

# Co-culturing and Assaying Spheroids in the Corning® Spheroid Microplate

## Application Note

CORNING

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### Introduction

Two-dimensional (2D) cell culture models are frequently used in drug discovery for the development of cancer therapeutics due to their ease of use and established compatibility with high throughput screening. More elaborate, three-dimensional (3D) cell culture models have been developed, which more closely mimic the *in vivo* tumor microenvironment, to bridge the gap between *in vitro* studies and success in clinical trials<sup>1-3</sup>. Understanding the complex interactions between cancer cells and other cell types in the tumor microenvironment, such as fibroblasts, endothelial cells, and immune cells, is critical to predict therapeutic efficacy. Many factors, such as hypoxia, extracellular matrix and gradients of various soluble factors have been associated with both drug resistance and sensitization<sup>2-4</sup>. Conventional methods for 3D cell culture are often time consuming, display increased variability and may lack the throughput required for screening. Corning spheroid microplates are cell culture microplates with opaque walls and unique clear, round well-bottom geometry that utilize Corning Ultra-Low Attachment surface coating. The coating is hydrophilic, biologically inert, and non-degradable, which enables the rapid and highly reproducible formation of a single multicellular tumor spheroid, centered in each well.

In this study, A549 cells, a human lung carcinoma cell line, were cultured in spheroid microplates with and without co-culture conditions, including primary human lung fibroblasts [FB] and peripheral blood mononuclear cells [PBMC]. Tumor 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 therapeutic outcome.

### Materials/Methods

#### Mono- and Co-culture Spheroid Formation

A549 cells (ATCC® Cat. No. CCL-185) and normal human lung fibroblasts (Lonza Cat. No. CC-2512) were cultured in 2D format 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 20 µL of FB media with 2,000 cells per well. For co-culture, cell ratios were varied for optimal culture conditions. 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 37°C, 5% CO<sub>2</sub> 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 re-suspended 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 10 µL of FB media per well. As a control for mono- and co-culture conditions without PBMC, 10 µL of FB media was added per well. Spheroids were incubated for an additional 48 hours prior to fixing or staining.

#### Staining/Imaging Co-culture Spheroids

Media was aspirated and spheroids were fixed in 30 µL per well of 4% paraformaldehyde (Electron Microscopy Sciences Cat. No. 157-SP) at room temperature, then stored at 4°C.

For immunohistochemistry (IHC), fixed cells were embedded and sectioned at 10 µm thickness via cryostat prior to staining and imaging with a Leica confocal microscope. Cells were stained for DAPI nuclei counterstain, anti-cytokeratin 7 (Abcam Cat. No. ab9021) and anti-fibroblast activation protein (Abcam Cat. No. ab53066) following vendor's protocols. Cryostat sectioning, staining and imaging were performed at the University of New England (Biddeford, Maine).

For live and dead cell staining, A549 and FB were seeded in mono- and co-culture conditions as described previously. After 48 hours of culture, 10 µL per well of FB media was added followed by the addition of 10 µL per well of doxorubicin (Sigma-Aldrich Cat. No. D1515) in a dose-dependent manner. The 1X top dose was 862 µM with subsequent 1:5 dilutions in FB media with 10% dimethyl sulfoxide (DMSO) (Corning Cat. No. 25-950-CQC). For vehicle control wells, 10 µL of FB media with 10% DMSO was added. Spheroids were incubated for an additional 48 hours post-treatment. After a total of 96 hours of culture, cells were stained using LIVE/DEAD® Viability/Cytotoxicity Kit following vendor's protocols (Thermo Fisher Cat. No. L3224). Images were obtained using an EVOS® fluorescent microscope at 10X objective.

## 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, 10  $\mu$ L per well of compounds in FB media with 10% DMSO were added and mixed twice using a CyBi®-Well Pipettor with the parameters listed in Table 2. Vehicle control consisted of 10  $\mu$ L 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). 40  $\mu$ L per well of CellTiter-Glo 3D reagent was added directly to the spheroid microplates 48 hours after compound addition. Microplates were shaken for 5 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 ( $TC_{50}$ ) for each compound.

