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INSPIRED CELL SOLUTIONS

APPLICATION NOTE

Detecting the onset and kinetics of apoptosis

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Introduction

Apoptosis or programmed cell death is a highly regulated process, essential for the development and homeostasis of all multicellular organisms. It is also an important mechanism of tumour suppression: the failure of cells to undergo apoptosis is a key element of tumorigenesis. Conversely, an excess level of apoptosis is a feature of pathologies such as neurodegenerative disease and autoimmunity.

Two main pathways are known to trigger apoptosis: an intrinsic and an extrinsic pathway. The key event downstream of both pathways is the activation of the proteolytic caspases cascade, which causes specific molecular changes together with morphological changes, such as cell shrinkage, nuclear condensation and fragmentation, membrane blebbing and the production of apoptotic bodies. In healthy tissues where levels of apoptosis are low, dying cells are efficiently phagocytosed. In diseases where levels of apoptosis are high, not all apoptotic cells are cleared by phagocytosis and these cells undergo secondary necrosis.

In this study, we analysed the ability of two drugs, Staurosporine and Etoposide, to induce apoptosis in different cancer cell lines.

Case Study: Detecting Apoptosis *in vitro*

In this study we used three different readouts to assess the kinetics of apoptosis in response to Staurosporine and Etoposide:

• IncuCyte® S3 Live-Cell Imaging System (Sartorius) to track the appearance of a fluorescent caspase-3/7 dye

• Multiplexing a fluorescent cell viability dye with an IncuCyte® Caspase-3/7 luminescent dye for apoptosis in a plate reader-based assay

• Analysing early and late apoptotic cell markers using western blotting and flow cytometry

Cell Lines Used

Cell Line	Genotype	Cat. No.
MDA-MB-231	Parental	HD PAR-402
HCT116	Parental	HD PAR-007
HCT116	*CHEK2-/-*	HD R02-017
HCT116	*CHEK2-/-; TP53-/-*	HD R02-018

Results and Discussion

Monitoring apoptosis using live cell imaging

The IncuCyte® Caspase-3/7 Green Apoptosis reagent (Sartorius) couples the activated caspase-3 and caspase-7 amino acid recognition motif (DEVD) to a green fluorescent DNA intercalating dye. This substrate is non-fluorescent unless the DEVD sequence is cleaved by active caspase-3 and/or caspase-7, so it can be added directly to the cells without perturbing their growth or morphology. Once in the medium, the substrate crosses the cell membrane where it is cleaved by activated caspase-3 and/or caspase-7 resulting in the release of the dye and green fluorescent staining of nuclear DNA. Therefore, the kinetic activation of caspase-3 and/or caspase-7 can be monitored morphologically following the variation of the green fluorescence with live cell imaging using an IncuCyte® microscope.

The IncuCyte® Annexin V Red reagent (Sartorius) contains a cyanine fluorescent dye that selectively recognises phosphatidylserine (PS). In healthy cells PS is located on the inner surface of the membrane, but becomes externalised during apoptosis. When not bound the dye is non-fluorescent and can be added directly to the cells without perturbing their growth and morphology. By monitoring for changes in red fluorescent signal level the kinetic externalisation of PS can be detected with live cell imaging using an IncuCyte® microscope.

The fluorescent object count increases with increasing drug doses (A, B), compared with the confluence of the cells detected using phase contrast (C). Both Staurosporine (Figure 1) and Etoposide (Figure 2) induce apoptosis, but with differing kinetics. Staurosporine induces detectable caspase-3/7 activity and externalisation of PS by 24 hours (1D). Fluorescent cells become detectable between 24 and 72 hours post treatment with Etoposide (2D).

