A Plate Based Real-Time Annexin V Method for Monitoring Antibody Drug Conjugate Induced Apoptosis and Cell Death

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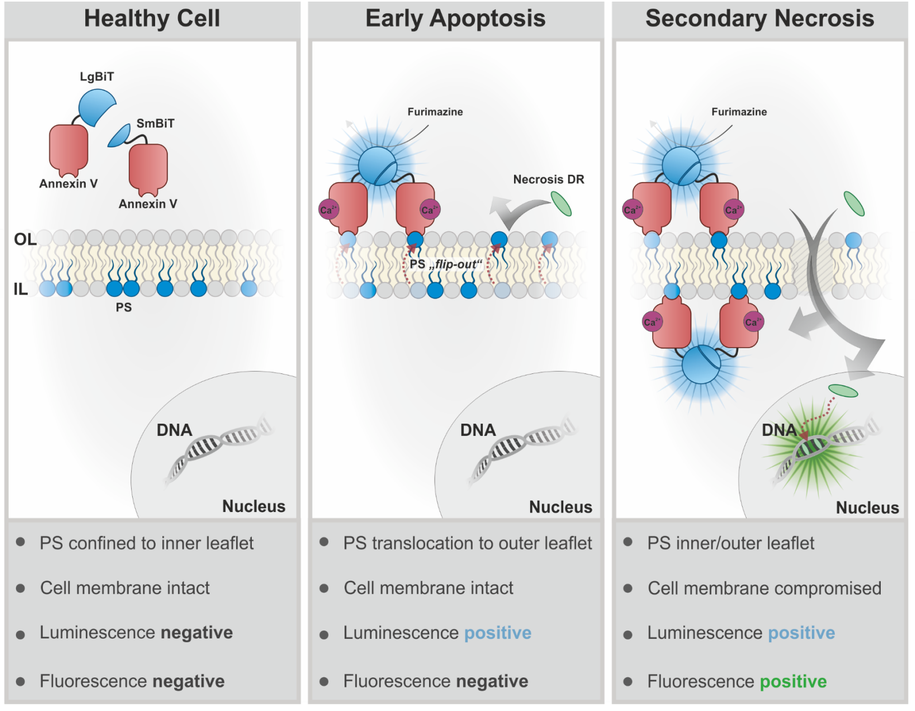
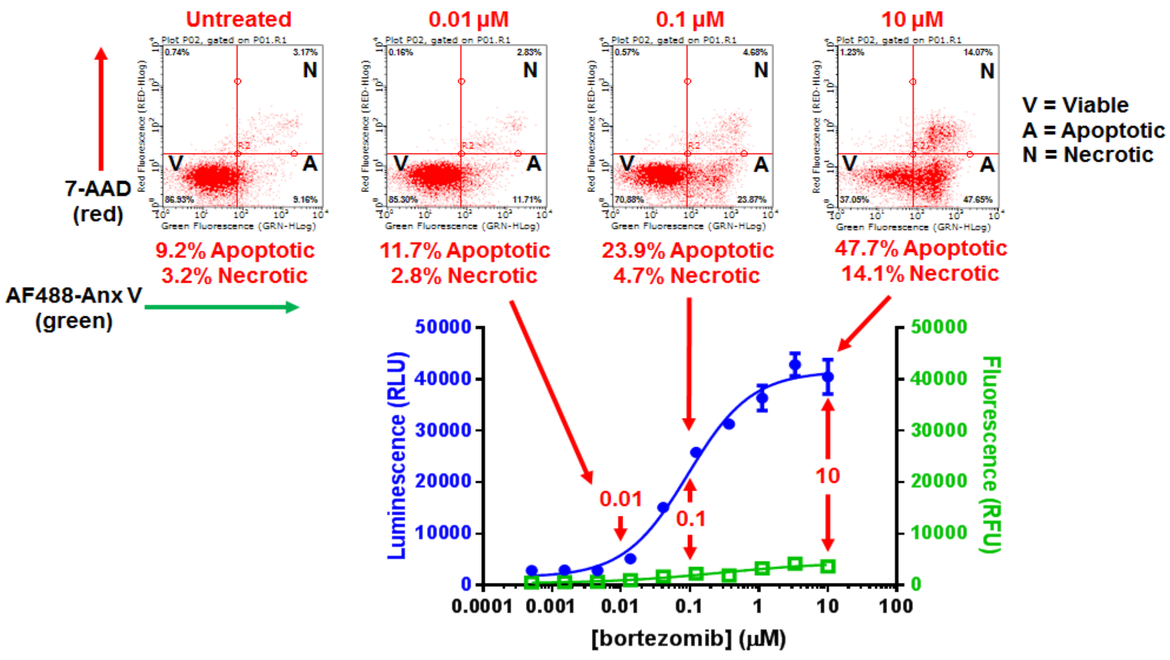
1. Introduction