## Results and Discussion

### Forming Mono-, Co-, and Tri-culture Spheroids

Mono-, co-, and tri-culture spheroids were formed by culturing cells in the spheroid microplate for a total of 96 hours, with PBMC addition for tri-culture conditions at 48 hours. Reproducible single spheroids were formed in each well for all culture conditions. As shown by immunohistochemical analysis, all three spheroid types displayed high levels of cytokeratin 7 (Cyt7) staining (green), which is expressed in A549 cells, and the co- and tri-culture spheroids also displayed staining for fibroblast activation protein (FAP)

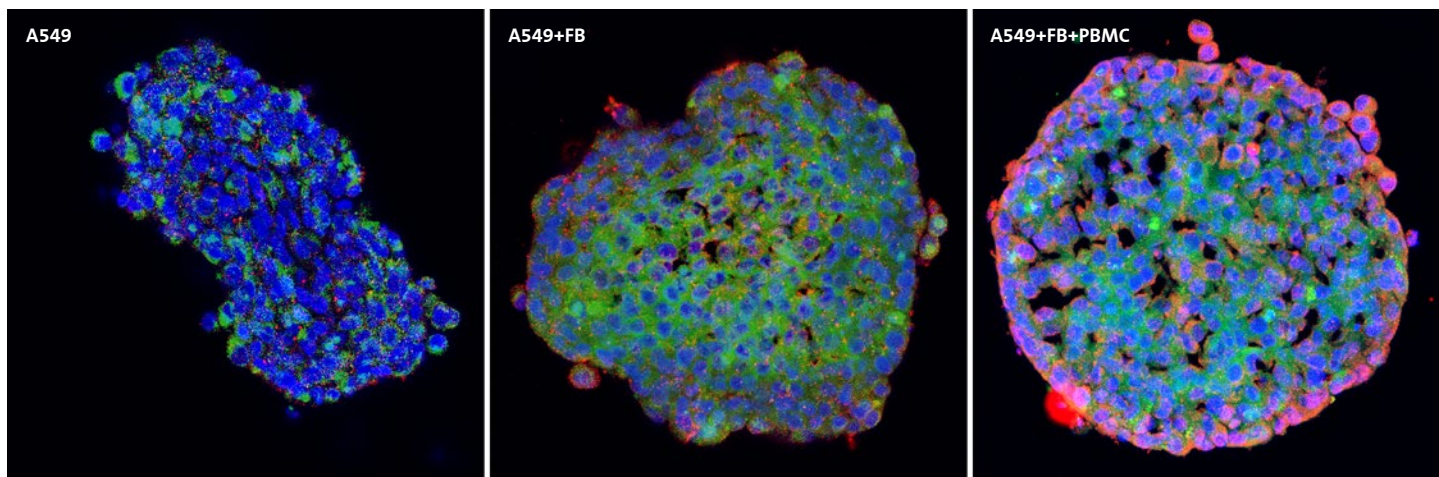
**Table 1.** Compounds Utilized for Chemotherapeutic Screening

Compound	Vendor	Cat. No.
Doxorubicin	Sigma-Aldrich	D1515
Carboplatin	Tocris Bioscience	2626
Cisplatin	Tocris Bioscience	2251
Etoposide	Tocris Bioscience	1226
Vinorelbine	Tocris Bioscience	3401
Erlotinib	LC Laboratories	E-4997
Paclitaxel	LC Laboratories	P-9600
Gefitinib	LC Laboratories	G-4408
Vincristine	LC Laboratories	V-8400

**Table 2.** Parameters for Compound Addition Using Automated Liquid Handler

Dispense Volume	10 $\mu$ L
Dispense Height	2 mm
Dispense Speed	8 $\mu$ L/sec
Number of Mixes	2
Mix Volume	15 $\mu$ L
Mix Height	2 mm
Mix Speed	8 $\mu$ L/sec

(red), confirming the incorporation of FB into the co- and tri-culture spheroids (Figure 1). Of note, the PBMC, which are suspension cells, were applied as a treatment and were not evaluated for incorporation into the reproducible single spheroids that were formed in each well; however, the presence of PBMC in these cultures appeared to affect the amount of FAP expression, as seen through IHC staining.



