000 (Thermo Fisher Scientific) and rabbit anti-β-actin 1:1,000 (Thermo Fisher Scientific) antibodies. After washing, the membranes were incubated with IRDye 800CW anti- Rabbit IgG 1:15,000 (LI-COR Biosciences, Lincoln, NE, USA) and imaged on the Odyssey Infrared Imaging System (LI-COR Biosciences) followed by densitometric analysis.

Immunofluorescence microscopy

Spheroids were grown for 48 h and then treated as described above. Live-dead staining: after treatment, the spheroids were placed in a new 96 well plate without agarose and were stained with calcein AM (CA, 3 μM) and propidium iodide (PI, 3 μM) in 200 μl of phosphate-buffered saline (PBS). Imaging with a Leica TCS SP5 Confocal Laser Scanning Microscope was performed 3h after the staining. Immunolocalization: spheroids were placed in Optimal Cutting Temperature (OCT) mounting media and frozen immediately. After that, blocks were cut in sections of 10 μM. The sections were then dried at room temperature and fixed for 10 min with cold acetone. After washing with PBS, the spheroid was permeabilized with 0.25% v/v Triton X-100 in PBS with 0.02% w/v BSA in a humidified chamber and blocked with 10% v/v normal goat serum for 2h at room temperature. The spheroids were then incubated overnight at 4 °C with HIF-1α-488 (Abcam; 1:100), CAV1 (Abcam; 1:500), GLUT1 (Sigma-Aldrich, 1:100) or ALP (Abcam, 1:250). The spheroids were then rinsed with 0.02% BSA, 0.02% (w/v) sodium azide (NaN_3) in PBS. Secondary fluorescent antibody Alexa Fluor 488 (Abcam, 1:1000) was applied for 1h at room temperature, except for HIF-1α-488, which is fluorescent-labeled. The spheroids were rinsed with 0.02% (w/v) NaN_3 and were incubated with DAPI (Abcam, 1:1000) for 15 min at room temperature, followed by rinsing with 0.02% (w/v) NaN_3. The spheroids were mounted in glycergel mounting medium and were imaged using a Leica TCS SP5 Confocal Laser Scanning Microscope. Sections were also submitted to MSKCC Molecular Cytology Core Facility for hematoxylin and eosin staining. In all experiments, untreated spheroids were used as controls and manipulated following the same protocols as for the treated ones.

Fluorescence quantification

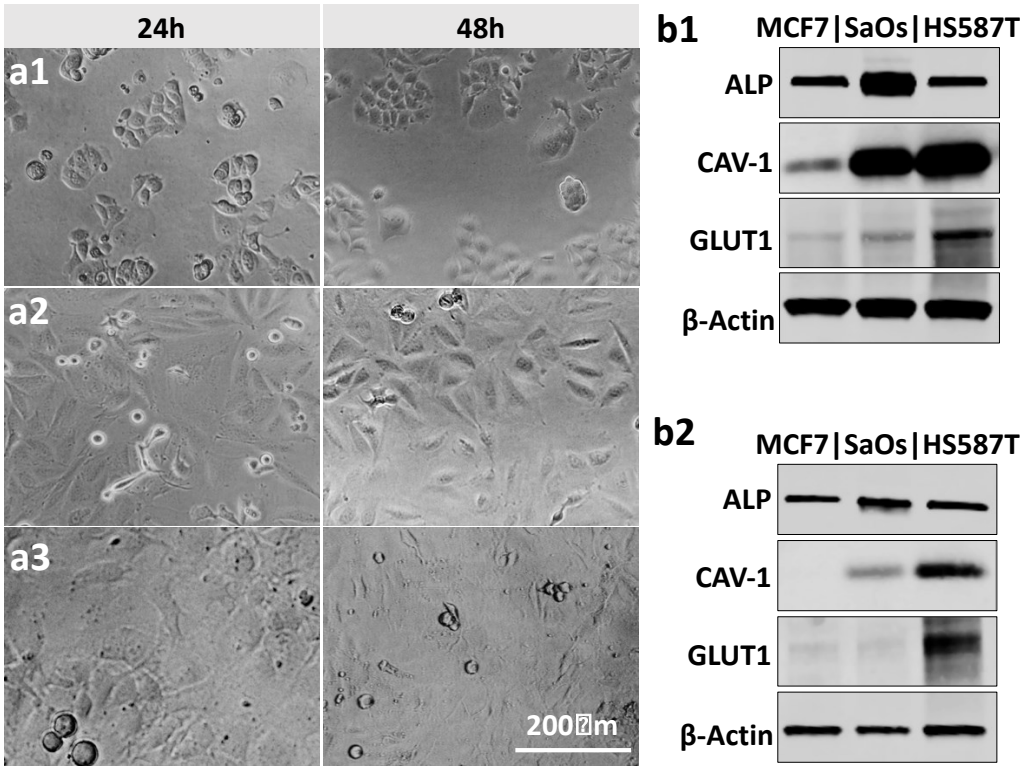
Each of the channels (green and blue) of the images obtained by immunofluorescence microscopy after staining for ALP, GLUT1 or CAV1 was converted to gray scale. To quantify the expression of each protein, we used Cell Profiler 3.0 Software. The threshold was optimized for each signal to separate it from the background and the respective maximum intensity per total area was determined. The obtained value for the green signal was divided by the blue one*, i.e.* protein signal was normalized by the number of cells. For each condition 6-10 images were analyzed.

