

CHARACTERIZING RESPONSES TO TREATMENTS USING HOMOGENEOUS CASPASE ACTIVITY AND CELL VIABILITY ASSAYS

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Promega now offers a complement of fluorescent and luminescent methods to measure caspase activities and correlates of cellular health. Homogeneous caspase assays are available to measure caspase-3/-7, caspase-8 and caspase-9. These caspase assays can be used in parallel with Promega cell viability assays to provide a more complete picture of how a treatment or compound affects cell viability. Furthermore, the add-mix-measure format provides a simple and efficient means to scale the assays for increased volumes or high-throughput use.

Introduction

The use of cell-based assays has grown in recent years to become an integral part of both basic research and the drug discovery process (1). Primary, well-characterized or transgenic cell lines are chosen and used for specific physiological attributes relating to metabolism, lineage phenotype or susceptibility/resistance to exogenous stimulation (e.g., hypoxic stress, radiation, growth factors or other signaling ligands). Valuable information not obtainable in biochemical assays, such as compound permeability, stability, and target availability, can be gathered by using these systems.

Unfortunately, the same biological complexities that make cell lines so desirable also introduce experimental variability. It is therefore necessary to measure multiple biological markers at different compound or treatment dosages over various time frames (2). Several convenient parallel combinations of homogeneous viability or cell death assay reagents can be used to address application issues of scale, throughput and time required to conduct the assays.

Characterizing the Response

Cells exposed to a treatment or compound commonly show one of four responses: Proliferation, adverse responses, cell death or no response. Often the response is a combination of these outcomes because in vitro cultured cells demonstrate population heterogeneity with respect to stage of cell cycle, receptor display and culture density. Therefore, any endpoint measurement from cell-based assays represents an average effect of that treatment on the population of cells in that well. Furthermore, any conclusion drawn relating to treatment is often specific to a particular cell type as well as the duration of drug exposure. To mitigate spurious results and to make meaningful comparisons, researchers need to conduct testing in replicate wells and include vehicle- or mock-treatment controls.

Focusing on Cell Death

With the advent of convenient and scalable homogeneous viability assays, it is now possible to gain information relating to cell toxicities from sizable compound libraries in multiple cell models or panels before testing those new chemical entities in specific functional assays or animal models. The compounds found to be toxic may be either culled from the

library based on their undesirable effects or advanced to further characterization in drug discovery efforts for cancer therapeutics. Cell-based assays allow researchers to evaluate compounds for characteristics such as permeability and solubility and focus additional studies on those compounds that not only show specificity but also have qualities that will allow them to be effective in vivo (3).

Viability assays using ATP or dye reduction as correlates of cell health can be powerful tools to define a compound's or treatment's cytotoxic effects, but they provide little or no information regarding the mechanism by which the cell population has died. For instance, cells that have undergone apoptosis in vitro eventually undergo secondary necrosis.

Choosing when to sample can often be as important as choosing what marker to measure when using cell-based assays.

Apoptosis is a normal energy-dependent process for eliminating compromised or unnecessary cells while circumventing the detrimental inflammatory aspects of necrosis. The caspase family of cysteine proteases are the central mediators of this process and perform their specialized functions through different initiator pathways that converge on the same effector enzymes. The extrinsic pathway involves receptor engagement or cross-linking of the tumor necrosis factor (TNF) superfamily of "death receptors," which promotes assembly of the death-inducing signaling complex (DISC), including the autocatalytic caspase-8 zymogen (4,5). The resulting active caspase-8 enzyme is capable of further processing and activating the "downstream" death-effector caspases (-3, -6, -7), leading to destruction of structural elements and repair enzymes (6).

The intrinsic or mitochondrial pathway is initiated by viral, ultraviolet or cell-permeable insults leading to cytochrome c release into the cytosol. Procaspase-9, Apaf-1, dATP and cytochrome c assemble to form a more catalytically active complex known as the "apoptosome" (7,8). As in the case of caspase-8, this caspase-9 complex can lead to activation of the death-effector caspases that mediate apoptosis.

Cell Death and Viability Assays

Two Experimental Models

In the present study, we examined the action of a known small molecule kinase inhibitor and two protein inducers of the apoptotic pathway in two cell lines. We were interested in defining both the specific pathways leading to apoptosis, as well as the kinetics associated with caspase induction and the loss of cell viability at maximal dosages.

