Inhibition of Hypoxic Tumor Cells using a Three-Dimensional Spheroid Model

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Introduction

Discovering innovative cancer therapies requires a deep understanding of the heterogeneous microenvironment of solid tumors. The Warburg effect describes the metabolic shift from oxidative phosphorylation to aerobic glycolysis (VanderHeiden *et al.*, 2009) that takes place in tumors, perhaps, even before the inevitable hypoxia that occurs as the tumor outgrows its insufficient and often irregular vasculature. The culmination of metabolic changes in cancer cells contributes significantly to increased metastasis and drug-resistance, aspects known to increase patient mortality (Bennewith *et al.*, 2011), (Brown *et al.*, 2001). Based on these observations, proteins that mediate metabolism and directly target hypoxic cells in primary, metastatic tumors are attractive targets for therapeutic intervention.

Spheroids, self-assembled microscale aggregates of cells, generated in hanging drop plates (HDPs) are a superior model of avascular microtumors. Because of their 3D structure, spheroids contain mass-transfer gradients of oxygen, nutrients, wastes, and therapeutic drugs that are highly comparable to what is observed in tumors within the human body. The metabolic gradients drive proliferation gradients and spheroids contain quiescent cells hypothesized to mimic drug resistant populations within tumors. Given sufficient cell numbers and time in culture, spheroids can develop hypoxic cores that can progress to necrosis, closely mimicking what is observed *in vivo* (Hirschhaeuser *et al.*, 2010).

Here we show the ability to interrogate compounds that alter the hypoxic microenvironment of tumors, using spheroids grown in HDPs and a simple fluorescent dye that specifically measures the activity of hypoxic cells. For the initial test we chose an inhibitor of carbonic anhydrase IX (CA IX), a hypoxia inducible factor 1a (HIF-1a)-regulated protein that functions to maintain intracellular pH (Bennewith *et al.*, 2011). This family of inhibitors has been shown to reduce cancer cell growth and tumor metastasis (Lou *et al.*, 2011). Imaging of spheroids in the hanging drop, following treatment, was performed to observe the inhibitory effects of the compound. The results demonstrate the validity of the 3D cell model, and the use of this method to predict potential downstream anti-metastatic effects of lead molecules.

Instrumentation

MultiFlo™ FX Microplate Dispenser: The MultiFlo™ FX Microplate Dispenser contains Parallel Dispense™ Technology, including non-contact peristaltic and syringe pump dispensing, as well as an optional wash module to perform medium exchanges and buffer washes. The instrument was used to dispense cells and medium to the wells of the HDPs.

Cytation™ 5 Cell Imaging Multi-Mode Reader: Cytation 5 is a modular multi-mode microplate reader that combines automated digital microscopy and microplate detection. Cytation 5 includes filter- and monochromator-based microplate reading; the microscopy module provides up to 60x magnification in fluorescence, brightfield, H&E and phase contrast. With special emphasis on live-cell assays, Cytation 5 features temperature control to 65 °C (37 ± 0.2 °C), CO_2/O_2 gas control and dual injectors for kinetic assays. Shaking and Gen5 software are also standard. The instrument was used to image spheroids within each hanging drop during the aggregation and assay process.

Perfecta3D® 96-Well Hanging Drop Plates

3D Biomatrix's Perfecta3D HDPs (Figure 1) facilitate 3D spheroid formation in 96- or 384-well formats. A drop of cell suspension is pipetted into the top of each well, and the plate geometry causes it to hang stably below the well. Spheroid diameter can be controlled with the type and number of cells added. Access holes at the top of each well allow for media exchange and the addition of compounds, reagents or additional cells or spheroids to establish co-cultures. Without contact with any surfaces or matrices, cells aggregate together to form one spheroid per well.

Hypoxia Red Detection Reagent

Hypoxia Red Detection Reagent, part of the Cyto-ID® Hypoxia/Oxidative Stress Detection Kit (Catalog No. ENZ-51042) was provided by Enzo Life Sciences (Farmingdale, NY). The reagent is a weakly-fluorescent compound containing a nitro (NO_2) moiety. Due to nitroreductase activity present in hypoxic cells, the nitro group is converted to hydroxylamine and amino groups. The original molecule then degrades releasing the fluorescent probe.

