Measuring Cytotoxicity in Real Time with a Highly Stable Green Dye

ABSTRACT

The cell-based assay community now enjoys numerous options to conduct routine in vitro cytotoxicity testing. Sensitive and homogeneous reagents exist with colorimetric, fluorescent and luminescent detection formats. Although these assays have a proven track record of improving toxicity predictions and altering subsequent decision making, there are opportunities available to increase assay efficiency and productivity. For instance, most viability and cytotoxicity reagents are applied at a terminal endpoint, which limits the determination of cytotoxic mechanism of action unless employed in time-course studies. Here we describe the development and use of a non-activity-based cytotoxicity probe that can be added at the beginning of the experiment and employed in real time for the kinetic determination of cytotoxicity.

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Introduction

In vitro cytotoxicity is largely influenced by test article concentration and the exposure period with cells. Depending upon these parameters and the mechanism of action by which cells ultimately die, cells may lose viability early in the exposure (<2 hours) by primary necrosis or at any point thereafter, typically by apoptotic mechanisms. This diversity of kinetic response can greatly complicate conventional cytotoxicity endpoint assay determinations because most assay reagents are formulated to measure enzymatic biomarkers that are susceptible to time-dependent decay (1) . Therefore, conventional “add-mix-measure” cytotoxicity assays provide the most value when they are employed in parallel time-point studies (2) . Cognizant of these kinetic cytotoxicity complications, we sought to develop an assay system to address this challenge by measuring cytotoxicity in real time.

Identification of a Suitable Cytotoxicity Probe

Membrane integrity, or lack thereof, has been an operational definition for viability and cytotoxicity for decades (3) . Therefore the ability to differentially exclude dye from viable cells served as a starting point for the evaluation of over one hundred novel asymmetric cyanine dyes. These dyes, designed and synthesized by Promega Biosciences, demonstrated affinity for binding genomic DNA, which led to an increase in fluorescence in the presence of dead cells (Figure 1). The dyes were screened using artificially blended ratios of viable and non-viable cells and proved to reliably measure incremental differences in viability across the cytotoxicity spectrum (Figure 2, Panels A and B).

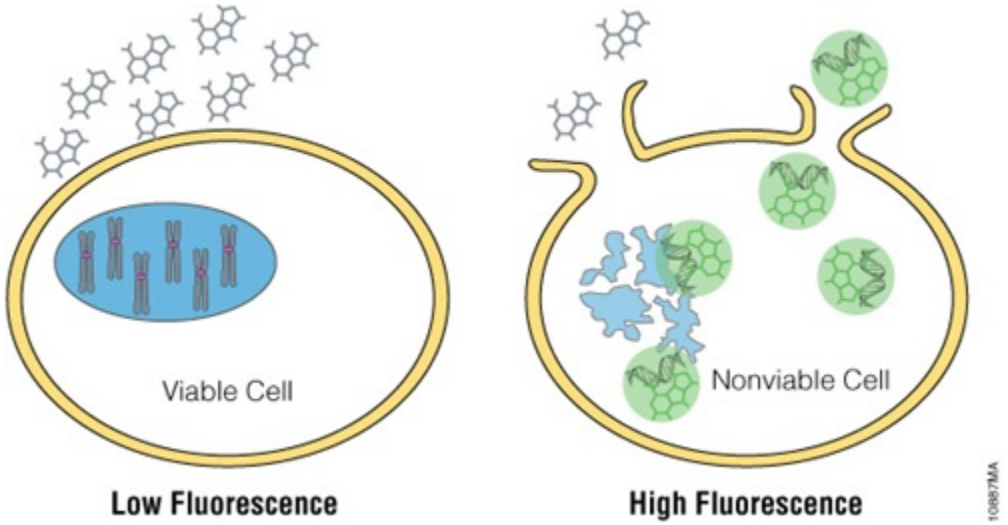

Figure 1. CellTox™ Green Cytotoxicity Assay. CellTox™ Green Dye is excluded from viable cells, but it binds to DNA from dead cells with compromised membrane integrity.

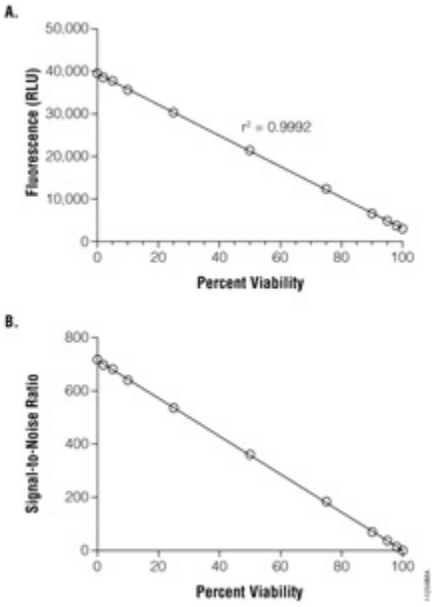

Figure 2. Assay proportionality and sensitivity. The spectrum of a prototypical cytotoxic response was simulated by blending mildly sonicated K562 cells (nonviable population) with viable cells at various ratios to represent 0–100% viability. Each ratio contained a total of 10,000 cell equivalents per 100µl in a 96-well assay plate.

