

High-throughput spheroid culture

The cost of drug development is becoming unsustainable, partly due to the high attrition rate of drugs in clinical trials. The predominant reasons for drug failure are lack of efficacy, poor pharmacokinetics and adverse toxic side-effects not identified during preclinical studies. These challenges of optimising drug discovery mandate a need for improved preclinical models – specifically those that better recapitulate the pathophysiology of the underlying disease, as well as more accurately predict *in vivo* responses to therapeutic compounds.

Two-dimensional (2D) cell culture models are frequently used in drug discovery due to their ease of use and established compatibility with high-throughput screening automation and detection instrumentation. While 2D cell cultures are widely used in drug research, it is clear that they exhibit different structural and functional properties than observed in the body. Therefore, three-dimensional (3D) spheroid cell culture models that closely mimic *in vivo* conditions are gaining acceptance, thus bridging the gap between *in vitro* studies and predictive activity in the clinic.

Cellular spheroids are 3D models generated from many cell types; spheroids form because of the tendency of adherent cells to aggregate. Common examples of spheroids include embryoid bodies, mammospheres, tumour spheroids, hepatospheres and neurospheres. Furthermore, adherent cells naturally aggregate and form spheroids, particularly under circumstances that impede adhesion to cell culture substrates. Common matrix-free methods employed for generating spheroids include the use of attachment-resistant cell culture surfaces or by maintaining the cells as suspension cultures in media (eg hanging drop technology, rotary cultures

and bioreactors). Several cell types also form spheroids when grown in hydrogels and, to a lesser extent, in solid scaffolds, depending on the structural and physical properties of the material.

Taken together by recapitulating cell-cell interactions, metabolic gradients and cell polarity observed *in vivo*, 3D multicellular spheroids provide more physiologically-relevant models compared to two dimensional (2D) cell culture. For this reason, the use of 3D technologies, already established in basic research, is now gaining acceptance in drug discovery.

Spheroids mimic various aspects of solid tissues and possess inherent gradients for optimal diffusion of oxygen and glucose, for example, as well as the removal of metabolic wastes. These cellular aggregates can emulate avascular, solid tumour behaviour more effectively than standard 2D environments because spheroids, much like tumours, contain a heterogeneous population. These can comprise surface-exposed and deeply-buried cells, proliferating and non-proliferating cells, as well as highly-oxygenated and hypoxic cells.

Spheroids thus represent good physiological 3D models for studying solid tumorigenesis. In terms

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of assessing compound action, spheroids can be readily analysed by imaging using light, fluorescence and confocal microscopy – a major advantage over other, more complex, 3D cell culture models, including organ-on-a-chip approaches. As interest grows in these areas, technologies are now being developed to enable mass production of uniformly-sized 3D spheroids, making them highly amenable for *in vitro* high throughput and toxicity screening applications.

Despite the advantages of spheroid culture, nonetheless, limitations of current technologies to enable simple and reliable assays have impeded the rapid adoption of 3D models for high-throughput screening (HTS). Indeed, conventional methods for 3D cell culture are time-consuming, display increased variability and lack the throughput required for automated HTS.

Below, we describe applications of high-throughput screening using 3D cell culture to assess cell viability. In addition, we highlight the importance of including multiple cell types in 3D assays to more accurately assess potency of chemotherapeutic drug candidates.

Co-culture with cells of the microenvironment

Understanding the complex interactions between cancer cells and other cell types in the tumour microenvironment, such as fibroblasts, endothelial cells and immune cells, is critical to predict therapeutic efficacy. This is important as many factors, including the secretion of cytokines and extracellular matrices, may impact drug resistance and sensitisation¹⁻³. 3D cultures develop hypoxic cores and demonstrate gradients of various soluble factors and a diffusion profile for drugs similar to tumours.

As shown in the following studies, the presence of primary cell types, as well as endothelial cells together with tumour cells, in a spheroid can affect the potency of chemotherapeutic compounds.

