Real-Time Apoptosis and Necrosis Detection in 3D Spheroid Cell Models

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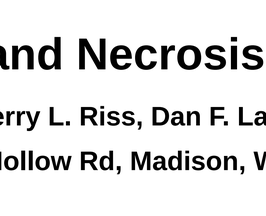
Abstract # 710

1. Introduction

Cancer spheroids grown in vitro often share many of the same structural and biological complexities of native tumors and have become a convenient means to approximate the relative efficacies of new chemical entities. In this work, we examined the pharmacodynamics of apoptosis induction in spheroids using a multiplexed bioluminescent and fluorescent, real-time assay reagent which utilizes annexin V fusion proteins containing binary elements of a complementing luciferase, a time-released luciferase substrate, and an excludable DNA membrane integrity dye. The assay reagent was added to HCT-116 and HepG2 spheroids dosed with serial dilutions of bortezomib, paclitaxel, or panobinostat to detail the response magnitude, potency and kinetics resulting from continuous exposure. Luminescent and fluorescent data were continuously collected every 30 minutes during a 48h exposure using a conventional multimode plate reader equipped with atmospheric control. After the exposure period, the spheroids were imaged by fluorescence microscopy to assess necrotic burden as well as assayed for remaining cellular viability using ATP content. All measures were compared to untreated control spheroids. Secondary necrosis resulting from completion of the apoptotic program lagged PS exposure in both time and magnitude. Imaging results for necrosis and ATP determinations provided orthogonal and inversely complementary verification of data values obtained with the apoptosis assay reagent. Taken together, this workflow may help define the in vitro efficacies of new chemical entities with respect to their capability to penetrate and induce apoptosis in spheroids and/or other more complex multicellular models.

2. Assay Concepts and Experimental Work Flow

RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay



CellTiter-Glo® 3D

Step1. Dose Cells, add RT Reagent

Measure luminescence/fluorescence (repeat every 30 min)

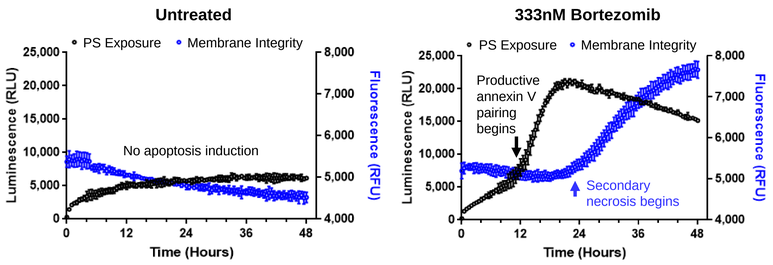
0 6 12 18 24 30 36 42 48

Exposure (Hours)

Step 2. Image fluorescence

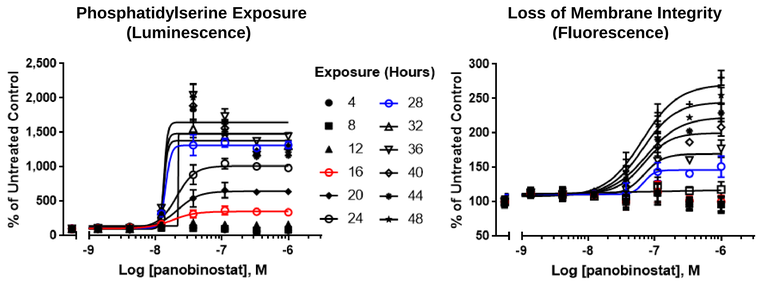
Step 3. Add ATP Reagent/Measure

3. The Apoptotic Response Phenotype



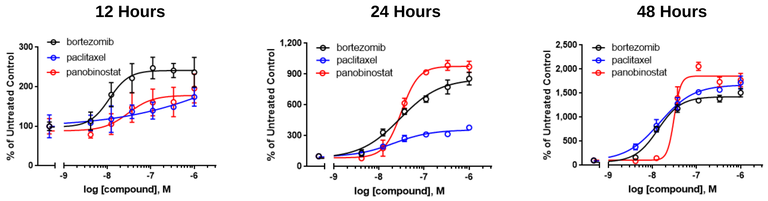
HepG2 spheroids (~250µm diameter) created in black, ultra-low attachment plates (Costar 4515) were dosed with 333nM bortezomib or vehicle control and RealTime-Glo™ Annexin V Apoptosis and Necrosis reagent added. Luminescence values associated with PS exposure and fluorescence associated with loss of membrane integrity were collected every 30 minutes for 48 hours using a BMG CLARIOstar® equipped with atmospheric control. Increases in PS exposure prior to increases in secondary necrosis values are consistent with the apoptotic phenotype for bortezomib treatment (N=3).

4. Exposure-Dependent Dose Reponses



HCT116 spheroids (~300µm diameter) were formed as described in panel 3, and dosed with three-fold dilutions of panobinostat in the presence of the RealTime-Glo™ Annexin V and Necrosis Assay reagent. Dose-dependent phosphatidylserine exposure began at 16 hours (left graph, red) and increased in magnitude (relative number of apoptotic cells) with a near maximal response at 28 hours (blue). Secondary necrosis resulting from completion of the apoptotic program produced dose-dependent increases beginning at 28 hours (right graph, blue). N=3 for each data point.