Flexibility in CellTox™ Green Dye Delivery

Lead candidates that demonstrated exceptional cell impermeability and quantum yield (a measure of “brightness”) were evaluated further for biological inertness by long-term (72-hour) exposures with cells followed by orthogonal viability measures.

These studies, performed with both primary and transformed cell lines, suggest that a particular dye, the CellTox™ Green Dye, can be delivered either at dosing or at the exposure endpoint with satisfactory signal windows and robustness. Furthermore, for endpoint application, the dye can be delivered in the provided buffer (or medium) in an equal volume or at more concentrated, lower volumes. This range of protocol options (Figure 3) allows significant downstream, same-well multiplexing opportunities for more informative data per well (4) .

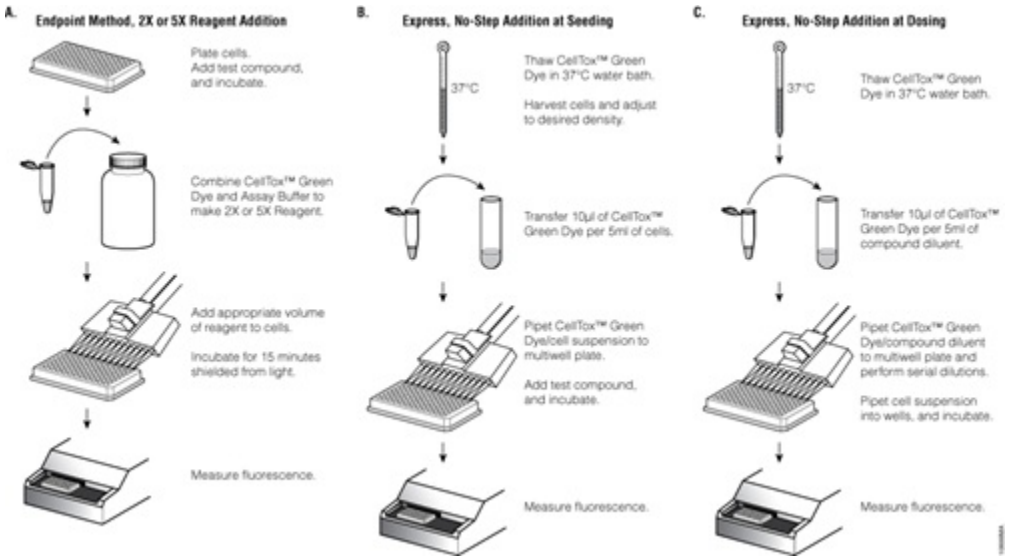

Figure 3. Schematic diagram of methods for delivering the CellTox™ Green Dye.

Kinetic Measurements of Cytotoxicity

Next, we sought to determine the utility of the profluorescent probe in kinetic measures of cytotoxicity. In the first experiment, we treated K562 cells at a density of 10,000 cells per well in a black 96-well plate in the presence of CellTox™ Green Dye with increasing concentrations of the potent proteasome inhibitor, bortezomib. Fluorescence was measured with an excitation source at 485nm and emission at 520nm using a BMG PolarStar™ reader at 4, 24, 48 and 72 hours post-compound addition. The plate was returned to a 37°C humidified incubator between measurements. The resulting data were plotted as raw fluorescence vs. dosage and demonstrate the cytotoxicity relationship between dosage and exposure time (Figure 4). Most notably, loss of membrane integrity is evident by 24 hours and persistent and accumulative out to 72 hours after compound addition. This indicates that genomic DNA, although actively degraded by inherent nuclease activity, is sufficiently stable to act as a reliable biomarker for cytotoxicity. These data also demonstrate that time course data can be collected from the same assay plate instead of requiring parallel plates, thereby reducing unnecessary duplication of effort required for endpoint measures. Furthermore, same-well time-point analysis allows application of multiplexed, spectrally distinct assay chemistry such as Caspase-Glo® 3/7 Reagent at the first indication of cytotoxicity (Figure 5). This concurrent analysis allows researchers to establish mechanism-of-action for cytotoxicity.

