Development and Validation of a Standardized In Vitro Cytotoxicity Assay

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1. Abstract

Several governmental agencies have explored and developed innovative testing methods for toxicity liability. Although their data, methods, and assays used to characterize toxicity are readily available, no fully standardized method has been endorsed for industry use. We sought to bridge this need and develop a completely integrated and standardized primary testing system that could be employed with low inter-and intra-assay variability and high predictive value. The system consisted of rigorously qualified, thaw-and-use HepG2 cells which were plated at a defined, sub-confluent density in a standardized culture medium containing an inert, profusion-sensitive membrane integrity dye. After cellular attachment and acclimation, the plates were serially dosed with two model control toxins. Digitonin, a pore-forming glycolide, served as the fast-acting primary necrosis control, whereas digitonin, a histone deacetylase inhibitor, served as the apoptosis-inducing control. After 48hrs of compound exposure, fluorescence data resulting from cytotoxicity was collected, followed by the application of a luminescent viability assay. Together, the data were analyzed for anti-proliferative or cytotoxic dose dependence, relative to an untreated vehicle control, using commercial software. We observed near inverse concordance between the viability and cytotoxicity measures with digitonin whereas dose-shifted anti-apoptotic and cytotoxic responses were measured with trichostatin A exposure. Signal to background dynamic responses for the two chemistry measures were robust, and inter- and intra-assay variation for EC50 values were typically far less than 10%. The data we collected are sufficiently invariant and encouraging to provide a tangible framework for the standardized method has been endorsed for industry use. We sought to bridge this need and develop a fully integrated and standardized primary testing system that could be employed with low inter-laboratory variability.

2. Thaw-and-Use Cells and Culture System

Thaw HepG2 and add to diluted CellTox Green Medium. Thaw HepG2 and add to Growth Medium. Digitonin is a potent histone deacetylase inhibitor which produces an apoptotic cascade which results in secondary necrosis. TSA also causes dose-dependent cell cycle arrest. Data from exposure to this control established viability criteria for the cell model component as well as chemistry robustness.

3. Test Article and Control Toxin Dosing

Test article(s) and control toxins are two-fold serially diluted in Growth Medium and applied to equal volumes to plated cells. The plate(s) are returned to a humidified CO2 incubator for 48hrs.

4. Multiplexed Viability and Cytotoxicity Measures

CellTox™ Green is an asymmetric cyanine dye delivered directly into cell cultures at cell seeding. The dye is excluded from viable cells with intact membranes but preferentially stains the DNA from cells with impaired membrane integrity. When the dye binds genomic DNA, its fluorescence properties are substantially enhanced. These attributes allow the dye to be used as a stable endpoint measure of cytotoxicity.

ATP is tightly regulated in healthy cells and therefore serves as an excellent surrogate for host cell viability and number. Cells with impaired viability are unable to maintain ATP levels. CellTiter-Glo® 2.0 is a stabilized lytic formulation of luciferin and Ultra-Glo™ luciferase which when added to cells measures ATP in a manner that is proportional to visible cell number.

5. Representative Data with Control Toxins

Digitonin produced near inverse concordance between the viability and cytotoxicity measures (EC50 of 3.05µM vs 3.75µM) whereas dose-shifted anti-proliferative and cytotoxic responses were measured with trichostatin A exposure (EC50 67h/8µM, 120hM). Signal to background dynamic responses for the two chemistry measures were robust, and inter- and intra-assay variation for EC50 values were typically far less than 10% owing to the consistent growth attitudes of the thaw and use and the optimized assay components.

6. Formal Validation of System Variability

Trichostatin A (TSA) is a potent histone deacetylase inhibitor which produces an apoptotic cascade which results in secondary necrosis. TSA also causes dose-dependent cell cycle arrest. Data from exposure to this control established viability criteria for the cell model component as well as chemistry robustness.

7. EU-Banned Cosmetic Ingredients

The European Commission, Health and Consumers-Cosmetics currently contains, a list of 1377 ingredients prohibited in cosmetic products. We chose five compounds from that list and tested them for cytotoxic and anti-proliferative potential to demonstrate sensitivity and the predictive nature of our standardized in vitro cytotoxicity assay system. TSA demonstrated largely anti-proliferative effects, whereas cotinine produced both cytotoxicity and anti-proliferative effects. Metformin, metothrexate, and cadmium chloride (data not shown) also produced toxicological effects which would warrant concerns regarding their safety.

8. Withdrawn Pharmaceuticals (US)

An alarming number of pharmaceuticals have been removed from the US Market in the last couple of decades due to unexpected adverse effects observed in clinical trials. We chose to test a panel of compounds in vitro cytotoxicity assay system to retrospectively examine potential safety concerns. Although no in vitro assay system can fully recapitulate the complexity of in vivo and chronic administration, the clearance of these effects with terfenadine and anti-proliferative effects with nelazosine are at least partially consistent with in vivo safety concerns leading to their de-listing. It is not inconceivable that the observed in vitro effects would only be exacerbated in patients presenting with existing morbidities such as hepato- or renal-insufficiencies.

9. Conclusions

The standardized in vitro cytotoxicity assay system proposed here offers an alternative paradigm to reduce conventional mammalian testing models, especially in early stage evaluations. The system and method offer a complete and convenient means of addressing the variation associated with traditionally divergent in vitro methods by providing:

-Protocol
-Growth serum
-Control toxins
-Viability chemistry
-Cytotoxicity probe

The data collected using the provided model toxins can be used to internally validate or reject test data based on dynamic cell responses and EC50 variability criteria.

The data collected using the system are sufficiently robust and invariant (typically better than 10%) to allow comparison of data collected at different institutions during different chronological periods.

Although our training set of toxic and non-toxic compounds currently remains limited (<50), the cross-referencing of our in vitro cytotoxicity assay system with proctor and illness demonstrates largely anti-proliferative effects, whereas cotinine produced both cytotoxicity and anti-proliferative effects. Metformin, metothrexate, and cadmium chloride (data not shown) also produced toxicological effects which would warrant concerns regarding their safety.

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