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Application Note

ADME/Tox (Mitochondrial Toxicity)

A Simple, Robust Automated Multiplex Assay for Mitochondria Toxicity Testing

Promega Celsis | In Vitro Technologies

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Introduction

Toxicology testing during drug discovery now includes a close look at how cell constituents, particularly mitochondria, react to potential drug compounds. Mitochondria generate most of an eukaryotic cell's energy, adenosine triphosphate (ATP), via oxidative phosphorylation, and are found in various sizes and quantities within each cell depending on the organism and overall cell function. Drug compound mitotoxicity, or toxicity within mitochondria, may cause damage to the cell, key organs such as the liver and heart, or even the overall organism.

Microplate- and cell-based mitochondrial assays offer a more realistic environment to test a drug compound's effect on mitochondrial function compared to isolated mitochondria-based tests. However, when monitoring a lead compound's effect on the mitochondria alone, this may lead to inaccurate data interpretation. This is due to the fact that mitochondrial function may be impaired not only via mitochondrial toxicity, but also from the compound's toxicity on the entire cell, leading to primary necrosis. This concern may be alleviated through a multiplexed assay that allows for the determination between primary necrosis and true mitochondrial toxicity. Combining mitotoxicity assays in a multiplex format also increases the amount of data per well while decreasing data variability arising from running the assays separately.

Additionally, highly proliferative cells, such as immortalized cell lines and cancer cells, use glycolysis instead of oxidative phosphorylation, effectively bypassing use of mitochondria and significantly altering test results. This phenomenon is known as the Crabtree Effect, and must be countered by growing cells using a glucose substitute such as galactose, thus forcing cells to use their mitochondria. However, as we will prove, this correction may not yield results similar to those of a primary cell model.

Here, we present the utility of an automated multiplex assay to assess cytotoxicity and mitochondrial function as measures of cell membrane integrity and ATP levels, respectively in primary hepatocytes and immortalized HepG2 liver carcinoma cells. Automated assay validation, glucose effects on mitochondria function, and pharmacology experiments were performed.

In the multiplex assay, cytotoxicity is assessed by measuring necrosis-related protease activity using a fluorogenic peptide substrate (bis-AAF-R110). The substrate cannot cross live, intact cell membranes, and therefore provides no signal for viable cells. An ATP detection reagent is then added that lyses the cells, and an emitted luminescent signal is proportional to ATP levels as a measure of mitochondrial function. The two assay results provide a distinction between mitochondrial toxicity and general cytotoxicity.

Materials and Methods

Materials

Liverpool™ Cryopreserved human suspension hepatocytes (Cat. No. X008052) were obtained from Celsis In Vitro Technologies. HepG2 hepatocellular carcinoma cells (Cat. No. 85011430) were obtained from Sigma-Aldrich. InVitroGro HT Medium (Cat. No. Z99019) was attained from Celsis In Vitro Technologies. High Glucose DMEM (Cat. No. 11995) and No Glucose DMEM (Cat. No. 11966) were obtained from Life Technologies. Mitochondrial ToxGlo™ assay (Cat. No. G8000) was donated by Promega Corporation. Antimycin (Cat. No. A8674), CCCP (Cat. No. C2759), and Digitonin (Cat. No. D141) were purchased from Sigma-Aldrich. Staurosporine (Cat. No. 1285) and Tamoxifen (Cat. No. 0999) were purchased from Tocris Bioscience.

Cell dispensing, compound serial titrations and reagent dispensing were performed with the Precision™ Microplate Pipetting System (BioTek Instruments, Inc.). The Precision combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. Results were read using the Synergy™ H4 Hybrid Multi-Mode Microplate Reader, which combines a filter-based and monochromator-based detection system in one compact unit. The filter-based system was used to read the ATP Detection Assay luminescent signal and the Cytotoxicity Assay fluorescent signal per the settings in Table 1 and Table 2.

Instrument Detection Component		BioTek Catalog Number
Excitation Filter #1	485/20 nm	7082221
Emission Filter #1	528/20 nm	7082247
Dichroic	510 nm Cutoff	7138510

Table 1. Synergy H4 Cytotoxicity Assay Instrument Setup.

Optimized Instrument Settings			
Cytotoxicity Assay			
Light Source	Xenon Flash Lamp	Delay After Plate Movement	0 mS
Measurements per Datapoint	20	Lamp Energy	High
ATP Detection Assay			
Integration Time	0.5 Sec	Delay After Plate Movement	0 mS
Dynamic Range	Extended		

Table 2. Synergy H4 Optimized Instrument Settings.

