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A Facile, *In Vitro* 384-Well Plate System to Model Disseminated Tumor Cells in the Bone Marrow Microenvironment

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Summary/Abstract

Bone marrow disseminated tumor cells (DTCs) are dormant cancer cells that harbor themselves in a bone marrow niche for years after patient remission before potentially returning to a proliferative state, causing recurrent cancer. DTCs reside in bone marrow environments with physiologically important mesenchymal stem cells that are often negatively affected by chemotherapy treatments. Currently, there are very few models of DTCs that recapitulate their dormant phenotype while producing enough samples to accurately quantify cancer and surrounding stromal cell behaviors. We present a three-dimensional spheroid-based model system that uses dual-color bioluminescence imaging to quantify differential cell viability in response to various compounds. We successfully screened for compounds that selectively eliminated cancer cells versus supportive stromal cells and verified results with comparison to efficacy *in vivo*. The spheroid co-culture system successfully modeled key aspects of DTCs in the bone marrow microenvironment, facilitating testing for compounds to selectively eliminate DTCs.

Keywords

Bioluminescence; Bone Marrow; Breast Cancer; Disseminated Tumor Cells; Dormancy; Spheroids

1 Introduction

Around 30% of breast cancer patients present with bone marrow (BM) disseminated tumor cells (DTCs) at the time of diagnosis (1). These DTCs are usually in a non-proliferative state and present with possible immune escape and cancer stem cell phenotypes (2). Undetectable by standard clinical imaging modalities, DTCs may remain dormant for years or decades before returning to an aggressive state (2,3). The phenomenon of tumor dormancy has not only been observed in breast cancer, but also in other epithelial cancers such as colon, lung, and prostate cancers, thus expanding the clinical relevance of DTCs (2). In breast cancer

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patients, these BM DTCs are associated with an increase in lymph node metastasis and more sizeable, higher histological grade, and hormone-receptor-negative tumors (1). The patients harboring DTCs in their bone marrow at the time of initial surgery have a significantly higher risk of recurrent disease, skeletal metastases, distant disease, and death when compared to patients without BM DTCs (1–6).

Bone marrow is a specific niche that aids DTCs in maintaining a potential stem cell phenotype, surviving chemotherapy treatment, and withstanding various microenvironmental stresses (6). The extracellular matrix, DTC-recruited bone marrow stromal cells, and bone marrow mesenchymal stem cells (MSCs) are fundamental aspects of this metastatic niche and increase the likelihood of DTC survival (7,8). MSCs have been shown to secrete exosomes containing assorted microRNAs that promote the dormant phenotype of DTCs (8). The stem cell properties of MSCs also aid in restructuring the bone marrow niche to enable long-term DTC survival (9). However, MSCs also provide vital support to hematopoietic stem cells (HSCs) by secreting various molecules (10). As DTCs residing amongst MSCs are in a non-cycling state, they are often unaffected by chemotherapies whose mechanism of action targets proliferating cells (8,11). In fact, over 60% of BM DTC positive breast cancer patients that underwent neoadjuvant chemotherapy still contained DTCs after 12 months of treatment (12). Such therapies are more likely to inadvertently harm MSCs whose elimination may lead to HSC death, in turn causing immune deficiency, a reduction in hematopoiesis, and hemorrhages (10). At some point, dormant, protected DTCs must become aggressive to begin forming clinically-detectable tumors (13). Treatments that successfully eliminate DTCs while maintaining the health and vital function of MSCs, and in turn HSCs, remain an unmet clinical need.