Experimental Procedure

Cell Preparation and Automated Dispensing into Hanging Drop Plates: HCT116 and fibroblast cells were harvested and diluted in HCT116 media to concentrations of 6.25 x 10^4 cells/mL. Prior to dispensing, the HDP plate and tray reservoirs were filled with 3 and 5 mL of sterile Dulbecco's phosphate buffered saline (DPBS), respectively. The cells were combined together and a volume of 40 µL was then dispensed by the MultiFlo FX to create spheroids containing 5000 cells per spheroid and equal numbers of each cell type. Following dispensing, the plate assembly was placed at 37 °C/5% CO_2.

Image-Based Spheroid Formation Monitoring: Spheroid formation was monitored every 24 hours. The HDP assembly was placed into the Cytation 5, previously set to 3 7°C/5% CO_2 using Gen5 as well as a gas control module. Focusing was performed using the brightfield channel. Manual imaging was performed using a 4x objective to determine the state of aggregation.

Carbonic Anhydrase Inhibitor Dosing: Upon completion of cell aggregation, media was removed from the hanging drops and replaced with media containing the carbonic anhydrase inhibitor U 104, R&D Systems Catalog No. 4540 (Minneapolis, MN), at concentrations of 0, 100, 1000, and 10,000 nM. The procedure involved slow removal of 10 µL of media from the top of each access hole, followed by a 10 µL addition of media with compound, which was repeated 5-6x to ensure a complete media exchange. Dosing was repeated on a daily basis over the two-week incubation period.

Analysis of Spheroid Hypoxia: Hypoxia detection reagent (5x) was added to appropriate wells following 1, 4, 7, and 14 days of compound treatment, incubated for the optimized time period, and then washed with DPBS as previously described. Fluorescent and brightfield imaging were then performed using a 4x objective and consistent LED excitation, integration time, and gain settings to ensure that changes in hypoxia levels were captured. Cellular analysis was performed using the Gen5™ Data Analysis Software on the 4x images of the spheroid captured. This was done in order to analyze only the fluorescent signal from the cells in the spheroid itself, and to ignore all other portions of the image.

The brightfield image was used as the detection channel in order to properly define the boundaries of the spheroid. The fluorescence from the hypoxia reagent within the spheroid boundaries, captured with the Texas Red channel, was then used for analysis.

Cell Suspension Dispensing and Aggregation Tracking

In order to accomplish proper cell dispensing by the MultiFlo FX into the hanging drop plates, optimization of peristaltic pump cassette usage, and dispense tip coordinate positioning was performed. This allowed cell suspensions to be dispensed in a droplet rather than a stream of liquid, and also ensured contact between the well sides and the droplet.

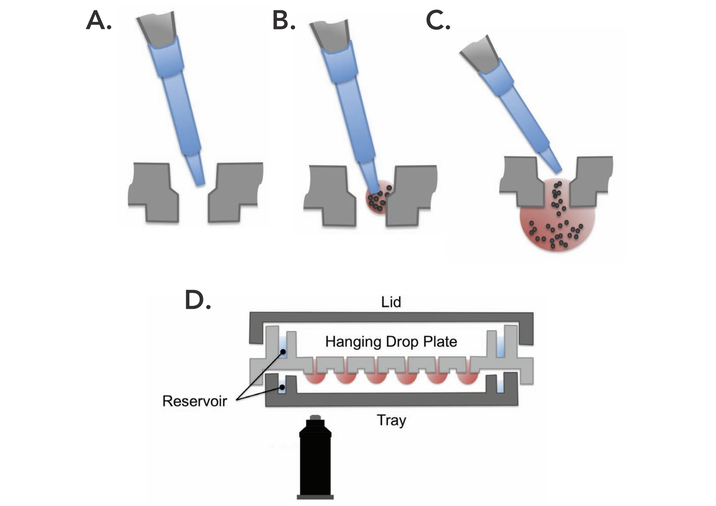

Figure 1. Automated Cell Suspension Dispensing and Image-Based Spheroid Aggregation Analysis. Peristaltic pump cassette tips, filled with 40 µL of cell suspension, are inserted halfway into the well **(A)**. Cell suspension is slowly dispensed **(B)**, and a drop begins to form on the bottom side of the well. Tips are then removed **(C)**, while the drop fully forms below each well with the cells inside. **(D)** Perfecta Hanging Drop Plate Assembly with Cytation 5 4x objective positioned for spheroid imaging. **(E)** HCT116/fibroblast cell aggregation following 0, 12, and 48 hour incubations within hanging drop.