Metabolically active and growing Jurkat and HL-60 cell lines were harvested and seeded into white-walled, clear-bottomed 96-well plates (Corning/Costar® Cat.# 3610) at a density of 25,000 cells per well in 50µl of RPMI 1640 with 10% FBS. Either staurosporine, anti-Fas mAb (clone CH-11) or recombinant TNF-Related Apoptosis Inducing Ligand (rTRAIL) was added in 50µl aliquots by staggered additions to replicate wells every hour over a time course of 10 hours at final concentrations of 5µM, 400ng/ml and 125ng/ml, respectively. The drug concentrations chosen represent dosages above the predetermined ED₅₀. Similarly, vehicle consisting of either RPMI 1640 with 10% v/v FBS and 0.1% DMSO or RPMI 1640 with 10% FBS were added to control wells.

At the end of the time course, CellTiter-Glo^{®(a,b)} (viability), Caspase-Glo^{™ 9(a,b)} (intrinsic pathway), Caspase-Glo^{™ 8(a,b)} (extrinsic pathway) or Caspase-Glo^{™ 3/7(a,b)} (effectors) Reagent was added to parallel plates containing experimentally treated and vehicle-treated cells. Luminescence was measured using a BMG FLUOstar luminometer.

Effect of Two Protein Inducers on Jurkat Cells (Extrinsic Pathway)

rTRAIL treatment of Jurkat cells effected a pronounced decline in cell viability as measured by ATP (Figure 1, Panel A). This loss of ATP began in the cell populations within two hours of treatment and resulted in a reduction of viability to 50% between 3 and 4 hours. Fas receptor cross-linking by a specific IgM antibody produced a less robust but more sustained and gradual killing of the cells over the time course (Figure 1, Panel A). Viability as indicated by ATP content remained above 50% for most of the 10 hours.

The activation of caspase-8, or -3/7 as measured by the Caspase-Glo^{™ 8} and Caspase-Glo^{™ 3/7} Assays, respectively, shared similar kinetic profiles to the viability data. The emergence of detectable caspase-8 activity just prior to caspase-3/7 is consistent with its role as an initiator caspase. rTRAIL induction of caspases-8, or -3/7 (Figure 1, Panel B) began within 1–2 hours and peaked at 3–4 hours. Anti-Fas Ab treatment (Figure 1, Panel C) displayed similar early kinetics, including a similar caspase-8 response, but caspase-3/7 activity was largely maintained until a late surge in activity at the end of the time course. The characteristic decline in caspase-8 activity at later time points reflects the function of caspase-8 as an initiator caspase. Furthermore,

caspase-8 measurements below those of vehicle-treated cells at 10 hours illustrates the difference between degraded caspase-8 from eliminated cells and the inherent basal/pro-enzyme activity of caspase-8 in the vehicle population. This observation emphasizes the importance of timing when making meaningful initiator caspase activity measurements.

The differing profiles gathered using these specific combinations of assays cannot be attributed to nonoptimal dosing, as both the Anti-Fas mAb and rTRAIL proteins were used well above their ED₅₀. The differential drug action is likely attributable to receptor affinity, cell density or temporal display in a nonsynchronous population.

Effect of Staurosporine on Two Cell Lines (Intrinsic Pathway)

Staurosporine caused substantial and precipitous cell death in Jurkat and HL-60 cell lines as revealed by ATP depletion (Figure 2, Panel A). Although the viability progress curves share near transposable death rates, they are separated by a 1 hour lag in induction of cell death.

The activation of caspase-9 precedes that of caspase-3/7 in both cell lines (Figure 2, Panels B and C), while their peak activities essentially mirror the kinetics observed in the viability assay. The decline of caspase-9 activity to near basal level at late time points is consistent with a completed initiator enzyme function and lack of viable cells.

Summary

Employing multiple homogeneous caspase assays in parallel and in combination with cell viability assays allowed systematic dissection of the nature of drug effects on the Jurkat and HL-60 cell lines. As shown in these time-course assays, the duration of drug exposure and a rudimentary understanding of the kinetics are crucial when interpreting data obtained by end-point cell-based assays. For example, *in vitro*, secondary necrosis is indistinguishable from cells having undergone necrosis without some sort of apoptosis determination to confirm the mechanism of cell death. Choosing when to sample can often be as important as what marker to measure when using cell-based assays. These sensitive and simple apoptosis assays, in conjunction with cell viability assays, can provide a more complete picture of the drug's potential maximal action and cell death.