There are currently very few models available to study DTCs and drug efficacy which encompass integral aspects of DTCs, such as extensive cell-cell adhesion in three-dimensional growth, inhomogeneous drug and nutrient distributions, and cancer cell quiescence (14). Other groups have performed two-dimensional cell culture experiments to investigate the relationship between stromal and cancer cells, as well as the anticancer drug effects on the system (15,16). These models encompass aspects of an ideal system such as being high-volume, easy to execute, and readily analyzable. However, two-dimensional culture models fail to recapitulate many features of DTCs and their surrounding environment as cells are grown in a uniform, homogenous manner on plastic dishes. DTCs have also been modeled in an *in vitro*, three-dimensional gel co-culture system that recapitulates breast cancer cells in both growth inhibitory and supportive bone marrow niches. This experimental system provides an accurate model for cancer cell quiescence but unfortunately prohibits bulk screening of cancer and stromal cell population viabilities (17).

The system to model DTCs in the bone marrow environment described in this protocol is simple, high-volume, and allows for multifaceted analysis of the tumor microenvironment. Human breast cancer cells and bone marrow stromal cells are plated in commercially-available 384-well plates that form many uniform, healthy spheroids. Samples can be analyzed for the growth phase of breast cancer cells, differential drug effects on breast cancer cells and bone marrow stromal cells, and cancer cell outgrowth. The growth phase of individual cancer cells can be easily observed with fluorescence ubiquitination-based cell

cycle indicator (FUCCI) expressing cells. FUCCI markers express different colors depending on the cell's current cell cycle phase; red (G1), yellow (G1 to S transition), or green (S/G2/M) (18). This dynamic mechanism verifies that breast cancer cells are truly in a growth-arrested phase and have the dormancy property fundamental to DTCs. Relative quantities of breast cancer cells and bone marrow stromal cells can be separately analyzed by dual-color bioluminescence imaging; a powerful tool that accurately measures relative cell numbers (19). Cancer and stromal cells in the system are tagged with different bioluminescent proteins that emit light at two distinct wavelengths depending on cell type when treated with the same substrate. Consequently, growth of individual cell types over time can be easily monitored, which is useful in observing the effects of drug treatments on both cell types and identifying compounds that selectively eliminate the breast cancer cells over the bone marrow stromal cells. Colony outgrowth of plated spheroids allows detection of delayed cytotoxicity that prevents subsequent growth of cells. To verify the three-dimensional spheroid culture system's ability to recapitulate important aspects of DTCs and the bone marrow microenvironment, results were compared to *in vitro*, two-dimensional models and mouse models.

2 Materials

2.1 Molecular Biology

1. pCBR-Basic plasmid (for CBR construct) (Promega)
2. pCBG99-Basic plasmid (for CBG construct) (Promega)
3. PCR primers (IDT® or similar vendor)
 - a. XbaI CBG99 forward 5' -
ATTATCTAGAACCGCCATGGTGAAGCGTGAGAAAAATGTC-3'
 - b. XbaI CBG99 reverse 5' -
ATTATCTAGACTAACCGCCGGCCTTCTCCAACAATTG-3'
 - c. XbaI CBR forward 5' -
ATTATCTAGAACCGCCATGGTAAAGCGTGAGAAAAATGTC-3'
 - d. XbaI CBR reverse 5' -
ATTATCTAGATTACTAACCGCCGGCCTTCACCAAC-3'
4. Lentiviral vector FUW (Addgene, cat. # 14882)
5. Fluorescence ubiquitination-based cell cycle indicators (FUCCI, Gifts from A. Miyawaki, *see* Note 1)
 - a. FUCCI C mKO2-hCdt1(30/120)/pCSII-EF-MCS plasmid
 - b. FUCCI D mAcGFP_hGeminin(1/110)/pCSII-EF-MCS plasmid
6. Enzymes, buffers, and equipment for PCR

¹The FUCCI construct used and described in these methods is not the most current and robust system available as it emits only red (G1) or green (S/G2/M) wavelengths. A newer construct is commercially available (18) and emits red (G1), yellow (G1 to S transition), or green (S/G2/M) wavelengths.