The imaging capability of the Cytation 5 allows focusing through the tray of the hanging drop assembly to the level of the spheroids within the hanging drop. This enables image-based analysis without sacrificing cell sterility and integrity. Following initial determination of spheroid height, automated imaging of each assay well can be completed.

Analysis of Hypoxia Red Reagent/ Spheroid Incubation

The fluorescent signal from the hypoxia dye was assessed within the spheroid after a 2 hour incubation. As seen from the image in Figure 2A, a relatively small concentration of reagent was integrated into the cells after two hours. Therefore the required image exposure to see the signal within the spheroid was set to near maximum. This led to high background fluorescence, making accurate cellular analysis difficult to accomplish.

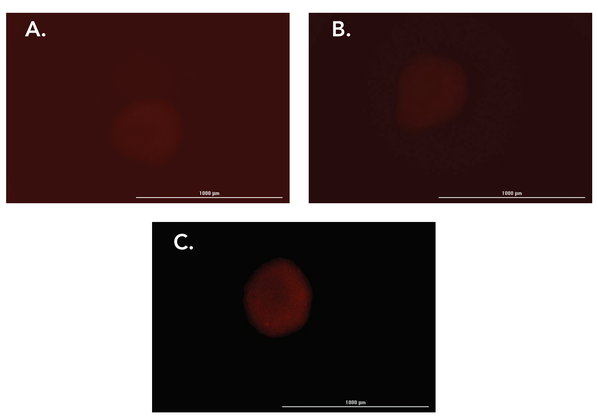

Figure 2. 4x images of Hypoxia Red Reagent signal captured after **(A)** 2 hours; **(B)** 4 hours; and **(C)** 6 hours of incubation with the spheroid at 37 °C/5% CO_2.

Examination of the image containing reagent incubated with the spheroid for four hours (Figure 2B) reveals increased reagent integration. However, exposure settings still generated relatively high background signal. Finally, when using a 6-hour incubation time, a sufficient amount of reagent was integrated, which allowed for exposure settings that yielded low background fluorescence while still giving a bright signal from the reagent within the spheroid. This incubation time was used for all subsequent experiments.

Analysis of Hypoxia Inhibition

Carbonic anhydrase IX (CA IX) is a hypoxia-inducible protein that promotes tumor cell survival and metastatic cell invasion (Lou *et al.*, 2011). Therapeutic inhibition of CA IX has been shown to decrease tumor growth and metastasis. The Hypoxia Red Reagent relies on the nitroreductase properties of active hypoxic cells. As these cells lose functionality less reagent is converted leading to a decrease in fluorescence. The ability of U 104 to affect nitroreductase activity was assessed over a two week period.

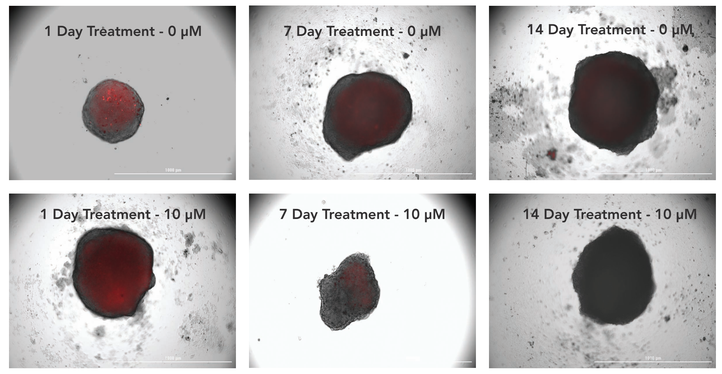

Figure 3. 4x brightfield and Texas Red overlaid images of HCT116/fibroblast spheroids, following Hypoxia Red Reagent addition and incubation.