Cell Death and Viability Assays

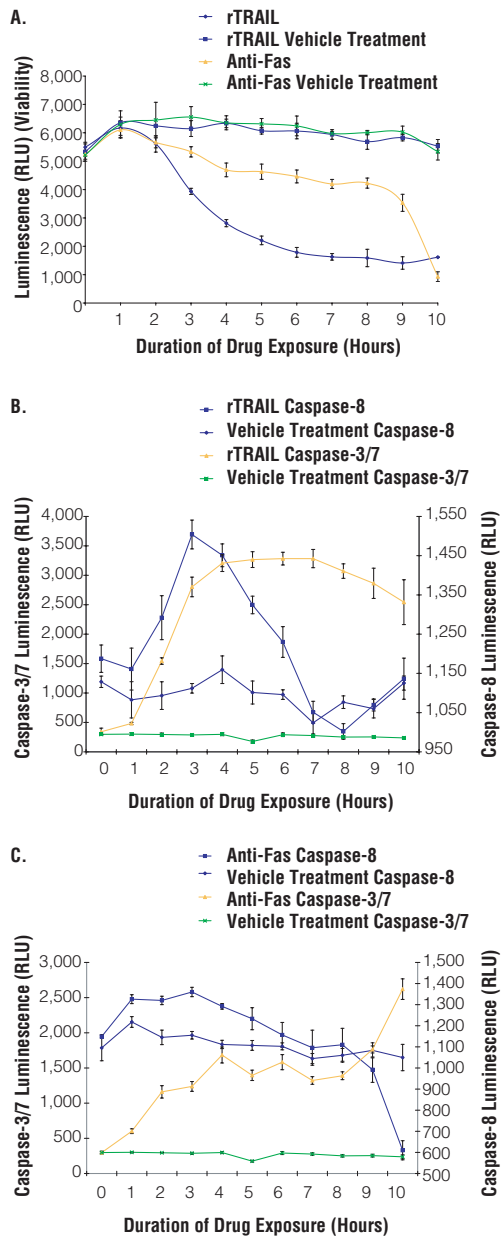


Figure 1. Panel A. rTRAIL and Anti-Fas mAb affect differential Jurkat cell viability kinetics. CellTiter-Glo® Reagent was added to triplicate wells of drug- and vehicle-treated controls in 100µl volumes. The plate was mixed briefly by orbital shaking, and luminescence was measured using a BMG FLUOstar luminometer. **Panel B. rTRAIL mediates the induction of a potent caspase-3/7, and -8 response.** Caspase-Glo™ 8 or Caspase-Glo™ 3/7 Reagent was added to triplicate wells for both rTRAIL- and vehicle-treated controls. After 1 hour of incubation on an orbital shaker, luminescence was measured as above. **Panel C. Fas receptor cross-linking by Anti-Fas mAb promotes a controlled apoptotic induction demonstrated by caspase-3/7 and -8 activity.** Caspase-Glo™ 8 or Caspase-Glo™ 3/7 Reagent was added to triplicate wells for both rTRAIL and vehicle-treated controls. Reactions were incubated and measured as in Panel B.