7. Restriction digests for DNA and ligations

2.2 Cell Culture

1. Immortalized human bone marrow mesenchymal stem cell line HS-5 (HS-5) (ATCC® CRL-11882™)
2. Breast cancer cell line MDA-MB-231 (231) (ATCC® HTB-26™)
3. Breast cancer cell line T-47D (T-47D) (ATCC® HTB-133™)
4. Media Supplies
 - a. Standard Fetal Bovine Serum (FBS, HyClone™ cat. # SH300088.03)
 - b. Dulbecco's Modified Eagle Medium with high glucose and pyruvate (DMEM, Gibco® cat. # 11995-065)
 - c. Penicillin Streptomycin Glutamine, 100X (Gibco® cat. # 10378-016)
5. 0.25% Trypsin-EDTA, 1X (Gibco® cat. #25200-056)
6. Sterile Phosphate Buffered Saline pH 7.4, 1X (Gibco® cat. # 10010-049)
7. Miscellaneous desired cell culture supplies such as plasticware, incubators, and sterile pipettes

2.3 Spheroid Co-Culture

1. 384-well low volume black round bottom polystyrene NBS™ microplate, nonsterile (Corning® cat. # 3676)
2. Polystyrene universal microplate lid, sterile (Corning® cat. # 3099)
3. Dulbecco's Modified Eagle Medium with high glucose and without phenol red (PRF DMEM, Gibco® cat. # 31053-036)
4. β -Estradiol powder, suitable for cell culture (Sigma-Aldrich® cat. # E2758-250MG, *see* Note 2)
5. Sodium Pyruvate, 100X (Gibco® cat. # 11360-070)
6. Multichannel pipettes with volumes from 1 to 200 μ L
7. Sterile pipette tips with low adherence

2.4 Bioluminescence Imaging

1. High sensitivity bioluminescence imaging system (*see* Note 3)
2. Software compatible with bioluminescence imaging system for data quantification (*see* Note 4)
3. D-Luciferin, potassium salt (Promega cat. # E1605, *see* Note 5)

² β -Estradiol for use in spheroid medium was dissolved in 100% ethanol for a 10nM stock solution.

³We used an IVIS Lumina Series III (Perkin Elmer, Waltham, MA) for all bioluminescence imaging.

⁴We used Living Image 4.3.1. for all bioluminescence image processing.

2.5 Fluorescence Microscopy

1. 2-photon imaging system with variable laser power and compatible 25x objective (*see* Note 6)
2. Transfer and imaging (TRIM) plate (*see* Note 7)
3. Epifluorescence microscope with compatible 10x objective and red and green filter cubes (*see* Note 8)

2.6 Three-Dimensional Spheroid Treatment

1. Compounds (*see* Note 9)
 - a. Cisplatin (SelleckChem © cat. # S1166)
 - b. Doxorubicin (SelleckChem © cat. # S1208)
 - c. Paclitaxel (SelleckChem © cat. # S1150)
 - d. PD0325901 (SelleckChem © cat. # S1036)
2. Sterile ultrapure water, type 1 (Milli-Q™ water or similar)

2.7 Quiescence, Dissociation, and Colony Outgrowth from Spheroids

1. 6-well plates (Corning ® cat. # 3506)

2.8 Cytotoxicity Assays in Two-Dimensional Culture

1. 96-well plate (Corning® cat. # 3599)

2.9 Animal Models of Bone Marrow Metastasis and Drug Treatment

1. Small animal shaver (Wahl compact cordless trimmer or similar instrument)
2. Depilatory solution such as Nair™
3. 28- to 30-gauge insulin syringes for intraperitoneal and intracardiac injections
4. Stereotaxic manipulator for intracardiac injections (optional, Stoelting © cat. # 51625 or similar)
5. Isoflurane

⁵Prepare a stock solution of 15mg/mL D-Luciferin in sterile PBS. Filter solution through a 0.2 µm syringe filter (Corning ® cat. # 431248) and store at -20°C until use.