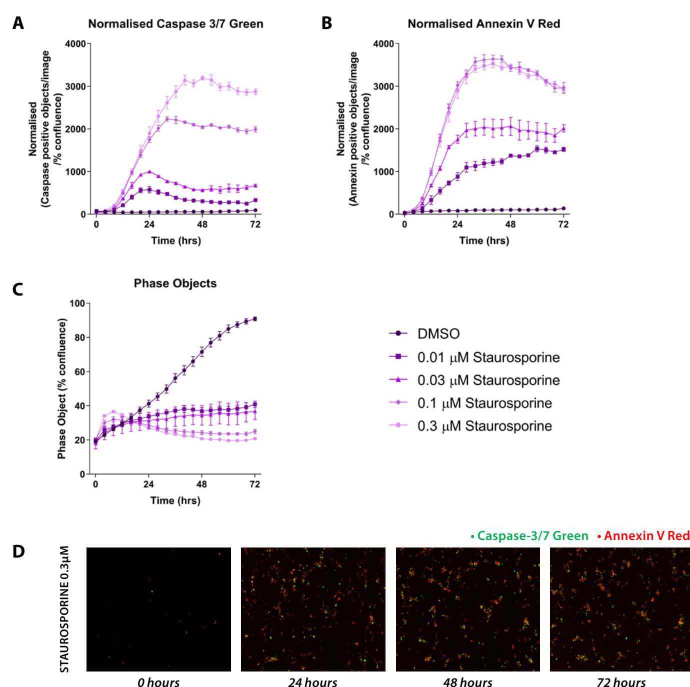

Figure 1. MDA-MB-231 cells were treated with Staurosporine in the presence of Caspase 3/7 Green Apoptosis reagent and Annexin V Red reagent. The plate was imaged every 4 h using the IncuCyte® S3 timelapse microscope. For cells treated with Staurosporine 0.3 µM representative images were taken of individual wells at 0, 24, 48, 72 h after treatment (IncuCyte® S3 microscope images of cells from individual wells).

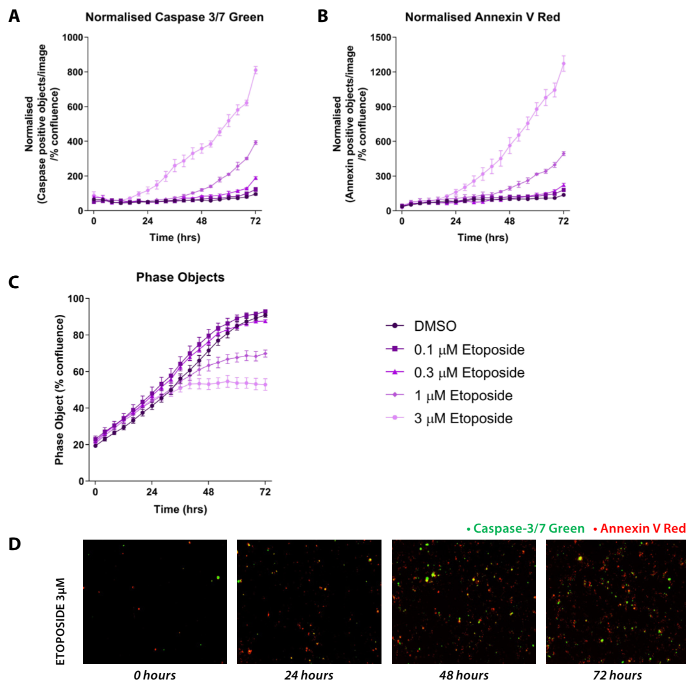

Figure 2. MDA-MB-231 cells were treated with Etoposide in the presence of Caspase 3/7 Green Apoptosis reagent and Annexin V Red reagent. The plate was imaged every 4 h using the IncuCyte® S3 timelapse microscope. For cells treated with Etoposide 3 µM representative images were taken of individual wells at 0, 24, 48, 72 h after treatment (IncuCyte® S3 microscope images of cells from individual wells).

Multiplexing apoptosis with viability detection

Multiplexing cell viability and apoptosis endpoints requires two sequential steps. First, the CellTiter-Blue® Viability Assay (Promega) is used to determine the number of viable cells. When added to cells in culture, the resazurin substrate is reduced to resorufin, which is the fluorescent end substrate of the assay. The number of metabolically active cells is determined by the level of resazurin reduction. Second, the Caspase-Glo® 3/7 Assay (Promega) is used to measure caspase 3/7 activity using a DEVD tagged luciferine substrate. Adding the reagent to the wells results in cell lysis, followed by caspase cleavage of the substrate. This cleavage liberates free aminoluciferin, a substrate for luciferase, resulting in the generation of a luminescent signal that is proportional to the caspase 3/7 activity present.

The addition of increasing concentrations of etoposide decreases cell viability and increases apoptosis.