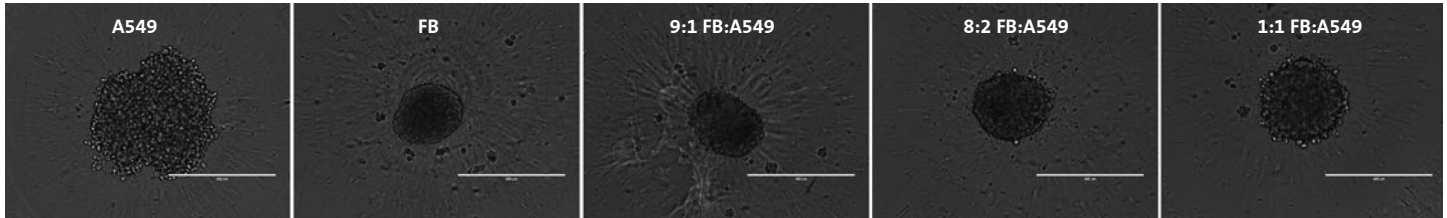
**Figure 1.** Immunohistochemical images of mono-, co-, and tri-culture spheroids. A549 cells were seeded at 750 cells/well; A549 and fibroblast cells [A549+FB] were seeded at 2K cells/well at a ratio of 1:1; and A549, fibroblast, and peripheral blood mononuclear cells [A549+FB+PBMC] were seeded at 2K cells/well at a ratio of 2:1:1. Cells were cultured in the spheroid microplate for 96 hours prior to fixation in 4% PFA. Spheroids were stained with DAPI nuclei counterstain (blue), anti-Cyt7 (green), and anti-FAP (red).

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 displayed in Figure 3, 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

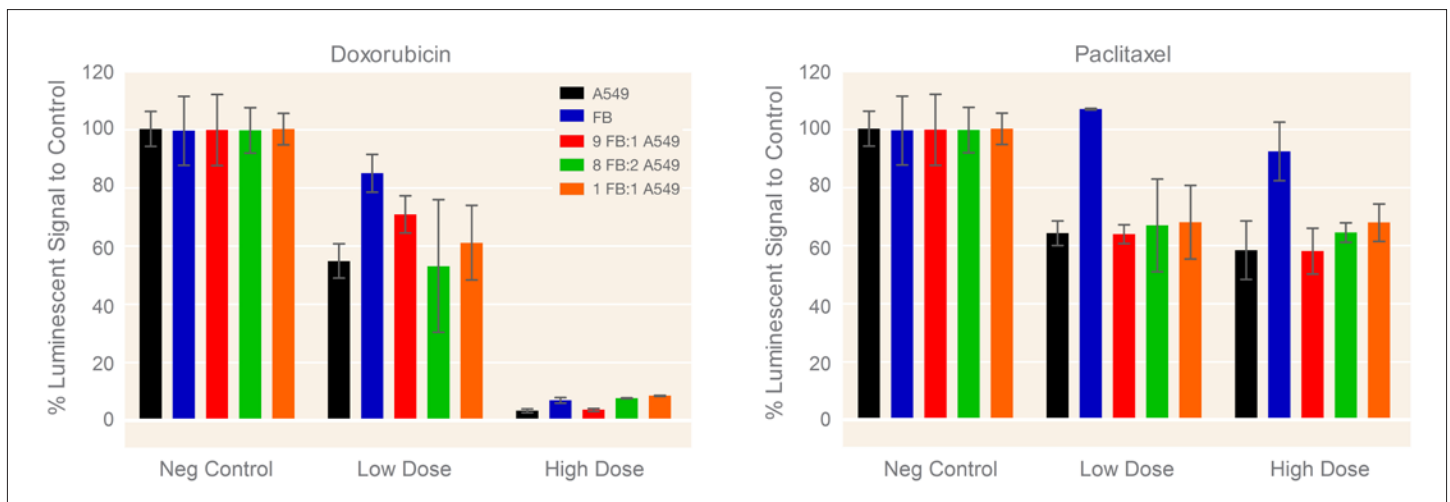
FB on A549 cells in the presence of a low dose of doxorubicin can also be visualized using viability and cytotoxicity stains (Figure 4).