⁶We used an Olympus FVE1000 MPE microscope for all 2-photon microscopy. Alternatively, confocal microscopy with lasers at 543 nm (red) and 488 nm (green) and can be used. However, confocal microscopy does not excite both Fucci proteins with the same wavelength. A 25× NIR corrected objective (XLPLN25XWMP, NA=1.05, Olympus, Tokyo, Japan) was used in conjunction with the microscope.

⁷We designed the TRIM (transfer, imaging, and analysis) plate to facilitate transfer and stabilization of spheroids for fluorescence microscopy. The protocol for fabricating the TRIM plate has been described previously (21).

⁸We used an Olympus IX70 microscope for all epifluorescence imaging. Images were taken through custom red and green filter cubes (Olympus ©) using a 10X objective.

⁹Cisplatin (NDC-0703-5748-11), doxorubicin (NDC-0069-3030-20), and paclitaxel (NDC-55390-304-50) were purchased as clinical formulations from the University of Michigan Hospital Pharmacy because UM IACUC requires pharmaceutical grade drugs when possible for animal studies. Cell culture studies do not require pharmaceutical grade products and compounds may be purchased from other vendors.

6. Various desired surgical supplies
7. Sterile 0.9% w/v NaCl solution
8. Adult female NSG mice (*see* Note 10)
9. Compounds (*see* Note 9)
 - a. Doxorubicin (NDC-0069-3030-20 as clinical formulation, University of Michigan Hospital Pharmacy)
 - b. Trametinib (GSK112021, SelleckChem © cat. # S2673)
10. Dimethyl Sulfoxide (DMSO, Corning ® cat. # 25-950-CQC)
11. Carboxymethylcellulose, Sodium Salt, Low Viscosity (Calbiochem cat. # 217277)
12. Tween® 80 (Sigma-Aldrich® cat. # P4780-100ML)
13. Flow Cytometer capable of exciting at 561 and 488 nm (BD FACS Aria II (Becton Dickinson) or similar device)

3 Methods

3.1 Construct and Maintain Stably-Expressing Cells

1. To generate stably-expressing cell lines constitutively expressing desired cell markers (*see* Note 11), use lentiviral transduction methods. Then, select those cells that are stably expressing desired markers. We refer readers to standard molecular biology texts for instructions on how to transfer reporters to lentiviral vectors and select for the stably-expressing cells. HS-5 cells were generated to stably express CBRed, 231 cells were generated to stably express CBGreen and FUCCI, and T-47D cells were generated to stably express CBGreen and FUCCI.
2. Before utilizing transduced cells in experiments, verify expression of the reporter in stable cell lines. Methods to do so include qRT-PCR or Western Blotting (refer to standard texts for techniques), fluorescence (flow cytometry or microscopy) or bioluminescence imaging assays (detailed below).
3. Maintain cells in appropriate culture medium as recommended by the supplier. For cells described in this section, we use DMEM supplemented with 10% FBS, penicillin, streptomycin, and glutamine (standard growth media). Passage cells every 2 to 4 days by trypsinization and resuspension.

3.2 Spheroid Co-Culture Model

1. Sterilize 384-well plates (*see* Note 12) by UV radiating the plates for 90 seconds.

¹⁰NSG mice were used to promote the growth of human breast cancer xenografts. Alternative strains of immunocompromised mice may be used. However, the growth of human breast cancer cells is deterred in less immunocompromised mice.

¹¹Cells stably expressing fluorescent and bioluminescent reporters are needed for long term cell culture and animal studies. We employ lentiviral transduction to generate stably-expressing populations of cancer cells.

2. For each spheroid, place cancer cells (*see* Note 13) along with CBRed HS-5 for a total of 3×10^3 cells per well in 25 μ L of spheroid medium (*see* Note 14). These spheroids will grow to approximately 200 to 300 μ m diameter.
 - a. For 231 spheroids make the spheroid 1% 231 CBGreen and Fucci
 - b. For T-47D cells, make the spheroid 5% T-47D CBGreen and Fucci
3. Control wells were distributed throughout the plate to normalize for the effect of position on bioluminescence signaling. To decrease the amount of media evaporation in experimental wells, fill the outermost wells of the plate with 25 μ L of medium.
4. Spheroids were maintained in long term culture by carefully removing 20 μ L of media from each well and gently replacing used media with 18 μ L of new spheroid medium (*see* Note 15).