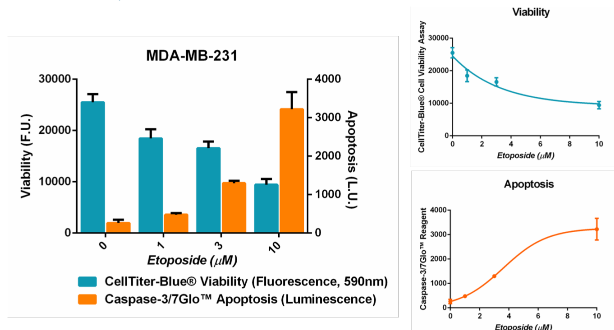

Figure 3. Multiplexed CellTiter-Blue (viability, fluorescence) and Caspase-Glo 3/7 (apoptosis, luminescence) endpoint assays MDA-MB-231 cells were exposed to Etoposide for 72hrs and apoptosis and cell viability were measured using respectively the Caspase-Glo™ luminescence assay (apoptosis) multiplexed with the CellTiter-Blue® fluorescence assay (viability).

Analysing apoptotic markers using flow cytometry and western blot

The Annexin V – Alexa Fluor™ 488 kit detects the external exposure of phosphatidylserine on the cell membrane, an early apoptotic change that occurs before the integrity of the cell membrane is compromised. Propidium iodide is an intercalating DNA dye that can only cross the cell membrane once its integrity has been lost, thus the level of signal is proportional to the degree of cell membrane integrity. Using both markers allows early and late markers apoptotic cell membrane integrity to be measured.

Cleaved poly ADP-ribose polymerase (PARP) and caspase 3, detected using western blot, are middle-to-late markers of apoptosis that are generated as a consequence of activation of the caspase cascade.

Flow cytometry: After 24 hours of drug exposure, levels of both Annexin V and Propidium iodide increase, indicating the presence of an early and late apoptotic cell population.

Western blot: After 24 hours of drug exposure, both cleaved PARP and cleaved caspase 3 increase in a dose dependent manner.

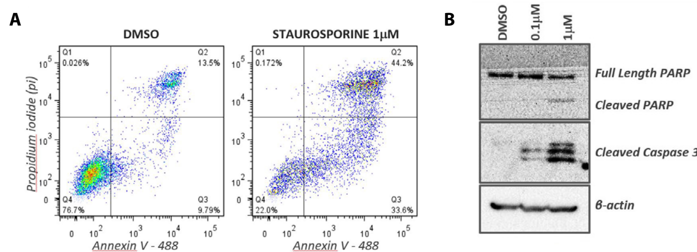

Figure 4A. Flow cytometry - MDA-MB-231 cells were treated with Staurosporine (0, 1 mM). After 24 h cells were harvested and stained using the Annexin V - Alexa FluorTM 488 conjugate (apoptosis, ThermoFisher) and Propidium iodide (Pi) Staining Solution (cell death). Data were collected using a FACS-verse instrument (BD Biosciences).

Figure 4B. Western blot - MDA-MB-231 cells were treated with Staurosporine (0, 0.1, 1 mM). After 24 h cells were harvested and total protein extracts probed for both PARP (full length versus cleaved) and activated caspase 3.

After 24 hours of treatment with Staurosporine, both cleaved PARP and cleaved caspase 3 levels increase in a dose dependent manner. The abundance of the cleaved proteins varies in the isogenic cell lines compared with the parental (PAR) cells (stronger in *CHEK2*-/- cells and weaker in *CHEK2*-/- *TP53*-/- cells).

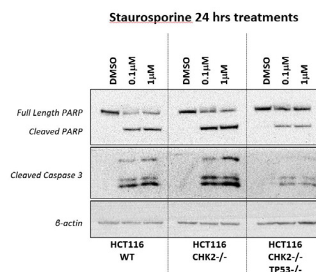

Figure 5. A panel of HCT116 isogenic cancer cells were exposed to Staurosporine for 24hrs.

Cells were harvested and total protein extracts probed for both PARP (full length versus cleaved) and activated Caspase 3 via western blot.

Conclusion

Horizon Discovery offers several assays and techniques that can assess the induction of apoptosis. We offer live cell monitoring to assess the kinetics of apoptosis induced by a compound of interest. Moreover, Horizon can multiplex assays, such as cell viability, cell proliferation and apoptosis, to attain more data from an individual experiment. Finally, Horizon can include a number of additional assays to detect early and late apoptotic markers (flow cytometry and qPCR, for example).

Other Assays of Interest

- T cell proliferation assays
- Drug combination screening service
- RNA-based immune cell screens

For more information

To find the contact information in your country for your technology of interest, please visit us at **horizondiscovery.com/contact-us**

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