### Chemotherapeutic Screening of Mono-, Co-, and Tri-culture Spheroids

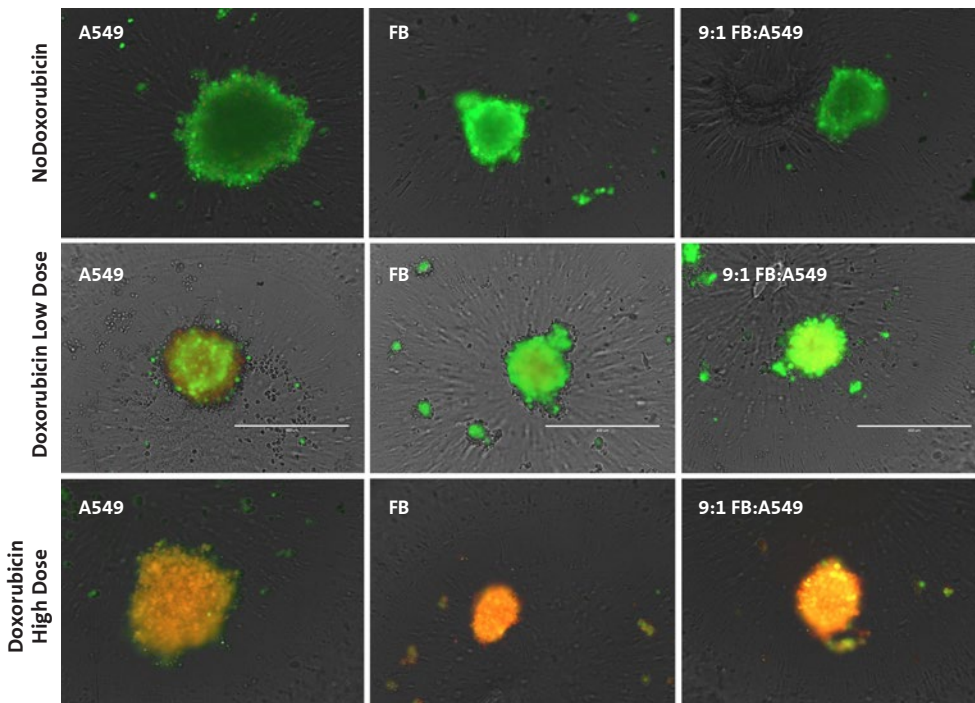
Dose series of several chemotherapeutic compounds were applied to mono-, co-, and tri-culture spheroids after 48 hours of culture in the spheroid microplate. Cell viability was assessed using CellTiter-Glo 3D cell viability assay and the luminescent signal from samples were plotted as percent compared to vehicle control to generate dose response curves (Figure 5). Toxicity potency (TC<sub>50</sub>) values were calculated and compared across the spheroid culture conditions (Table 3). Although the presence of FB and immune cells did not affect the potency of compounds carboplatin and cisplatin to A549 lung tumor 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.