Scanning electron microscopy

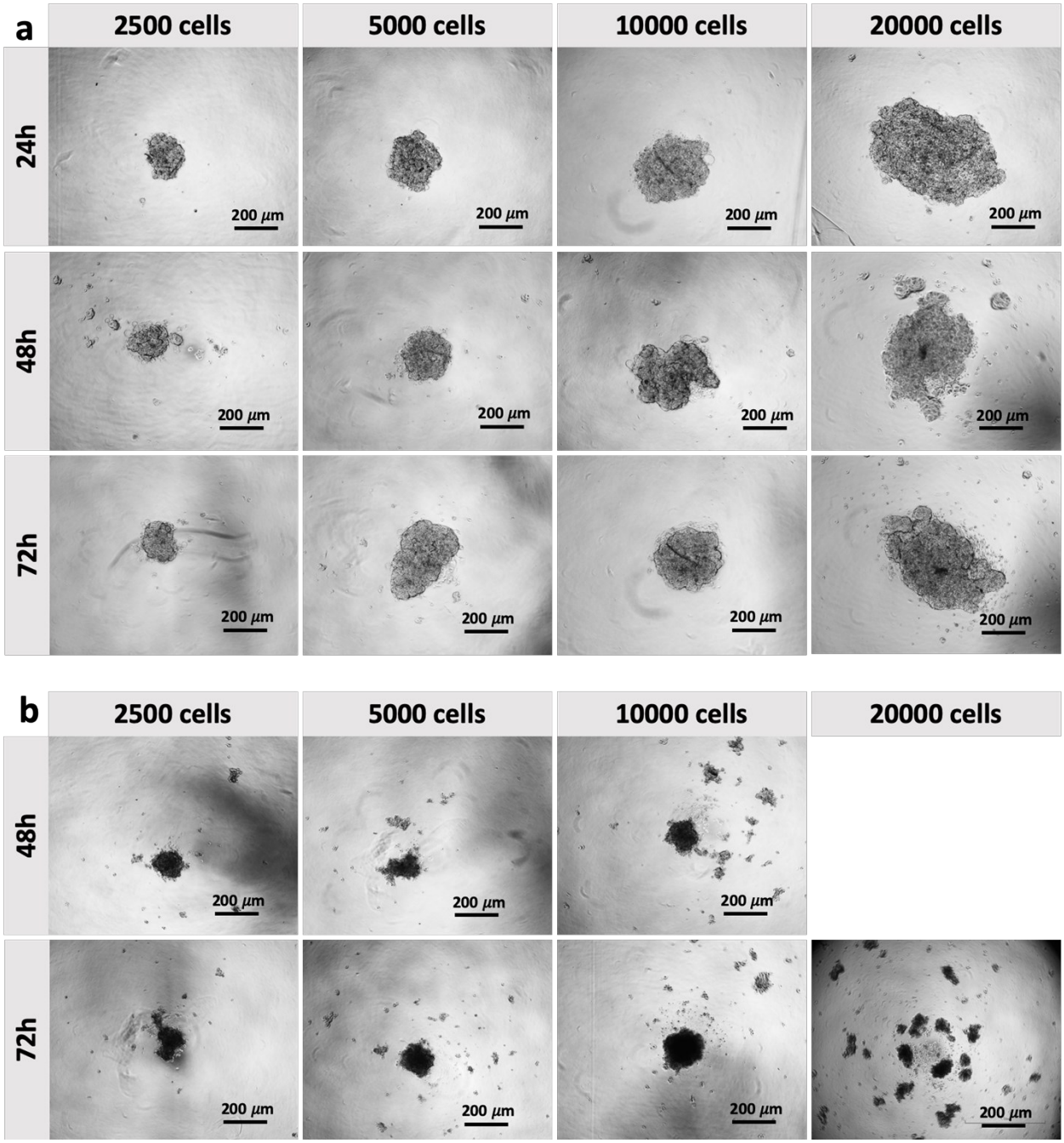
The spheroids were placed onto a silica-wafer, dried at room temperature and imaged using high-resolution scanning electron microscopy (HRSEM, Auriga Compact, ZEISS) at 5kV after coating with 1nm of platinum.