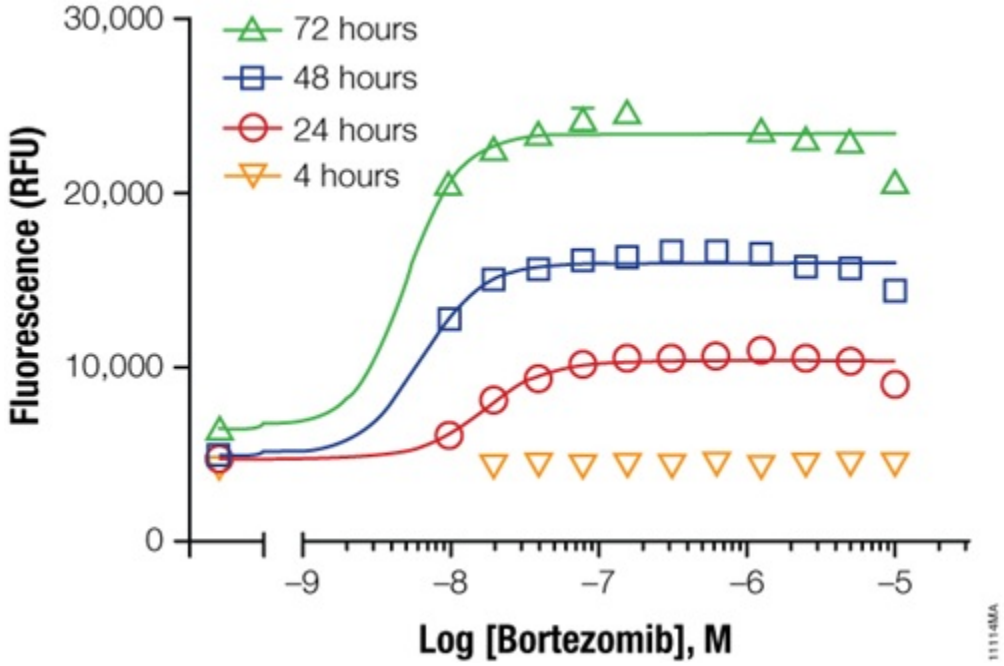

Figure 4. Dose- and exposure-dependent increases in cytotoxicity. Bortezomib caused appreciable cytotoxicity in K562 cells between the 4- and 24-hour time points. Addititve fluoresence at later time points indicates continued loss of membrane integrity occurring as a function of cytotoxicity against remaining cells. CellTox™ Green Dye was added at seeding.

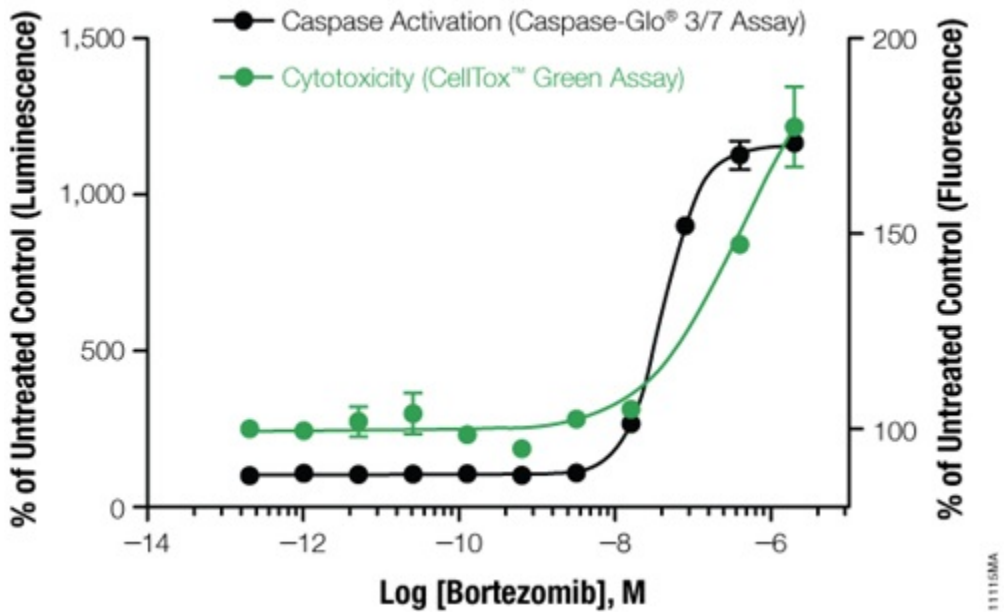

Figure 5. Determining mechanism of action through same-well multiplexing. K562 cells were treated with bortezomib in the presence of CellTox™ Green Dye (dye added at seeding). At the first indication of cytotoxicity, as defined by dose-dependent

increases in fluorescence (at 24 hours), Caspase-Glo® 3/7 Reagent was applied to all wells. Caspase activity, with commensurate increases in cytotoxicity fluorescence, strongly indicates an apoptotic mechanism.

