A Real-Time Annexin V Method for Monitoring Programmed Cell Death

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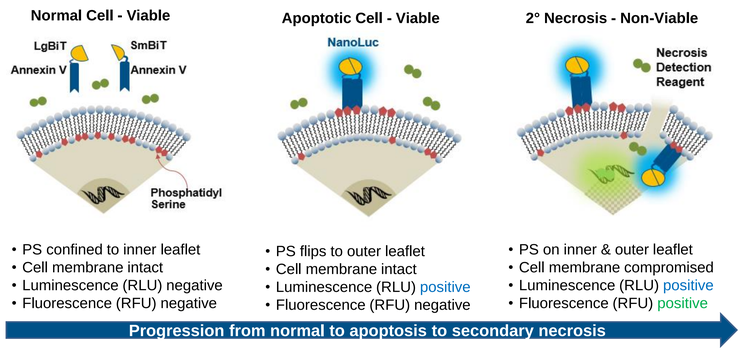
Abstract # 4296

1. Introduction

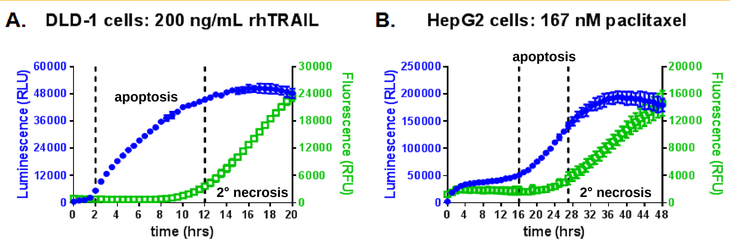
We developed a homogeneous luminogenic annexin V binding assay to detect the occurrence of apoptosis in real time using a multimode plate reader. The detection reagent has two different annexin V fusion proteins engineered to contain complementing domains of a binary luciferase, a substrate for luciferase and a cell impermeable fluorogenic DNA dye to detect necrotic cells. The annexin-luciferase fragment fusion pairs have only modest affinity for each other, thus luminescence remains low in the presence of viable cells. When annexin V in the fusion proteins binds in close proximity to the phosphatidylserine exposed on the surface of apoptotic cells, the luciferase fragments reconstitute to form an active enzyme and generate luminescence. The reagent is added directly to cells in culture providing a homogeneous protocol that does not require cell washing steps typically needed with fluorescent annexin V binding assays used for flow cytometry. The real time method has been used to monitor the kinetics of apoptosis and secondary necrosis in several model systems by repeatedly recording luminescence and fluorescence from the same samples of cells.

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



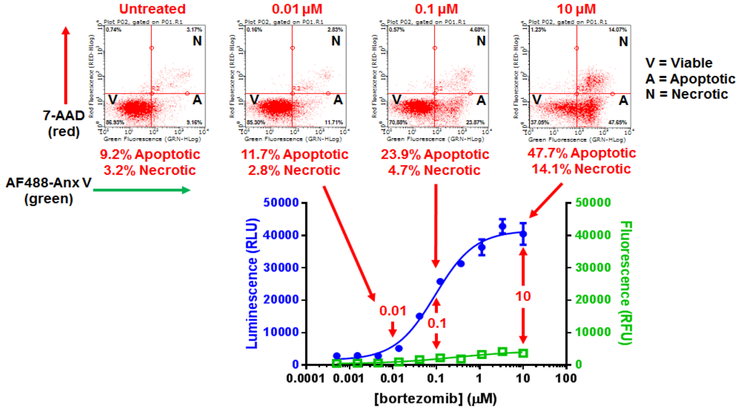
Phosphatidylserine (PS) is oriented toward the cytoplasm in normal viable cells (left). During apoptosis, PS translocates from the inner leaflet of the membrane to become exposed on the outer cell surface and available for annexin V binding. Annexin V fusion proteins bind in close proximity to enable the luciferase fragments to reconstitute to form an active enzyme and generate luminescence (center). Secondary necrosis is detected by fluorescent DNA staining (right).

3. Real-Time Detection of Apoptosis and 2° Necrosis



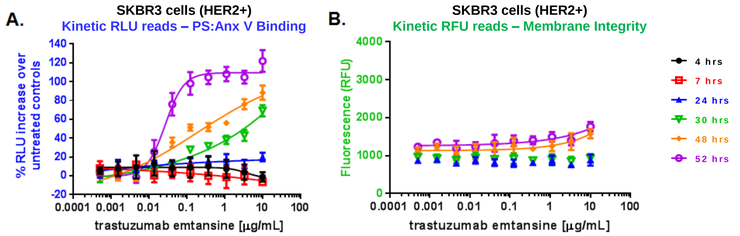
DLD-1 cells (panel A) or HepG2 cells (panel B) were seeded at 10,000/well in 96-well plates and treated as indicated. The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay reagent was added once at time zero and luminescence (RLU, PS:Anx V binding) and fluorescence (RFU, membrane integrity) was recorded repeatedly from the same sample wells. The lag (indicated by dotted lines) between the onset of increasing annexin V luminescence (blue) and the onset of 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.