Statistical analysis

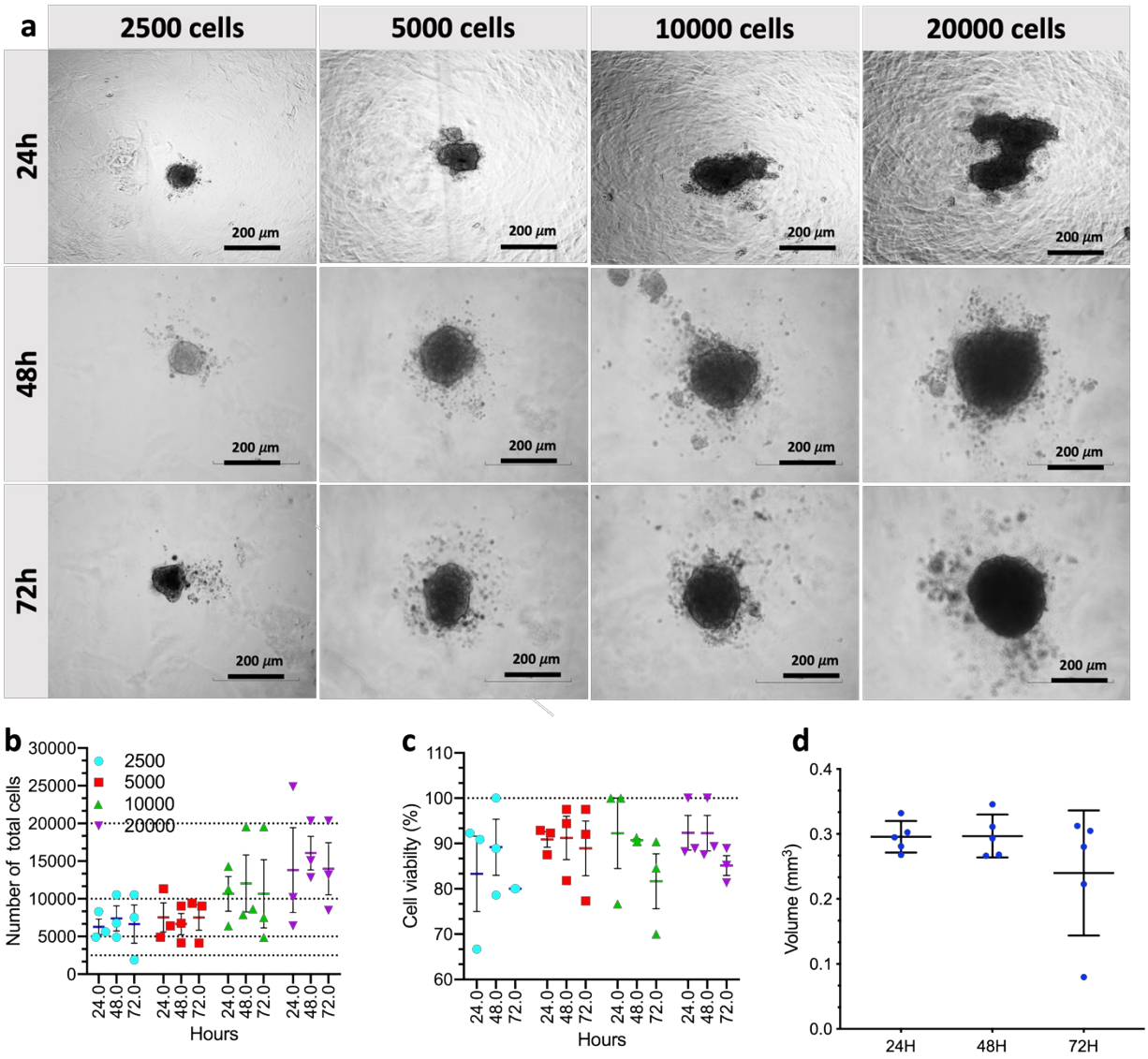
Statistical analyses were carried out using a statistical program (GraphPad Prism; GraphPad Software). Student's *t*-test was used to evaluate the effect of the treatment compared with the control. P- values were considered at the 5% level of significance to deduce inference of the significance of the data, were * $p < 0.01$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$.