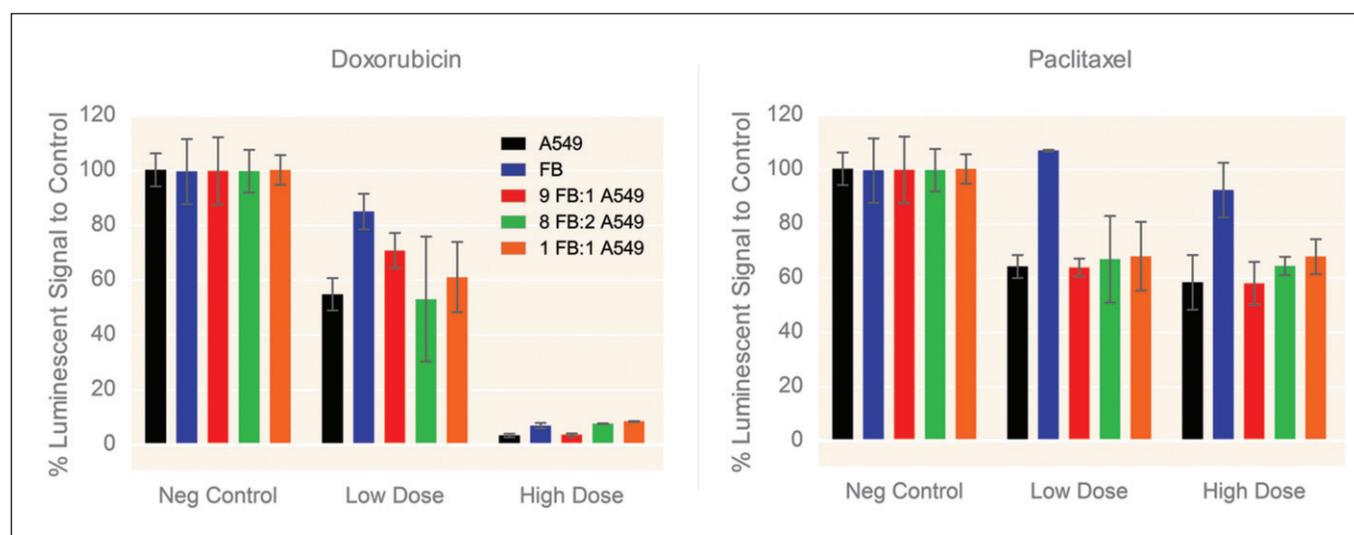
A549 cells, a human lung carcinoma cell line, were cultured in Corning spheroid microplates with and without co-culture conditions, including primary human lung fibroblasts (FB) and peripheral blood mononuclear cells (PBMC). Tumour spheroid viability was screened after treatment with various chemotherapeutics in both single culture and co-culture conditions. Dose-dependent responses of selected chemotherapeutics were compared, demonstrating the impact that including multiple cell types in 3D assays can have on experimental results.

Mono- and co-culture spheroid formation

A549 cells (ATCC® Cat. No. CCL-185) and normal human lung fibroblasts (FB; Lonza Cat. No. CC-2512) were subcultured following vendors' protocols. A549 cells were cultured in F-12K (Corning Cat. No. 10-025-CV) supplemented with 10% Fetal Bovine Serum (FBS) (Corning Cat. No. 35-010-CV) and FB were cultured in supplemented Fibroblast Basal Medium FGM-2 (FB medium) (Lonza Cat. No. CC-3132). Confluent cells were harvested and seeded into 384-well spheroid microplates (Corning Cat. No. 3830), adding 20µL of FB media with 2,000 cells per well. For chemotherapeutic dose response assays, A549 cells and FB were seeded at 2,000 cells per well in monoculture and in co-culture conditions with a 9:1 ratio of FB to A549 cells. Spheroid microplates were covered with breathable membrane sealing tape (Corning Cat. No. 3345) and pulse centrifuged at 130 x g prior to culture in a humidified