In the second experiment, we chose to examine the respective kinetics of cytotoxicity provoked by two unrelated agents. Frozen instant HepG2 cells (provided by Cell Culture Service) at a density of 10,000 cells/well were treated with tenfold serial dilutions of either nocodazole or terfenadine (Figure 6). The Tecan HP D300 Digital Dispenser was used for compound addition. CellTox™ Green Dye was introduced into the cultures at cell plating. Fluorescence associated with the progression of cytotoxicity was monitored, and data were collected in real time in an environmentally controlled Tecan Infinite® 200 PRO reader with Gas Control Module (GCM™) using bottom-read fluorescence, shaking the plate before each read. The real-time data reveal striking discordance in the kinetics and potency of the cytotoxic events both within individual concentration of the same drug and between two compounds. Ultimately, these types of cytotoxicity profiles are essential for understanding mechanism of action and addressing possible pharmokinetic concerns such as biotransformation of the compound during the exposure period.

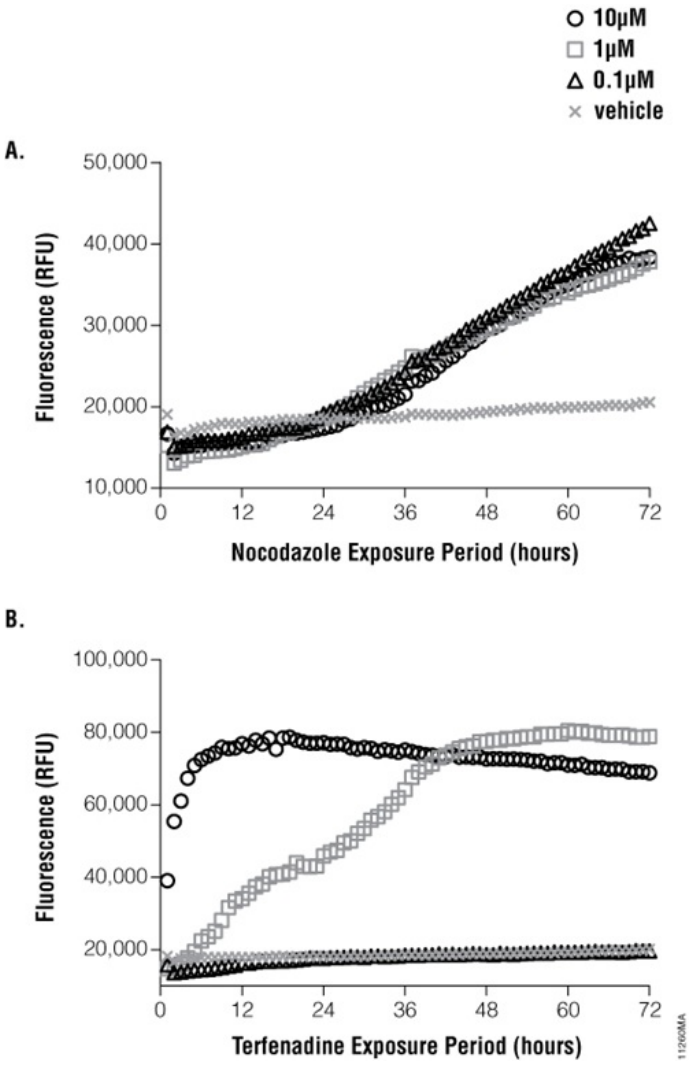

Figure 6. Real-time cytotoxicity measures. Frozen instant HepG2 cells (Cell Culture Service) were exposed to varying concentrations of either nocodazole or terfenadine, and cytotoxicity was subsequently measured every hour for 72 hours using a Tecan Infinite® 200 PRO reader with Gas Control Module (GCM™). **Panel A.** Nocodazole alters microtubule assembly necessary for cell division leading to cytotoxicity beginning at 24 hours with equal potency at dosages over two logs of concentration. **Panel B.** Terfenadine exhibits rapid-onset toxicity at high concentrations by a mechanism that is both titratable and time-dependent.

Summary

Conventional “add-mix-measure” endpoint cytotoxicity assays continue to be an integral aspect of biological experimentation because they are easy to use and provide exceptional value. However, they can be more costly to employ in terms of time and effort because they require parallel time-point studies to fully characterize a cytotoxic event. The CellTox™ Green Assay eliminates this unnecessary duplication of effort by allowing real-time cytotoxicity measurements, whether employed at specified time points using the same assay plate or by collecting cytotoxicity data in real time with specialized fluorometers.

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