A Real-Time *In Vitro* Safety Assessment Approach Utilizing a Simplified, Multi-Parametric Work Flow

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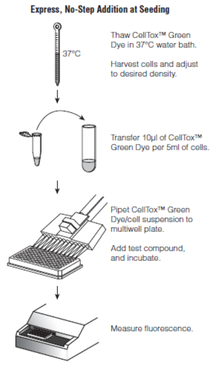
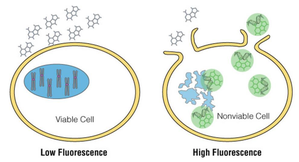
Abstract # 1628

1. Abstract

In vitro cytotoxicity is inextricably linked to a combination of compound dosage, exposure period, and intrinsic cell susceptibility. Current screening paradigms which utilize only endpoint measures in a defined cell type adequately address effects due to dosage, but often fail to define important toxicokinetic profiles or inherent mechanistic sensitivities. We investigated the use of a real-time cytotoxicity probe applied at the time of dosing with staurosporine, panobinostat, imantinib, terfenadine, colchicine, aflatoxin B1, bortezomib, camptothecin, valinomycin, nocodazole, methotrexate and ionomycin. Serial dilutions of these model compounds with the probe were delivered to iPSC-derived, terminally differentiated hepatocytes and proliferating hepatoma and erythroid leukemia cell lines. Cytotoxicity data were collected at 4, 24, 48 and 72hr followed by a same-well multiplexed viability assay. The collated data revealed striking differences in toxicokinetics, potency and magnitude of response which positively correlated with known mechanism of action for the model compounds. The multiplexed viability data further served to either confirm observed cytotoxicity by inverse signal concordance, or suggest replicative perturbation in susceptible replicating cells. Furthermore, the use of cell types with differential capacity for phase I metabolism, allowed us to stratify cytotoxic risk based on mode-of-action of parent molecule toxicity and/or metabolic by-products owing to biotransformation. Lastly, the experimental approach taken was sufficiently predictive and informative to merit consideration for adoption as a new safety screening paradigm for new chemical entities.

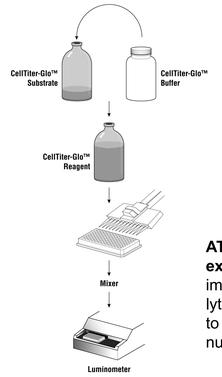
2. Real-Time Cytotoxicity Assay Method and Principle

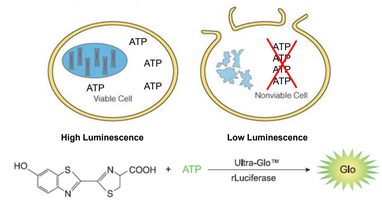




CellTox™ Green is an asymmetric cyanine dye that can be delivered directly into cell cultures at cell seeding or dosing. The dye is excluded from viable cells with intact membranes but preferentially stains the DNA from cells with impaired membrane integrity. When the dye binds genomic DNA, its fluorescence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

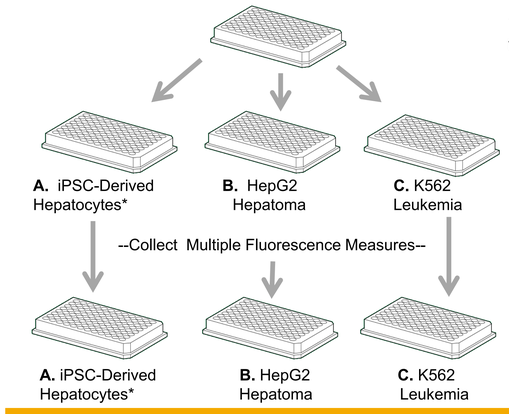
3. Viability Assay Method and Principle





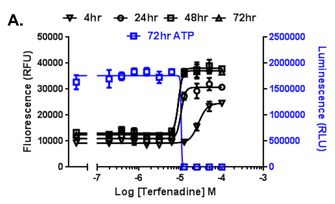
ATP is tightly regulated in healthy cells and therefore serves as an excellent surrogate for host cell viability and number. Cells with impaired viability are unable to maintain ATP levels. CellTiter-Glo® is a lytic formulation of luciferin and Ultra-Glo™ luciferase which when added to cells measures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

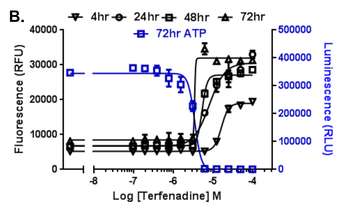
4. Multi-Parametric Workflow: Biomarkers and Cell Types

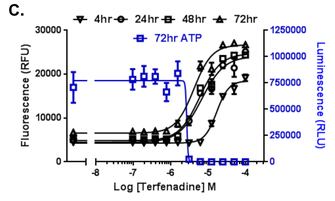


1. Prepare master dilutions of test articles
2. Transfer diluted test articles to plates containing cells and CellTox™ Green probe, incubate at 37°C
3. Measure cytotoxicity-associated fluorescence after 4, 24, 48 and 72hrs of exposure
4. Add CellTiter-Glo® Reagent, measure luminescence associated with remaining viable cell population