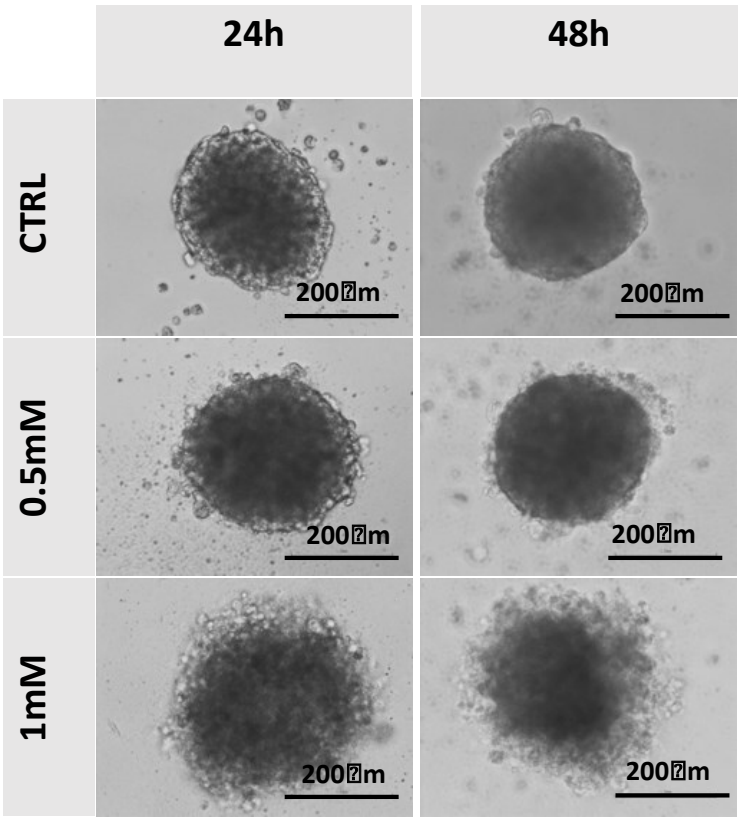
Supplementary figure S1. (a) Microscopy images of **(a1)** MCF7, **(a2)** SaOs2, and **(a3)** HS587T cells seeded on flat tissue culture plates for different time and **(b)** Western-Blot analysis of ALP, CAV-1, and GLUT1 expression by **(b1)** these cells and **(b2)** spheroids/aggreagates generated from them 48h after seeding. β-Actin was used to normalize the data in the densiometry analysis presented in Figure 1 of the main manuscript.