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



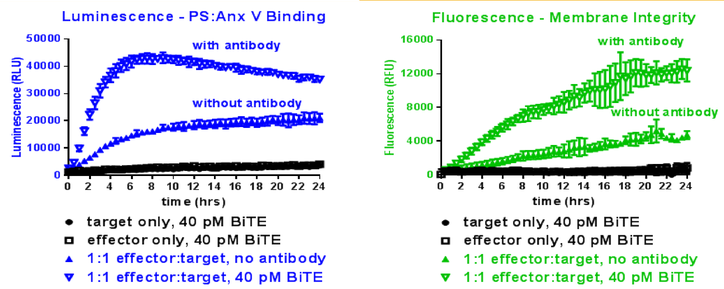
K562 cells were exposed to 0, 0.01, 0.1, or 10 µM bortezomib for 16 hours. Cells were harvested, washed, and labeled with Alexa Fluor® 488 (green fluorescence, PS:Anx V binding) and 7-AAD (red fluorescence, membrane integrity) and analyzed by flow cytometry (10,000 events, top panel). K562 cells (10,000/well) 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).

5. Real-Time Detection of Antibody Drug Conjugate Induced Apoptosis and 2° Necrosis



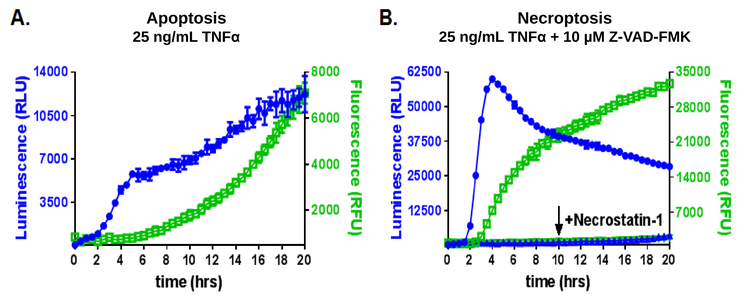
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay detection of apoptosis and secondary necrosis in antibody-drug conjugate (trastuzumab emtansine) treated SKBR3 HER2+ cells (10,000/well in 96-well plate). The plate was incubated in a plate reader with atmospheric control and luminescence from PS:Anx V binding (panel A) and fluorescence from DNA staining of necrotic cells (panel B) were recorded at the indicated times.
Note: T47D HER2- cells showed no increase in apoptosis under the same conditions (data not shown).

6. Real-Time Detection of Cytolytic T-Cell Mediated Cytotoxicity ± BiTE Antibody Treatment



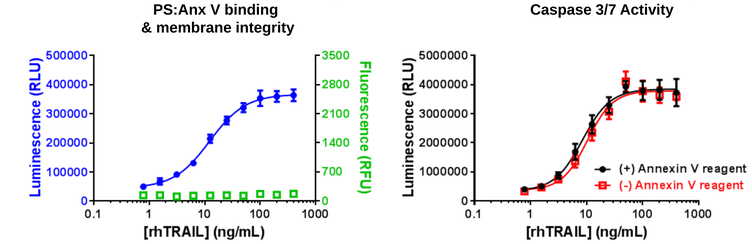
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay detection of cytolytic cell death in effector TALL-104 T-cell mediated cytotoxicity of target Raji cells in the presence and absence of a bi-specific T-cell engager (BiTE) blinatumomab. Target and effector cells were mixed at 1:1 with and without BiTE to examine kinetic progression and enhancement of cytolytic cell death by the BiTE, reflected by the concurrent rise in luminescent (left panel, blue) and fluorescent (right panel, green) signals.

7. Discriminating Cell Death Mechanisms Using Real-Time Assay Reagent and Selective Inhibitors



U937 cells (10,000/well) were exposed to 25 ng/mL TNFα to produce an apoptotic phenotype (panel A); 25 ng/mL TNFα + 10 µM Z-VAD-FMK to initiate a necroptotic response (panel B, top 2 lines); and 25 ng/mL TNFα + 10 µM Z-VAD-FMK + 10 µM necrostatin-1 to block both the apoptotic and necroptotic pathways (panel B, bottom 2 lines). The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay reagent was added at the time of adding treatments and luminescence (blue) and fluorescence (green) was recorded every 0.5 hour over a 20 hour time course.

8. Multiplexing Annexin V Binding and Caspase Detection as Orthogonal Apoptosis Assays



DLD-1 cells (10,000/well) were exposed to serial dilutions of rhTRAIL in a 96-well plate. The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay reagent was added once at time zero and incubated in a plate reader with atmospheric control. Luminescence (panel A, blue, PS:Anx V binding) and fluorescence (panel A, green, membrane integrity) was collected kinetically. Following the 6.5 hour RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay read, Caspase-Glo® 3/7 reagent was added to wells in a multiplex format (panel B, caspase 3/7 activity, black). As a control, parallel wells of DLD-1 cells not exposed to the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay reagent were assayed with the Caspase-Glo® 3/7 reagent (panel B, red line) suggesting any residual luminescence from the annexin V assay had no effect on the caspase assay reading.

9. Summary

- 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 biologics) to induce cell death
- The assay demonstrates concordance with fluorescent annexin binding methods used for flow cytometry
- The method can be used to differentiate modes of cell death with appropriate use of inhibitors
- The assay can be multiplexed with luminescent caspase-3/7 detection as an orthogonal method to confirm apoptosis

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