5. Non-Specific, Time Dependent Cytotoxicity

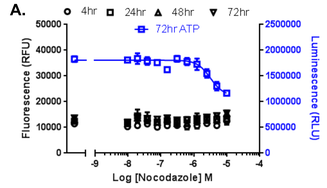


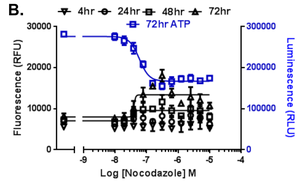


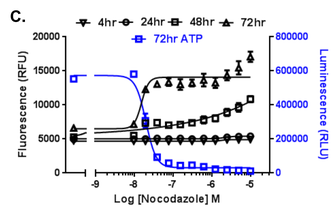


Terfenadine, a non-sedating anti-histamine, produced a progressive, exposure period dependent cytotoxicity in **A.** iPSC-derived hepatocytes **B.** HepG2 and **C.** K562 cells. This non-specific cytotoxic effect (no cell type bias) was inversely correlated with a commensurate loss of viability in all three cell types.

6. Replication Dependent Cytotoxicity

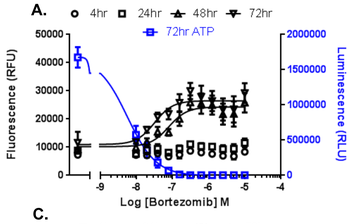


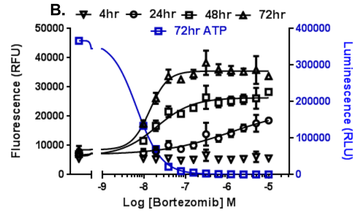


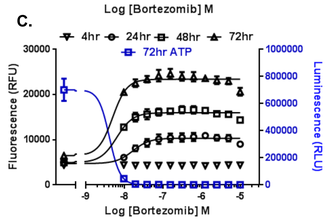


Nocodazole, a cytokinesis inhibitor, produced a replication dependent cytotoxic effect in cancer cells. **A.** The non-dividing iPSC derived hepatocytes were not susceptible to overt cytotoxicity, but demonstrated a dose-dependent reduction in ATP. **B.** HepG2 (hepatoma) demonstrated a modest increase in cytotoxicity over the exposure period marked by an inversely concordant decrease in viability **C.** The rapidly dividing K562 were profoundly susceptible to the compound after a prolonged cell cycle arrest, resulting in cytotoxicity and a decrease in ATP.

7. Non-Replication Dependent, Targeted Cytotoxicity

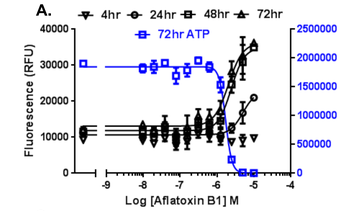


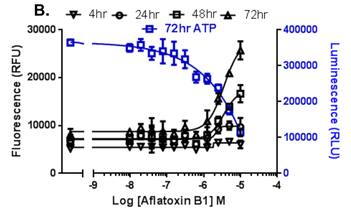


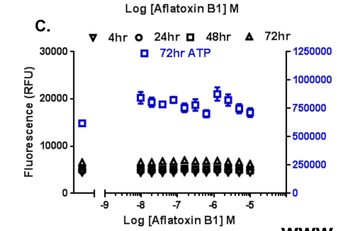


Bortezomib, a 26S proteasome inhibitor, produced a non-replication dependent cytotoxicity with a modest window of selectivity for the cancer lines. **A.** The iPSC-derived hepatocytes lost membrane integrity and viability with prolonged exposure (48-72hr) with a high nanomolar potency. **B** and **C.** HepG2 and K562 demonstrated progressive and substantial cytotoxicity With a commensurate loss of viability beginning before 24hr with a low nanomolar potency.

8. Biotransformation Dependent Cytotoxicity







Aflatoxin B1 is a pro-toxin actively biotransformed by inherent cytochrome p450 activity. **A** and **B.** Both iPSC-derived hepatocytes and HepG2 hepatoma cells demonstrated a progressive cytotoxic response which resulted in loss of cell membrane integrity with commensurate loss of viability. **C.** K562 were not susceptible to the pro-toxin owing to their inability to metabolize the compound into the toxic form.

9. Summary

- Introduction of the pro-fluorescent cytotoxicity probe at the time of cell seeding or dosing, allows for a facile and flexible means to measure real-time cytotoxicity. Measurement of cytotoxicity in real-time allows for the development of revealing toxicokinetic profiles for new chemical entities or other test articles.
- Application of the ATP viability chemistry at the terminal endpoint allows for an orthogonal measure of cell health in non-replicating cells, and a measure of overall cell number after xenobiotic exposure.
- Multi-parametric analysis using disparate test cell phenotypes can define mechanism of action for:
  - Non-specific cytotoxic compounds
  - Replication dependent cytotoxicity
  - Targeted anti-neoplastics with on- and off-target efficacy
  - Biotransformed compounds which produce reactive metabolite

* iCell® Hepatocytes were graciously provided through a collaboration with Cellular Dynamics International 525 Science Drive, Madison, WI 53711

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