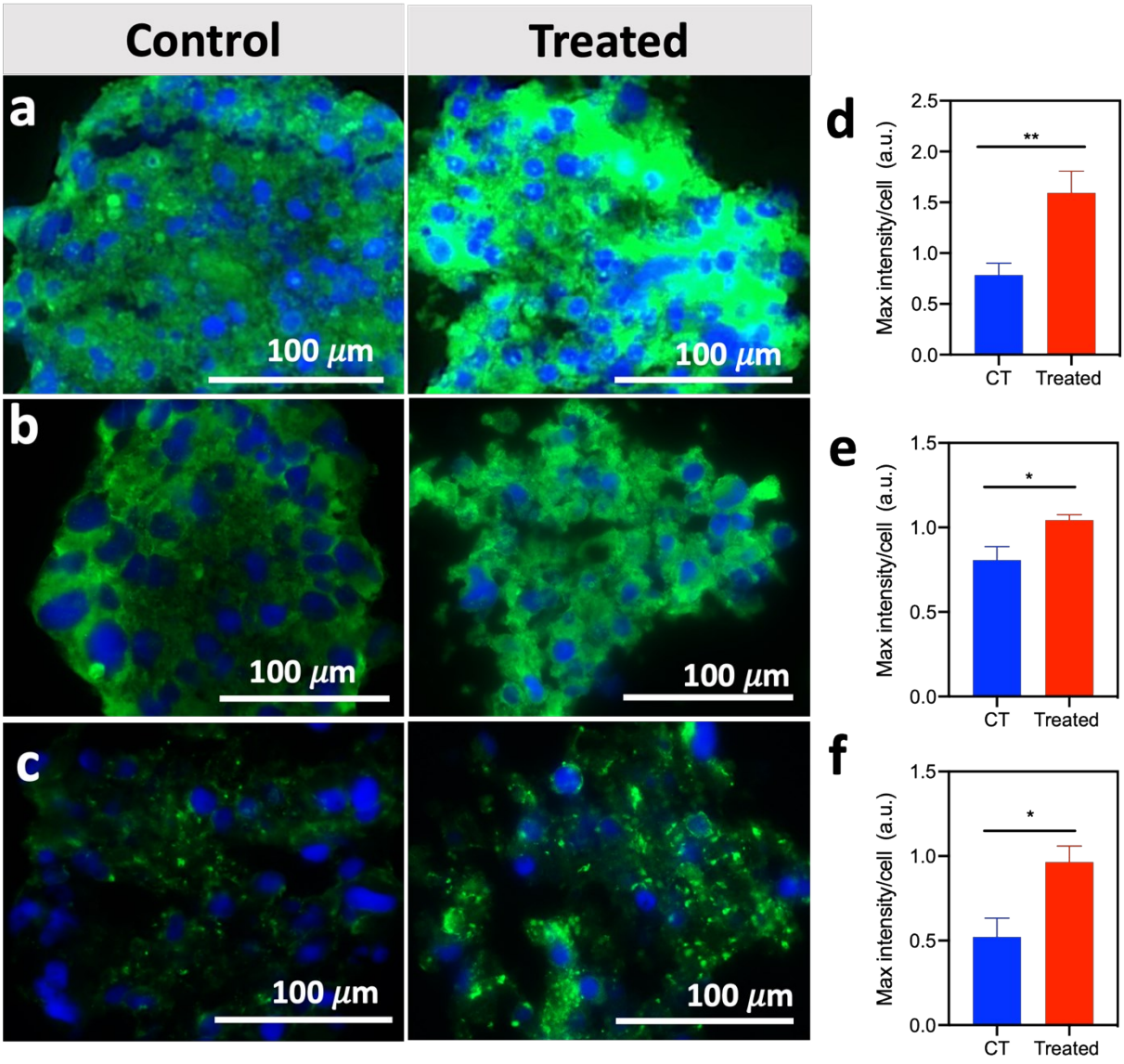
Supplementary figure S2. Ability of **(a)** MCF7 and **(b)** SaOs2 cells to form spheroids at different cell seeding desity (2,500 – 20,000 cells per well) and timeframe (24 – 72 h). No cells aggregates or spheroids were observed after 24 h for SaOs2 cells.