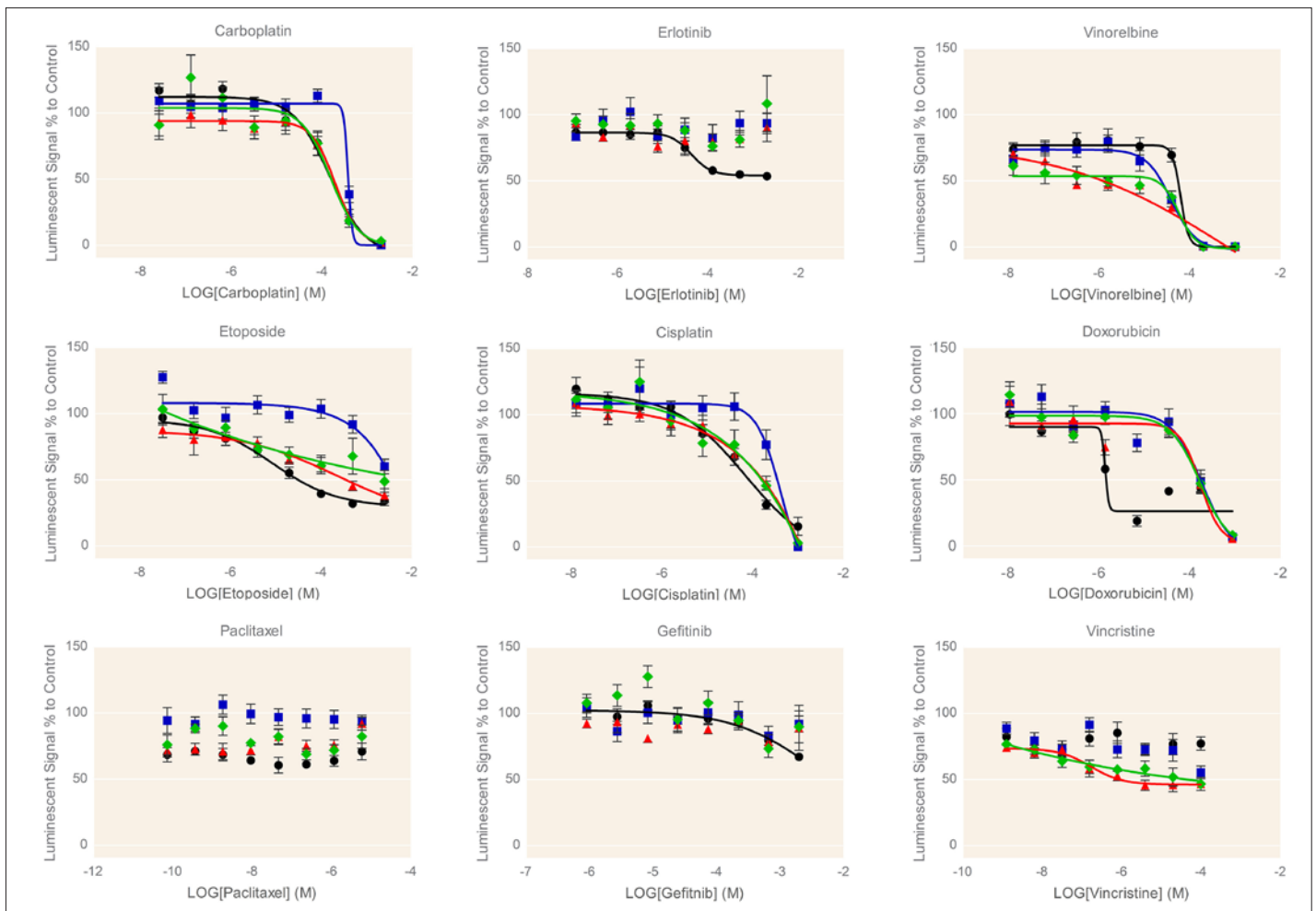
**Figure 2.** 48-hour images of mono- and co-culture A549 and lung fibroblast cells in Corning 384-well spheroid microplates. Cells were seeded in a total of 2K cells/well in mono-culture and in co-culture ratios of fibroblast (FB) to A549 cells of 9:1, 8:2, and 1:1. Images captured using an EVOS fluorescent microscope at 10X objective. Scale bar = 400  $\mu$ m.



**Figure 3.** 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 = 862  $\mu$ M, low dose = 1.3  $\mu$ M) and paclitaxel (high dose = 1.2  $\mu$ M, low dose = 9 nM) for 48 hours. FB monoculture demonstrated higher cell viability after 48 hour exposure to low dose doxorubicin, as measured using CellTiter-Glo<sup>®</sup> 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. Error bars represent standard deviation (SD). Assays were performed in triplicate.



**Figure 4. Live (green) and dead (red) stained 96-hour mono- and co-culture A549 and fibroblast spheroids.** Spheroids were exposed to high (34.5  $\mu\text{M}$ ) and low (27.6  $\mu\text{M}$ ) 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. 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  $\mu\text{m}$ .



**Figure 5. Dose response curves of chemotherapeutic compounds applied to mono-, co-, and tri-culture spheroids for 48 hours using CellTiter-Glo<sup>®</sup> 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. Assays were performed in triplicate two independent times. Error bars represent standard error of the mean (SEM).

**Table 3.** Toxicity Potency (TC<sub>50</sub>) Values of Chemotherapeutic Compounds Applied to Mono-, Co-, and Tri-culture Spheroids

Compound	A549 TC <sub>50</sub> (M)	FB TC <sub>50</sub> (M)	A549+FB TC <sub>50</sub> (M)	A549+FB+PBMK TC <sub>50</sub> (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-5
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

### Conclusions

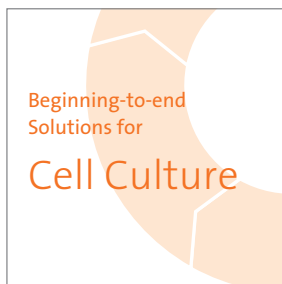
- ▶ The Corning® spheroid microplate can be used to generate mono- and co-culture spheroids and perform viability assays in an easy-to-use, high throughput format.
- ▶ Spheroids formed and cultured in the Corning spheroid microplate are amenable to immunohistochemical and fluorescent staining analysis.
- ▶ The presence of other primary cell types together with tumor cells in a spheroid affects the potency of several chemotherapeutic compounds.

### References

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