Antibody drug conjugates (ADC) induce cell death via cognate interactions with surface antigens, internalization, release of toxin, and the binding of the toxin to its cellular targets. The key factors that determine the effectiveness of an ADC include the antibody, linker, toxin, and conjugation method. Testing all possible combinations can be challenging with assays that simply readout a single time point. To address this need, we developed a real-time, live cell assay method that utilizes a fully homogeneous, bioluminescent annexin V reagent. The method does not require laborious washing and sample preparation steps associated with traditional annexin methods and is fully compatible with plate-based multimodal signal detection systems. The system contains two annexin proteins which have been engineered to contain separate and distinct complementing subunits of a binary luciferase. Additionally, the system contains a novel time-released luciferase substrate and a cell impermeable, fluorogenic DNA dye for monitoring necrosis. Because the annexin-luciferase fusion pairs have only modest affinity for each other, luminescence remains low until phosphatidylserine exposure, a hallmark of the programmed cell death phenotype, brings annexin monomers into close proximity facilitating complementation of the luciferase sensor. The assay reagent can be applied at dosing for real-time measurement of the dose-dependency and magnitude of programmed cell death progression. This work describes our efforts to characterize and compare the performance of the bioluminescent annexin assay to traditional endpoint assays after ADC exposure with target cells.

2. RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Schematic

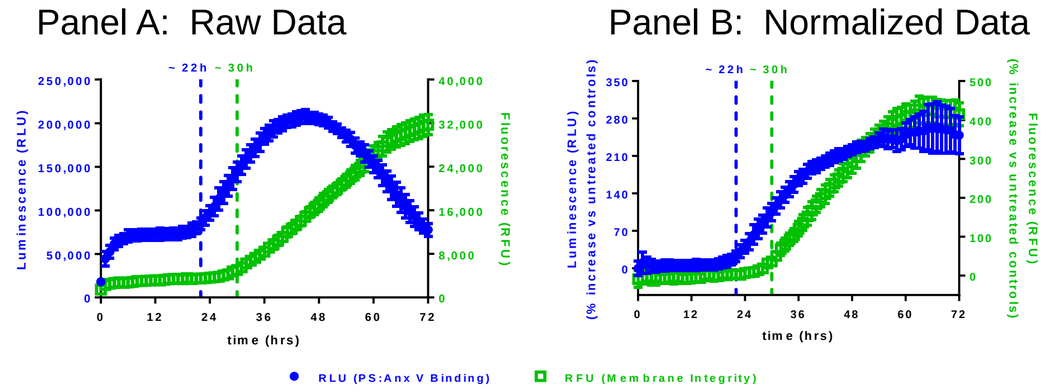


3. Concordance of Homogeneous Real-Time Assay with Multistep Flow Cytometry Method



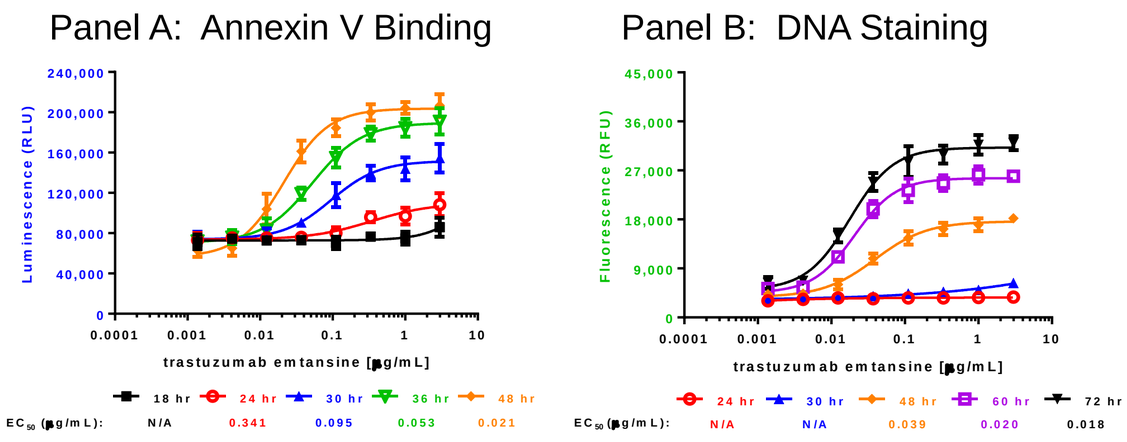
K562 cells were exposed to 0, 0.01, 0.1, or 10 µM bortezomib for 16 hours. Cells were harvested, washed, and labeled with Alexa Fluor® 488 (green fluorescence, PS:Anx V binding) and 7-AAD (red fluorescence, membrane integrity) and analyzed by flow cytometry (10,000 events, top panel). K562 cells (10,000/well) were exposed to serial dilutions of bortezomib in the presence of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay reagent. Luminescence (PS:Anx V binding) and fluorescence (membrane integrity) was recorded kinetically and the 16 hour incubation with bortezomib is shown (bottom panel).

4. Real-Time Detection of Antibody Drug Conjugate (ADC) Mediated Cytotoxicity



SKBR3 HER2+ cells were seeded at 10,000 cells/well in phenol red free growth medium with 10% FBS in a solid white TC-treated 96-well plate and treated with 1 µg/ml of the antibody-drug conjugate (ADC), trastuzumab emtansine. The 1X RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay detection reagent was added once at time zero and the plate was incubated in a plate reader equipped with atmospheric control (37°C/5% CO_2). The luminescence (RLU, PS:Anx V binding, blue solid circles) and fluorescence (RFU, membrane integrity, open green squares) was recorded repeatedly from the same sample wells (every 1 hr for 72 hrs) and both the raw RLU and RFU (panel A) and normalized (% increase vs untreated controls) RLU and RFU (panel B) was graphed over time. The lag (indicated by dotted lines) between the onset of increasing annexin V luminescence (blue) and increasing necrosis probe fluorescence (green) is indicative of apoptosis. The subsequent time period following this lag where there is a concurrent rise in luminescence and fluorescence is indicative of secondary necrosis.

5. Antibody Drug Conjugate (ADC) Dose Response Curves at Fixed Time Points



RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay detection of apoptosis and secondary necrosis in antibody-drug conjugate (trastuzumab emtansine) treated SKBR3 HER2+ cells (10,000/well in 96-well plate). The plate was incubated in a plate reader with atmospheric control (37°C/5% CO_2) and luminescence from PS:Anx V binding (panel A) and fluorescence from DNA staining of necrotic cells (panel B) were recorded every 1 hr for 72 hrs with the indicated time points shown.

Note: T47D HER2- cells showed no increase in apoptosis and secondary necrosis under the same conditions (data not shown)

6. Conclusions

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay detects the kinetics of apoptosis and secondary necrosis in real time resulting from a variety of treatments (including ADCs) to induce cell death

- The assay demonstrates concordance with fluorescent annexin V binding methods used for flow cytometry
- Following treatment of SKBR3 cells with trastuzumab emtansine:
  - maximal PS on the outer cell membrane (RLU) occurred between 36 and 48h
  - maximal cell membrane destruction (FLU) occurred at 60-72h
- The method can be used to differentiate modes of cell death
- The assay can be multiplexed with luminescent caspase-3/7 detection as an orthogonal method to confirm apoptosis (data not shown)

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