The 4x images in Figure 3 illustrate the inhibitory effect of the compound at a 10 µM concentration compared to the negative control. Using optimized Cellular Analysis parameters with the 4x brightfield and Texas Red images, the signal from the Hypoxia Red Reagent was evaluated within each spheroid analyzed. Figure 4 exhibits the cellular masks that were drawn around the spheroids evaluated after a 14 day U 104 treatment.

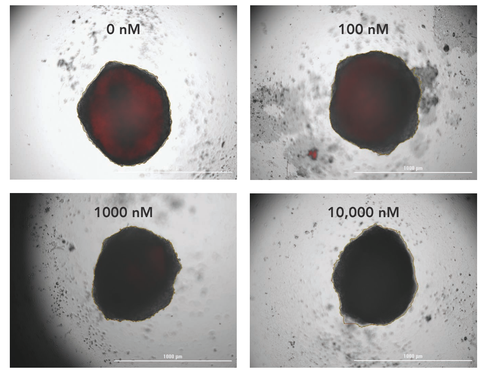

Figure 4. Object masks drawn by Gen5™ around HCT116/fibroblast spheroids following 14 day U 104 treatment, using the folllowing criteria. Detection Channel: Bright Field; Threshold: 10,000 RFU; Min. Object Size: 500 µm; Max. Object Size: 1000 µm.

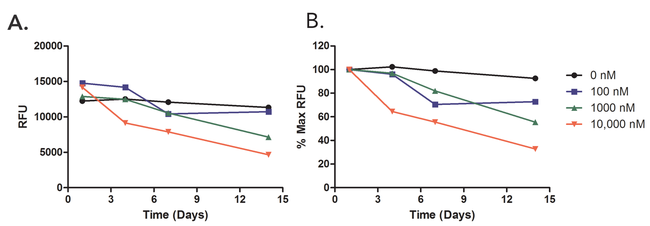

Figure 5. U 104 Induced Reduction of Hypoxic Cell Nitroreductase Activity. (A) Mean raw fluorescence values from Hypoxia Red Reagent using a 4x objective and the Texas Red Imaging Cube; **(B)** Normalization of fluorescent signal calculated by the following formula: (Mean $RFU_{Day\ X}$ / Mean $RFU_{Day\ 1}$).

The raw fluorescent signal generated from active hypoxic cells within each spheroid was plotted for each compound concentration tested over the two-week dosing period (Figure 5A). Normalization was then completed by comparison of fluorescent units from subsequent day analyses to values from the initial analysis after a one day incubation, and expressed as a percentage (Figure 5B).

Results from Figure 5 demonstrate that the carbonic anhydrase inhibitor U 104 specifically affects hypoxic tumor cells, as witnessed by the dose-dependent decrease in nitroreductase activity over the 14-day dosing period. We hypothesize that the reduction in fluorescent signal is due to increased cell death of hypoxic cells. This agrees with what has been shown previously in the literature, that therapeutic treatment of tumor cells by inhibition of CA IX is accomplished in part by decreasing the ability of cells to adapt to the low extracellular pH found in hypoxic regions of primary tumors (Li *et al.*, 2011).

Conclusions

1. Co-cultured cancer cell/fibroblast spheroids, created and assayed in Perfecta3D® Hanging Drop Plates, can be successfully used to observe the effects of test compounds on hypoxic tumor cells
2. Cells and media can be rapidly and efficiently dispensed to the hanging drop plates, where spheroid formation is accomplished and monitored via real time imaging
3. The activity of hypoxic cells can be assessed with the incorporation of the Hypoxia Red Reagent and experimental imaging taking place within the hanging drop
4. Cellular analysis of fluorescent signal emanating solely from within the spheroid increases assay sensitivity
5. The combination of plate, detection reagent, and image-based cellular analysis can provide initial information regarding the potential final effect of test compounds on tumor growth and metastatic activity