Compound	A549 TC ₅₀ (M)	FB TC ₅₀ (M)	A549+FB TC ₅₀ (M)	A549+FB+PBMC TC ₅₀ (M)
Carboplatin	1.48E-4	3.75E-4	1.95E-4	1.53E-4
Erlotinib	4.25E-5	N/A	N/A	N/A
Vinorelbine	6.26e-5	3.87E-5	6.92E-4	5.72E-4
Doxorubicin	1.38E-6	1.83E-4	1.70E-4	1.69E-4
Etoposide	8.51E-6	>5.00E-4	>5.00E-4	>5.00E-4
Cisplatin	7.07E-5	>2.00E-4	>2.00E-4	>2.00E-4
Paclitaxel	N/A	N/A	N/A	N/A
Gefitinib	>2.00E-3	N/A	N/A	N/A
Vincristine	N/A	N/A	N/A	N/A

Table 1: Toxicity potency (TC₅₀) values of chemotherapeutic compounds applied to mono-, co- and tri-culture spheroids



37°C, 5% CO₂ incubator for 48 hours. For tri-culture conditions, PBMC (AllCells Cat. No. PB003F) were thawed into IMDM (Corning Cat. No. 10-016-CM) with 10% FBS, centrifuged at 200 x g for 15 minutes, and resuspended in FB media. PBMC were added to the 384-well spheroid microplates, containing 48 hour mono- and co-culture A549 and FB spheroids, with 1800 cells in 10µL of FB media per well. As a control for mono- and co-culture conditions without PBMC, 10µL of FB media was added per well. Spheroids were incubated for an additional 48 hours prior to fixing or staining.

Chemotherapeutic screening

To determine the optimal seeding conditions, chemotherapeutic compounds doxorubicin and paclitaxel were used. The compounds included for chemotherapeutic testing are listed in Table 1. Immediately following media or PBMC addition, 10µL per well of compounds in FB media with 10% DMSO were added and mixed twice using a CyBi®-Well Pipettor. Vehicle control consisted of 10µL of FB media with 10% DMSO. Spheroids were cultured for an additional 48 hours prior to cell viability assays. For each compound, this was performed in triplicate two independent times.

For cell viability assays, spheroids were assayed in the spheroid microplate using CellTiter-Glo® 3D (Promega Cat. No. G9683). 40µL per well of CellTiter-Glo 3D reagent was added directly to the spheroid microplates 48 hours after compound addition. Microplates were shaken for five minutes and then incubated for 25 minutes at room temperature. Luminescence was measured using a Tecan Infinite® M1000 plate reader. The concentration at which half of the cells were no longer viable, as

measured by comparing luminescence to vehicle control wells, was calculated as toxicity potency (TC₅₀) for each compound.

A549 cells and FB can be incorporated into co-culture spheroids using different seeding ratios. To determine the optimal cell seeding ratio for a chemotherapeutic screen, co-culture spheroids were formed using FB to A549 cell ratios of 9:1, 8:2, and 1:1. After 48 hours in culture, a dose series of the chemotherapeutic agents doxorubicin and paclitaxel were added, and the spheroids were cultured for an additional 48 hours. Each mono- and co-culture condition resulted in a single spheroid per well (Figure 2). After a total of 96 hours in culture, cell viability was assessed using CellTiter-Glo 3D cell viability assay. As shown in Figure 1, FB mono-culture demonstrated higher cell viability after 48-hour exposure to low dose doxorubicin than A549 mono-culture spheroids. A 9:1 ratio of FB to A549 cells also displayed this protective effect of FB from the low-dose doxorubicin exposure. FB mono-culture also displayed resistance to paclitaxel treatment at both high and low doses compared to the effects seen on A549 mono-culture spheroids, however the presence of FB did not demonstrate protective effects on A549 cells. The protective effects of FB on A549 cells in the presence of a low dose of doxorubicin can also be visualised using viability and cytotoxicity stains (Figure 2).