3.3 CBGreen and CBRed Bioluminescence Imaging

1. Capture signals from bioluminescence imaging using a bioluminescence imaging system with optical filters to separate CBGreen and CBRed light emissions and analyze data using software compatible with the specific imaging system.
2. For spheroid imaging, gently remove 5 μ L of media from each well of the 384-well plate. Quickly add 5 μ L of a 1:4 dilution of 150 μ g/mL luciferin (*see* Note 16) for a final luciferin dilution of 1:20 or 7.5 μ g /mL of luciferin in each well.
3. For imaging standard two-dimensional culture systems, quickly add 10 μ L of a 1:10 dilution of 150 μ g /mL luciferin (*see* Note 16) to each well of the 96-well plate for a final luciferin dilution of 1:100 in or 1.5 μ g /mL of luciferin in each well.
4. After adding luciferin, incubate wells at 37°C for 5 minutes and place a single plate in the IVIS. Use medium binning, a three- to five-minute exposure time for each channel, a 520nm band pass filter for CBG imaging, and 680nm band pass filter for CBR imaging settings to acquire images (Fig. 1). Separate signals using previously discussed methods (*see* Note 17).

¹²384-well plates were used to culture spheroids because they are low-cost, easy to use, have low cell adhesion, and permit bulk experiments. The geometry of the well promotes quick (<24 hours) spheroid growth and production of a single, uniform, stable, and reproducible spheroid per well.

¹³Percentages of cancer cells were optimized to mimic relatively small numbers of DTCs in bone marrow and to read bioluminescence signal. There must be enough cancer cells to provide sufficient signal to be detected by imaging but not so many cells as to produce detectable signal CBGreen signal in the CBRed imaging filter window. The percentage of cancer cells per spheroid varies between cancer cell types due to differing growth rates of the T-47D and 231 cells.

¹⁴Spheroid medium is formulated as phenol red free DMEM supplemented with 1% FBS, 0.1nM β -Estradiol, penicillin/streptomycin/glutamine, and pyruvate to match all but the serum content and phenol in standard growth medium.

¹⁵Use a 20 μ L multichannel pipette to ease and quicken spheroid handling in the 384-well plates. Remove media from the wells by barely sticking the tips in the media to avoid aspirating the spheroids. Empty waste into a designated reservoir. To avoid wasting tips flush out the tips three times with sterile 1X PBS from a reservoir to remove media from wells within the same experimental group. Use the multichannel pipette with new tips to collect media from a reservoir (per experimental group) and gently add it to each well to avoid rupturing the formed spheroids.

¹⁶Further dilute luciferin in sterile 1X PBS at the desired concentration before adding to spheroids or cells.

¹⁷Images were taken with large binning and a two-minute exposure time. To gather both red and green signals, acquire images of the same plane using both 530–550 nm and 690–710 nm emission filters on the bioluminescence imaging system (22).

3.4 Fluorescence Microscopy

1. Using an upright Olympus FVE1000 MPE microscope with a 25× NIR corrected objective, acquire Z-stack slice sequences of spheroids. Two-photon microscopy has the benefit of minimal out of plane signaling and that both FUCCI proteins are excited at the same wavelength which decreases imaging time and photobleaching.
2. Since the microscope objective requires immersion in water, transfer spheroids to a TRIM plate to increase imaging efficiency and quality.
3. Use an excitation wavelength of 920 nm to excite both FUCCI proteins. FUCCI C protein emission was captured in the red channel (575 – 630 nm) and indicates the cell is in the G1 phase. FUCCI D protein emission was captured in the green channel (495 – 540 nm) and indicates the cell is in either S/G2/M phases (*see* Note 1).
4. Acquire 150µm deep stacks of spheroid images with a 5µm step size (30 images per spheroid). Two-photon signal decreases while imaging deeper into a spheroid and using the Olympus Bright-Z function helps compensate for this decrease in signaling. The Bright-Z function allows the user to adjust laser power and/or detector gain throughout a Z-stack for optimal imaging (unsaturated but maximized signals) throughout the spheroid. For optimal data analysis, keep spheroid image acquisition parameters consistent throughout a single experiment.
5. For two-dimensional culture experiments, use an Olympus IX70 epifluorescence microscope to easily visualize FUCCI markers. Signal is visible when using either red or green filter cubes.