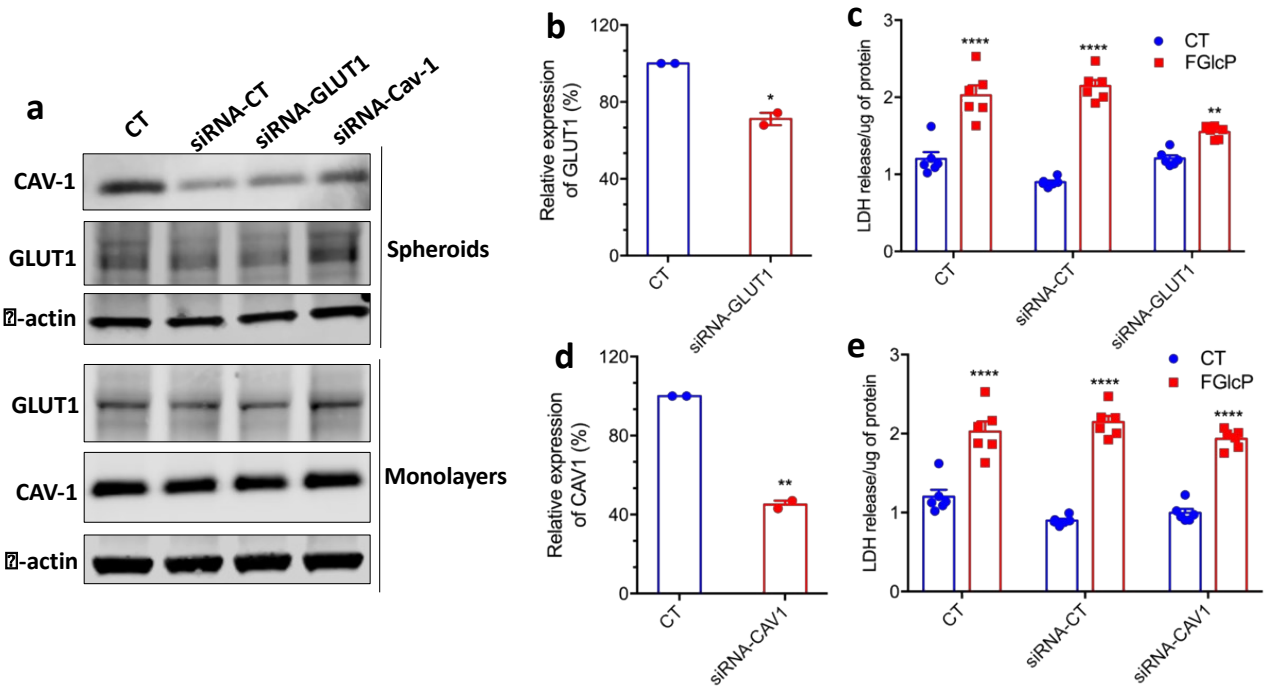
Supplementary figure S3. Effect of the conditions (seeding density and culture time) on the formation of HS578T spheroids: (**a**) representative optical microscopy images of the aggregates/spheroids; (**b**) number of total HS578T cells forming the aggregates/spheroids and (**c**) their viability (obtained from the ratio between live cells and the total number of cells within the 3D cell structure) as a function of time; (**d**) volume of the spheroids generated at a cell seeding density of 5,000 cells/well.



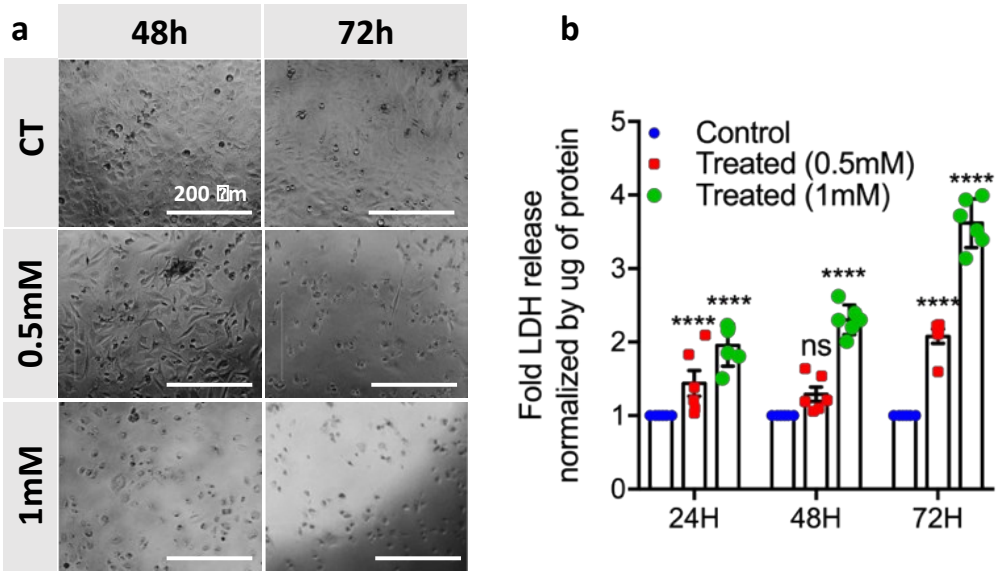
Supplementary figure S4. Light microscopy images of untreated spheroids (control, CTRL) and spheroids exposed to **1** at different concentration (0.5 and 1 mM) and for different timeframe (24 and 48 h).