Chemotherapeutic screening of mono-, co- and tri-culture spheroids

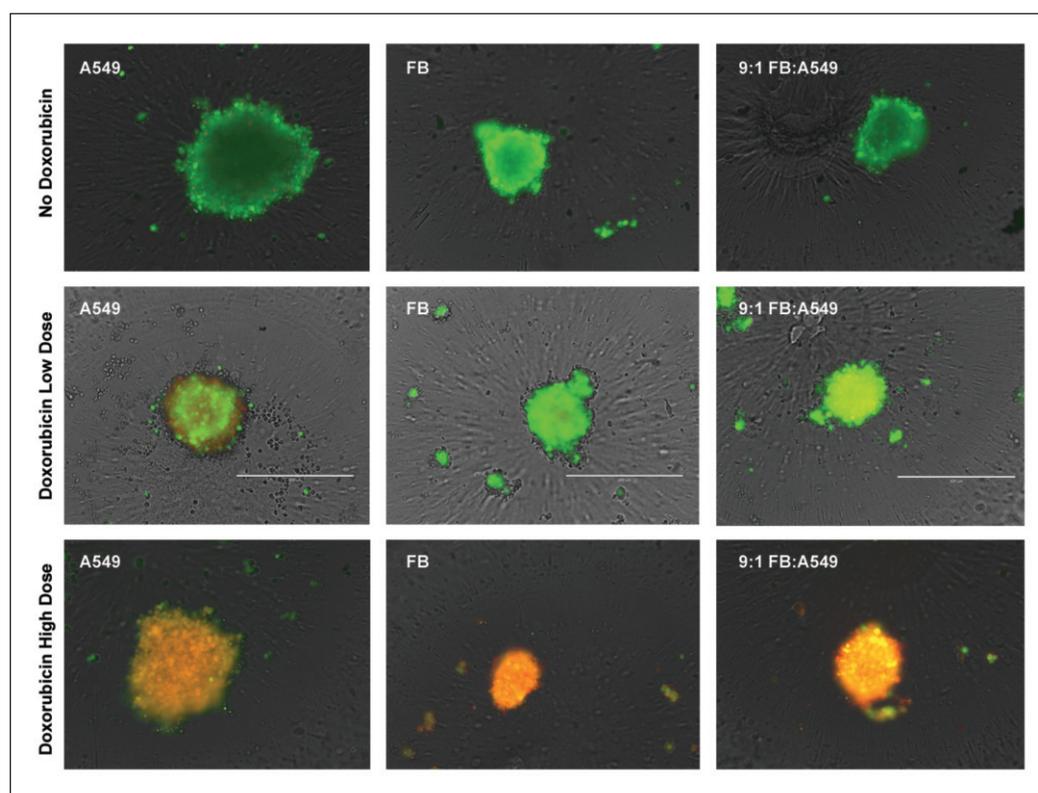
Dose series of several chemotherapeutic compounds were applied to mono-, co- and tri-culture spheroids after 48 hours of culture in the spheroid

Figure 1

A549 vulnerability to chemotherapeutics is affected by the presence of lung fibroblast cells. 48-hour mono- and co-culture A549 and lung fibroblast (FB) spheroids were exposed to doxorubicin (high dose = 862µM, low dose = 1.3µM) and paclitaxel (high dose = 1.2µM, low dose = 9nM) for 48 hours. FB monoculture demonstrated higher cell viability after 48-hour exposure to low dose doxorubicin, as measured using CellTiter-Glo® 3D cell viability assay, than A549 mono-culture spheroids. A 9:1 ratio of FB to A549 cells also displayed this protective effect from the low dose doxorubicin exposure. FB monoculture displayed resistance to paclitaxel exposure at both high and low doses compared to the effects seen on A549 mono-culture spheroids