3.5 Three-Dimensional Spheroid Treatment

1. Using the spheroid co-culture model protocol, develop spheroids for two days before starting treatment with specified compounds and concentrations.
2. Compound preparation of various concentrations
 - a. Dissolve cisplatin in sterile ultrapure water, type 1, to create concentrations between 10^{-1} and 10^5 nm.
 - b. Dissolve doxorubicin in sterile ultrapure water, type 1, to create concentrations between 1 and 10^5 nm.
 - c. Dissolve paclitaxel in sterile DMSO to create concentrations between 10^{-2} and 10^3 nm.
 - d. Dissolve PD0325901 in sterile DMSO to create concentrations between 10^{-2} and 10^3 nm.
3. On day two, use two to four columns of the plate to establish preliminary bioluminescence of spheroids to help establish the growth curve before beginning compound treatment in the remaining wells (*see* Note 18).

4. Exchange compound-containing spheroid media every other day using the same method as noted above.
5. Image spheroid bioluminescence after eight days of treatment (Fig. 2). After imaging, exchange the media in each well with 20µL of media three times to remove the majority (>99%) of remaining luciferin and compounds (*see* Note 18). In case of desired future cell-recovery, exchange media in each well for a total of six days (two media changes).

3.6 Quiescence, Dissociation, and Colony Outgrowth from Spheroids

1. Use the abovementioned 'Spheroid co-culture model' protocol to culture both types of cancer cell spheroids (231 CBGreen and FUCCI or T-47D CBGreen and FUCCI cells) and image for FUCCI markers two and 10 days after beginning culture. FUCCI reporters present orange when the cell is in the G1 phase and green when in the S/G2/M phases (*see* Note 1).
2. As well as imaging on days two and 10, dissociate parallel spheroids of each condition on both days to analyze two-dimensional colony outgrowth.
 - a. Use a 200µL pipette and tip to gently aspirate spheroids. Place spheroids undergoing the same treatment together into a well of a 6-well plate containing 2mL of 1X PBS. Make sure that each condition has the same number of spheroids per well.
 - b. Once spheroids are collected, carefully aspirate PBS and add 0.2mL of trypsin to each well.
 - c. Add 1.3mL of growth media per plate after spheroids are dissociated and gently swirl to evenly spread cells.
3. Image cells 1, 4, and 8 days after seeding cells with both epifluorescence, to visualize the growth phase of cells using FUCCI markers, and bioluminescence using the protocols described above. Replace media on cells after bioluminescence imaging to reduce luciferin toxicity on cells and 6 days after seeding to maintain optimum nutrient concentrations. The higher rate of growth of the cancer cells in two-dimensional culture (vs three-dimensional culture) requires only analyzing CBGreen signaling.

3.7 Cytotoxicity Assays in Two-Dimensional Culture

1. Place a total of 1×10^4 cells per well with 100µL growth media in a 96-well plate. Use a mixture of 4% 231 CBG and FUCCI cells and 96% HS-5 CBR cells. Make sure that the cell types are mixed together well to create a homogenous co-culture.
2. Grow cells for 24 hours and subsequently treat cells with compounds for 72 hours.

¹⁸Leaving the luciferin on the cells is toxic and will skew results. Removing and diluting the media also helps remove the compound from the system, which is desired as it is the end of the experiment.