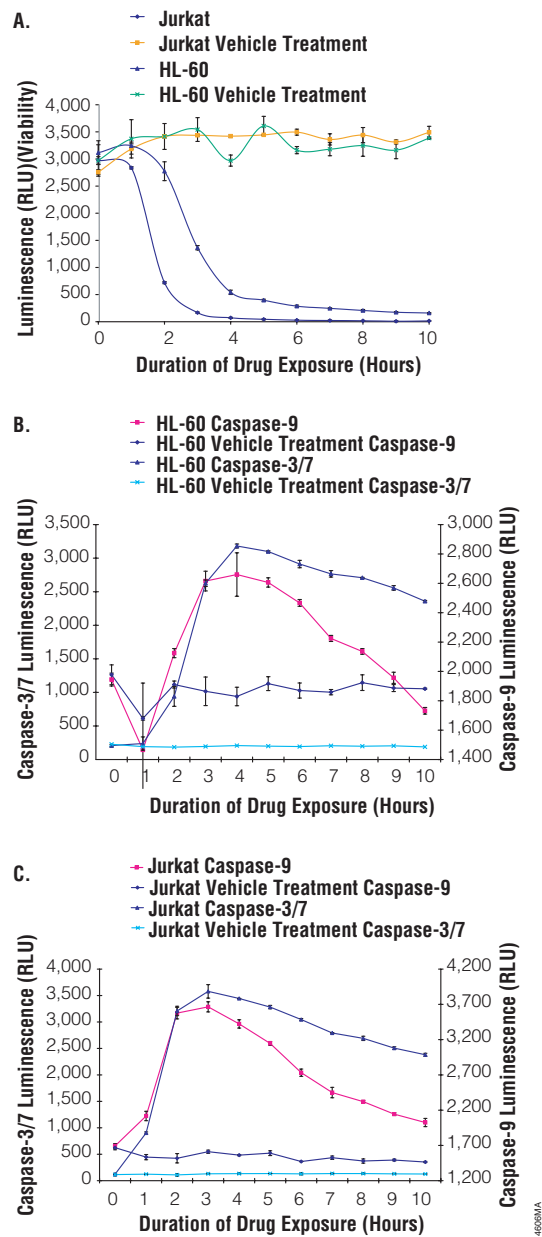


Figure 2. Panel A. ATP levels, and viability, fall as a function of time after staurosporine treatment of either Jurkat or HL-60 cells. CellTiter-Glo® Reagent was added in matched 100µl volumes to both drug and vehicle-treated wells. Plates were mixed briefly by orbital shaking, and luminescence was measured on a BMG FLUOstar luminometer. **Panel B. Peak induction of caspase-3/7, and -9 activities occurred 4 hours after addition of staurosporine to HL-60 cells.** Caspase-Glo™ 9 or Caspase-Glo™ 3/7 Reagent was added to triplicate wells containing either 5µM staurosporine- or vehicle-treated controls. After 1 hour of incubation on an orbital shaker, luminescence was measured as in Panel A. **Panel C. Rapid induction of Caspase-3/7, and -9 was observed in Jurkat cells after staurosporine treatment.** Caspase-Glo™ 9 Reagent or Caspase-Glo™ 3/7 Reagent was added to triplicate wells containing either staurosporine or vehicle-treated cells. Reactions were incubated and measured as in B.

Cell Death and Viability Assays

References

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Protocols

CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin #TB288
(www.promega.com/tbs/tb288/tb288.html)

Apo-ONE® Homogeneous Caspase-3/7 Assay Technical Bulletin #TB295
(www.promega.com/tbs/tb295/tb295.html)

Caspase-Glo™ 3/7 Assay Technical Bulletin #TB323
(www.promega.com/tbs/tb323/tb323.html)

Caspase-Glo™ 8 Assay Technical Bulletin #TB332
(www.promega.com/tbs/tb332/tb332.html)

Caspase-Glo™ 9 Assay Technical Bulletin #TB333
(www.proemga.com/tbs/tb333/tb333.html)

Ordering Information

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay ^(a,b)	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml	G7792
	10ml	G7790
	100ml	G7791
Caspase-Glo™ 3/7 Assay ^{*(a,b)}	2.5ml	G8090
	10ml	G8091
	100ml	G8092
Caspase-Glo™ 8 Assay ^{*(a,b)}	2.5ml	G8200
	10ml	G8201
	100ml	G8202
Caspase-Glo™ 9 Assay ^{*(a,b)}	2.5ml	G8210
	10ml	G8211
	100ml	G8212

*For Laboratory Use.

^(a)U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents pending.

^(b)The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

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Solution to crossword puzzle.

B	R	U	C	E		M	A	G	M	A		P	E	P
A	R	G	O	N		A	B	E	A	M		I	L	L
D	R	A	C	O		F	U	N	G	I		L	I	E
			K	B	P		T	U	N			S	O	D
O	R	E		L	E	A		S	E	D	A	T	E	D
M	A	G	N	E	G	S	T		H	E	W			
A	C	R	I	D		C	U	M	I	N		A	S	P
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G	A	Z	E	T	T	E		T	A	M		L	Y	S
I	D	E	A		H	E	L		X	E	R			
A	D	S		M	O	R	I	A		D	A	I	L	Y
N	E	T		P	R	I	S	M		A	R	R	A	Y
T	R	Y		I	N	E	P	T		Y	E	A	S	T