Figure 2

Live (green) and dead (red) stained 96-hour mono- and co-culture A549 and fibroblast spheroids. Spheroids were exposed to high (34.5 μ M) and low (27.6 μ M) doses of doxorubicin or vehicle control (no doxorubicin) for 48 hours in Corning 384-well spheroid microplates. Fibroblast (FB) monoculture displayed the most intense live staining upon low dose exposure to doxorubicin, while A549 monoculture showed increased cell death after high dose exposure. The 9:1 ratio of FB to A549 cells also displayed a protective effect at the low-dose doxorubicin exposure. All cell types displayed significant toxicity after high dose exposure. Images captured using an EVOS fluorescent microscope at 10x objective. Scale bar = 400 μ m



microplate. Cell viability was assessed using CellTiter-Glo 3D cell viability assay and the luminescent signal from samples were plotted as percent compared to vehicle control to generate dose response curves (Figure 3). Toxicity potency (TC₅₀) values were calculated and compared across the spheroid culture conditions (Table 1). Although the presence of FB and immune cells did not affect the potency of compounds carboplatin and cisplatin to A549 lung tumour cells, a right-shift in the potency of several other known chemotherapeutics was observed, demonstrating the protective effects of the presence of other cell types such as FB in a co-culture spheroid.

Co-culture with endothelial cells

In this study, an epithelial tumour cell line was cultured in 96-well Corning spheroid microplates with and without the presence of GFP-expressing mouse endothelial C166 cells. Tumour spheroid viability was screened after treatment with various chemotherapeutics in both single tumour cell line culture and endothelial co-culture conditions. Dose-dependent responses of selected chemotherapeutics were compared, demonstrating the importance of including multiple cell types in 3D assays to more closely mimic the *in vivo* tumor microenvironment.

Spheroid formation

HT-29 (ATCC® Cat. No. HTB-38) and C166-GFP (ATCC Cat. No. CRL-2583) were cultured in 2D format following ATCC's protocols. Confluent cells were harvested and seeded into 96-well spheroid microplates (Corning Cat. No. 4520) at 2,000 total cells per well either in mono-culture or in co-culture with a seeding ratio of 1:1. Cells were seeded in 100 μ L per well of culture medium (IMDM [Corning Cat. No. 10-016-CM] supplemented with 10% FBS [Corning Cat. No. 35-010-CV]). Cells were cultured in a humidified 37°C, 5% CO₂ incubator for 24 hours for the formation of a spheroid in each well. After 24 hours the spheroids were overlaid with 100 μ L per well of 1.76mg/mL Corning Matrigel matrix (Corning Cat. No. 354234) diluted in culture medium. The Corning spheroid microplates were centrifuged at 220 x g at 4°C for two minutes and returned to a humidified 37°C, 5% CO₂ incubator for four days.

Cell viability assays

Five days after cell seeding, 50 μ L per well of compounds in culture medium containing 0.5% dimethyl sulfoxide (DMSO) (Corning Cat. No. 25-950-CQC) were added. 50 μ L of culture medium containing 0.5% DMSO was used as vehicle control. Spheroids were cultured for an additional 48

hours prior to cell viability assays. For cell viability assays, cells were assayed in the spheroid microplate using CellTiter-Glo® 3D (Promega Cat. No. G9683) following vendor's protocols. Luminescence was detected using a PerkinElmer EnVision™ Multilabel plate reader.

Dose response curves of chemotherapeutic compounds applied to mono- and co-culture spheroids for 48 hours were generated using CellTiter-Glo 3D cell viability assay (Figure 4). The data were normalised to spheroids in the presence of 0.5% DMSO as 100% viability control.

Toxicity potency (TC₅₀) values of chemotherapeutic compounds applied to mono- and co-culture spheroids for 48 hours were generated using CellTiter-Glo 3D cell viability assay (Table 2). Although the presence of endothelial cells did not affect the potency of Paclitaxel on HT-29 colon tumour cells, a right-shift in the potency was

observed with Vinorelbine, and a left-shift in potency was observed with Cisplatin under co-culture conditions.