3. Prepare compounds at an appropriate range of concentrations to create a cytotoxicity curve for two-dimensional cell culture conditions.
 - a. Dissolve cisplatin in sterile ultrapure water, type 1, to create appropriate concentration range
 - b. Dissolve doxorubicin in sterile ultrapure water, type 1, to create appropriate concentration range
 - c. Dissolve paclitaxel in sterile DMSO to create appropriate concentration range
4. Use the bioluminescence imaging protocol detailed above to quantify drug toxicity.

3.8 Animal Models of Bone Marrow Metastasis and Drug Treatment (see Note 19)

1. Inject 100µl of 1×10^5 231 CBG and FUCCI cells suspended in 0.9% NaCl solution via intracardiac injection into the left ventricles of female NSG mice between 5 and 9 weeks old. Randomly assign mice to treatment groups and dose three days after cancer cell injection. Administer either
 - a. A single intraperitoneal injection of doxorubicin (5 mg/kg, *see* Note 20)
 - b. Five daily doses of trametinib by oral gavage (1 mg/kg, *see* Note 21)
 - c. Combined treatment of doxorubicin and trametinib
 - d. Vehicle controls
2. 13 days after beginning treatment, (i.e. seven days after the last dose of trametinib or vehicle is given) humanely euthanize mice per institutional protocols.
3. Harvest bone marrow from the lower extremities of mice by flushing through the interior of the femur and tibia with PBS (20).
 - a. To quantify cancer cell growth, plate recovered bone marrow in a 10cm tissue culture treated dish with standard growth medium. One week after plating bone marrow contents, use the bioluminescence imaging protocol described above to quantify growth of 231 cells.
 - b. To perform FLOW cytometry analysis of harvested bone marrow; keep each sample separate, centrifuge recovered bone marrow, re-suspend in 200µL of PBS. For each sample, analyze 5×10^5 events using an appropriate FLOW cytometer.

¹⁹All animal procedures should be approved by the local IACUC (our protocol was approved by the University of Michigan Committee for the Use and Care of Animals).

²⁰Only a single dose of doxorubicin was administered because multiple doses are toxic to NSG mice. As doxorubicin was obtained already in solution from the University of Michigan Hospital pharmacy, plain DMSO was used as a vehicle control.

²¹Formulate trametinib for gavage (23) by dissolving it in sterile 100% DMSO and diluting it 1:9 in sterile-filtered 1% carboxymethylcellulose and 0.4% Tween-80.

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Figure 1. Dual-Color Bioluminescence Imaging

Bioluminescence imaging of a 384-well plate section after treating spheroids with multiple different compounds. Cancer cell signals (CBGreen) and stromal cell signals (CBRed) are detected in the green and red channels, respectively. Pink bins show compounds toxic to both stromal and cancer cells, white bins show compounds that destroy stromal cells and not cancer cells, and yellow bins show compounds that eliminate cancer cells and not stromal cells. Scale bar depicts bioluminescence on a pseudo color scale with red and blue indicating high and low signaling, respectively.

Figure 2.

a: Drug Response Curves

Graph shows mean values $\pm$ SEM for bioluminescence fold change in response to varying compound concentrations for both T-47D cells (green curve) and HS-5 cells (red curve). Fold change was determined in comparison to untreated spheroids. Curves may be used to determine optimal drug dosing to differentially eliminate cancer cells over stromal cells.

b: Combinatorial drug dose response plot.

Cancer cell fold change in response to compound combination treatments. Fold change was determined by normalizing bioluminescent image data of treated cells to control cells after eight days of combination treatment. Color scale bar shows selectivity of compounds for

eliminating cancer versus stromal cells. Red and blue depict highest and lowest selectivity, respectively. The white circle delineates optimal combination drug treatments for selectively eliminating cancer cells over stromal cells. The black circle shows combination treatment concentrations that do not inhibit cancer cell growth and kill stromal cells.