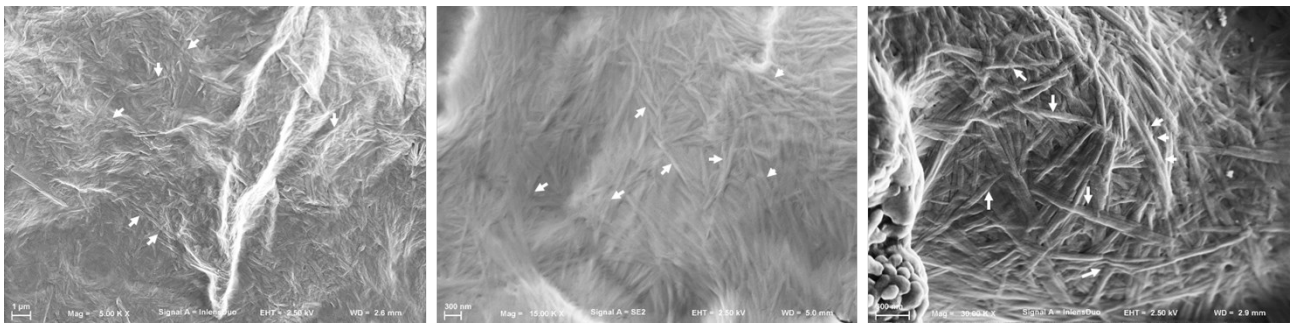
Supplementary figure S5. Immunolocalization of (**a**) ALP, (**b**) GLUT1, and (**c**) CAV1 showed by confocal microscopy images of untreated spheroids (control) and spheroids exposed to **1** (1 mM, 48 h). Expression of (**d**) ALP, (**e**) GLUT1, and (**f**) CAV1 obtained by quantification of the fluorescence using Cell Profiler 3.0 Software. Statistical data were obtained using t-test $^{**}p < 0.01$; $^{*}p < 0.05$.



Supplementary figure S6. (**a**) Western Blot analysis and respective densitometry analysis showing the relative expression of (**b**) GLUT1 and (**d**) CAV1 in HS587T spheroids after depletion of the respective genes. Controls (CT) for the data presented on Figure 3 in the main manuscript: effect of (**c**) GLUT1 and (**e**) CAV1 knockout on the cell viability determined by LDH release. siRNA-CT is abbreviation used for non-specific siRNA.



Supplementary figure S7. (**a**) Representative images of the monolayers of HS578T cells supplemented with **1** (0 - 1mM) for different time (48-72h) and the respective (**b**) cell viability indicated by LDH release; * $p < 0.01$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$.



Supplementary figure S8. Supplementary high-resolution scanning electron microscopy images of spheroids treated with **1** (1 mM, 48 h). White arrows indicate different fibers bundles.

References

1. R. A. Pires, Y. M. Abul-Haija, D. S. Costa, R. Novoa-Carballal, R. L. Reis, R. V. Ulijn and I. Pashkuleva, *J Am Chem Soc*, 2015, **137**, 576-579.
2. A. Brito, Y. M. Abul-Haija, D. S. da Costa, R. Novoa-Carballal, R. L. Reis, R. Ulijn, R. A. Pires and I. Pashkuleva, *Chem Sci*, 2019, **10**, 2385-2390.
3. L. S. Birchall, S. Roy, V. Jayawarna, M. Hughes, E. Irvine, G. T. Okorogheye, N. Saudi, E. De Santis, T. Tuttle and A. A. Edwards, R. V. Ulijn, *Chem Sci*, 2011, **2**, 1349-1355.
4. P. M. R. Pereira, N. Berisha, N. Bhupathiraju, R. Fernandes, J. P. C. Tome and C. M. Drain, *PLoS One*, 2017, **12**, e0177737.