Conclusion

In vitro 3D cell culture models are now becoming increasingly complex, and their usefulness in supporting cell growth, tissue morphogenesis, stem cell differentiation, disease modelling, drug discovery and toxicity testing is growing. It is evident that a range of 3D models with varying physiological characteristics are required to meet the needs of specific cell types or assays. Currently, many complex 3D models of tumorigenesis, stem cell differentiation and organoid formation employ naturally-derived ECM-based hydrogels. Combining newer technologies, such as microarray on a chip, microfluidics or even bio-printing with biologically relevant materials,

Figure 3
Dose response curves of chemotherapeutic compounds applied to mono-, co- and tri-culture spheroids for 48 hours using CellTiter-Glo® 3D cell viability assay. The presence of fibroblast (FB) and immune cells affected the potency of several compounds. Black = A549, blue = FB, red = A549 + FB and green = A549 + FB + PBMC

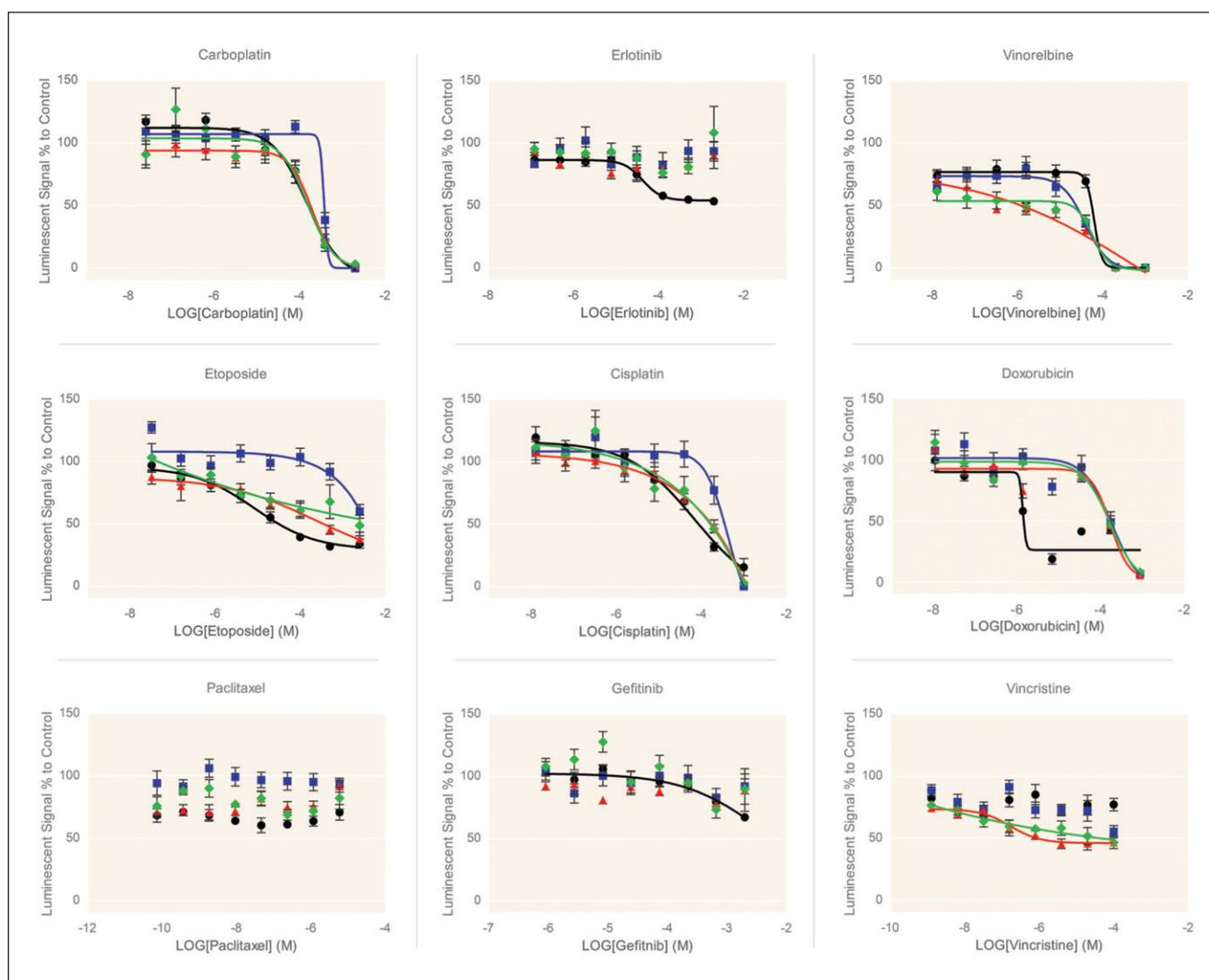
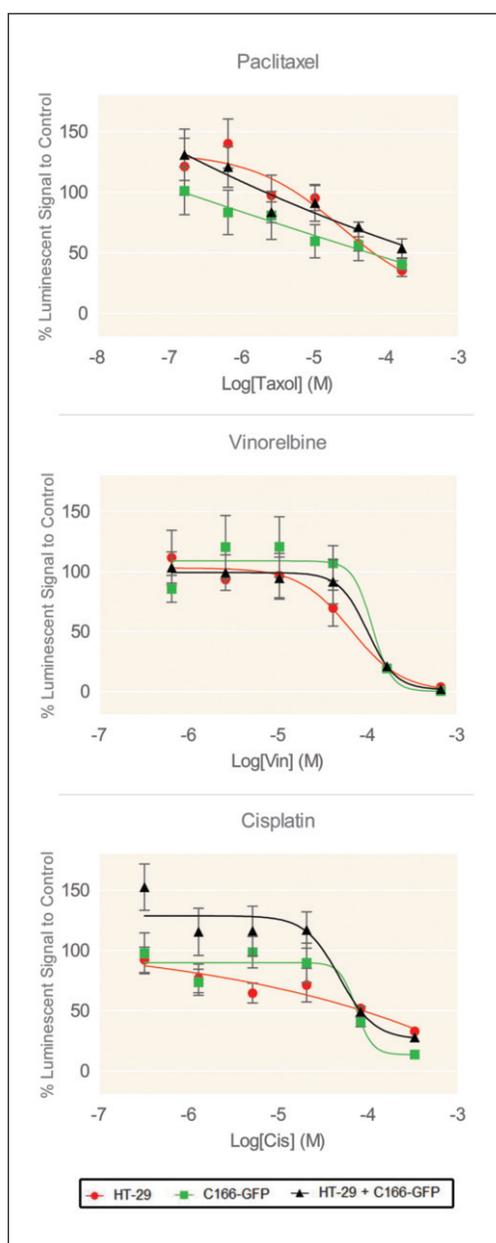