5. Apoptotic Progression by Potency and Magnitude

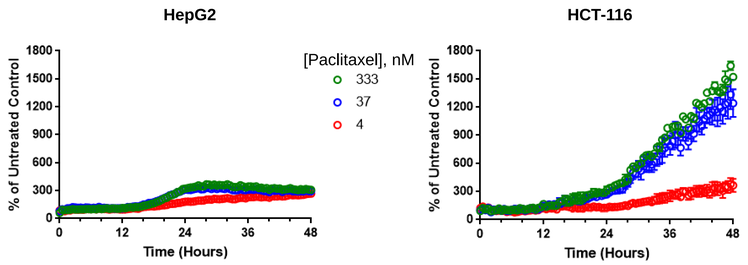


EC_{50} as a Function of Exposure

Treatment	12h	24h	48h
bortezomib	11nM	29nM	13nM
paclitaxel	> 1µM	21nM	14nM
panobinostat	30nM	31nM	31nM

HCT-116 spheroids were treated with bortezomib, paclitaxel, or panobinostat and data collected using the real-time apoptosis and necrosis reagent. Full dose-response curves were plotted from data collected at 12, 24 and 48 hours. Bortezomib produced the earliest response, followed by panobinostat and paclitaxel. Paclitaxel induced a progressive increase in potency whereas bortezomib and panobinostat were largely unchanged. Necrosis data are not shown.

6. Differential Induction Susceptibility of Spheroids



HepG2 and HCT-116 spheroids were dosed with paclitaxel in the presence of the real-time apoptosis and necrosis reagent and data collected at 30 minute increments for 48 hours. Paclitaxel initiated PS exposure in both cell types at ~18 hours but produced a more robust induction ratio with HCT-116 cells when compared with HepG2. Similar trends were observed for bortezomib and panobinostat, suggesting differential efficacies are inherent features of the spheroid masses. Necrosis data are not shown.

7. Imaging as a Complement to Fluorometry

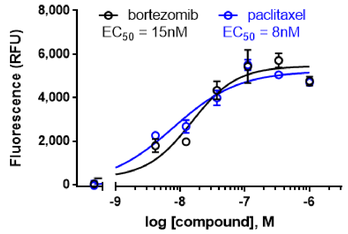
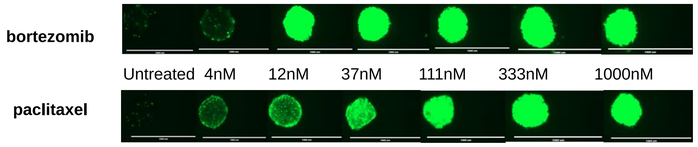
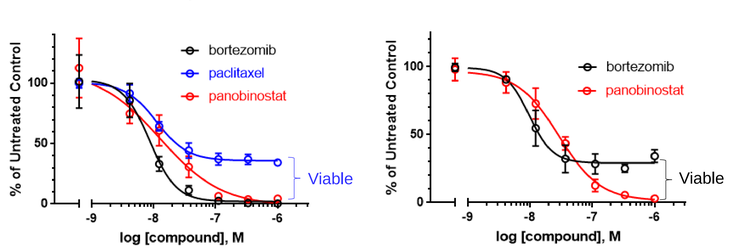


Plate reader-based fluorometry enables "hands off" continuous measurement of secondary necrosis by the Necrosis Detection Reagent. HepG2 spheroids at 48 hours of exposure with bortezomib and paclitaxel (left), produced similar dose-dependent cytotoxicity profiles. The probe also enables fluorescent imaging which can reveal morphological distinctions not possible with total fluorescence measures (bottom). Images were collected at 4x magnification after 48 hours of exposure using an EVOS FL Cell Imaging system. Scale bar = 1000µm.



8. Viability Measure Defines Degree of Tumor Persistence



CellTiter-Glo® 3D reagent was added to treated spheroids after 48 hours of drug exposure. ATP-derived luminescence was measured after 10 minutes of incubation. (Left) Paclitaxel produced a potency similar to bortezomib and panobinostat but left a substantial viable mass. (Right) Bortezomib is more potent than panobinostat, but did not produce complete cytotoxicity. Therefore, the endpoint viability measure is useful for defining cytotoxic efficacy.

9. Conclusions

Real-time Apoptosis and Necrosis Reagents can be employed with spheroids

- To establish the primary cytotoxic mode of action (apoptosis vs. other death modes)
- To characterize the link between dose and kinetic onset of apoptosis
- To define time-dependent, relative magnitudes of response
- To explore differential susceptibility of diverse spheroid models to test agent

The real-time Necrosis Detection Probe offers dual detection modalities

- To allow for continuous collection of plate-based fluorometry data
- To enable morphological examination of treated spheroids

The endpoint viability measure by CellTiter-Glo® 3D provides an orthogonal measure of efficacy

- To assess relationship between dose and cytotoxicity
- To reveal incomplete reductions in spheroid viability

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