Figure 4

Dose response curves of chemotherapeutic compounds applied to mono- and co-culture spheroids. The presence of endothelial cells affected the potency of several of the compounds tested. Assays were performed in triplicate two independent times



References

1 Xu, X et al. Three-dimensional in vitro tumor models for cancer research and drug evaluation. *Biotechnol. Adv.* 32(7):1256-1268 (2014).
 2 Nyga, A et al. 3D tumour models: novel in vitro approaches to cancer studies. *J. Cell Commun. Signal.* 5(3):239-248 (2011).
 3 Hirschhaeuser, F et al. Multicellular tumor spheroids: An underestimated tool is catching up again. *J. Biotechnol.* 148(1):3-15 (2010).

including hydrogels, will optimise cell scale-up. Collectively, these complex 3D cell culture models will provide the basis for more predictive and relevant drug screening assays and toxicity tests – as well as providing novel systems for basic research – and so aid in the identification of novel disease mechanisms, and reduce the attrition rate in early drug discovery. **DDW**

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Compound	HT-29 TC ₅₀ (M)	C166 TC ₅₀ (M)	HT-29+C166 TC ₅₀ (M)
Paclitaxel	>5E-5	>5E-5	>5E-5
Vinorelbine	6.56E-5	1.14E-4	1.01E-4
Cisplatin	>1E-4	7.41E-5	4.86E-5

Table 2: The presence of endothelial cells (C166) with tumour cells (HT-29) in a spheroid affected the potency of several chemotherapeutic compounds