Methods

Cryopreserved hepatocytes were thawed and re-suspended in InVitroGro HT Medium. The cultures were centrifuged, supernatant was removed, and the cells were resuspended in serum-free, glucose-free media consisting of No Glucose DMEM, 5 mM HEPES, 10 mM galactose, 2 mM glutamine, 1 mM Na-Pyruvate and 1% Penicillin/Streptomycin.

HepG2 cells were propagated in media consisting of High Glucose DMEM, 10% FBS and 1% Penicillin/Streptomycin. After removal from the growth flask, the cells were resuspended in the previously described serum-free, glucose-free medium.

All compounds were titrated in 100% DMSO to create an 11-point dose-response curve. The compounds were then diluted from the 1000X stock in non-serum/non-glucose medium to a final 2X concentration before addition to the assay plates.

Per each assay, 10 µL of hepatocytes or HepG2 cells were added to each well in a 384-well microplate. Compound, 10 µL, was added to each well, and the plates were placed in a 5% CO_2/37° C incubator for 1, 2, 3, 4, or 6 hours. After incubation, 5 µL of 5X cytotoxicity reagent was added to each well, and the plate was incubated using the same environmental conditions for thirty minutes. The fluorescent signal was then read using the aforementioned Synergy H4 settings.

The microplate was then equilibrated to room temperature for approximately 15 minutes. 25 µL of ATP Detection Reagent was added to each well, and the plate was incubated for five minutes at room temperature. The resulting luminescent signal was then read on the Synergy H4.

Data Analysis

Luminescent or fluorescent values from wells containing media, treatments, and assay reagents were subtracted from raw values detected from cell containing wells. % Unstimulated Control was then computed using the following formula:

$$\% \text{ Unstimulated Control} = (\text{Value(T)} / \text{Avg Value(U)})*100$$

Where Value(T) equals the background subtracted value from wells containing compound, and Avg Value(U) equals the average value from background subtracted basal wells containing no compound.

Results

Automated Assay Validation

Z'-factor analyses were first performed to validate the HepG2 and hepatocyte-based multiplex assays. The known mitochondrial inhibitor antimycin was used as a control mitotoxicant, and forty replicates of 100 µM or 0 µM compound were used as the positive and negative controls, respectively. Compounds were incubated for two hours with each cell type. As seen in Figure 1, Z'-factor values from the ATP Detection Assay are excellent for the automated assay using HepG2 and hepatocyte cell lines. As antimycin cannot completely kill hepatocytes, a higher signal background is seen in the negative control for this assay.

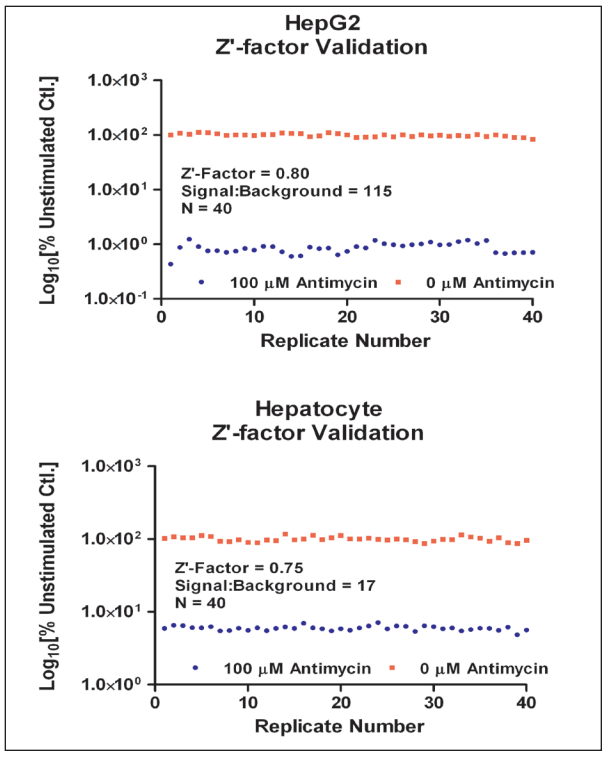

Figure 1. Z'-factor validation data of the automated system, showing excellent assay results.

Cell Model Effect on ATP Production

The Crabtree Effect, where cancer cells rely on glycolysis instead of oxidative phosphorylation via mitochondria to produce energy, is demonstrated by resuspending HepG2 cells and primary hepatocytes in either high-glucose medium or non-glucose medium, then exposing the cells to varying concentrations of antimycin for two hours. Results confirm that when performing the cytotoxicity assay and ATP Detection Assay on primary hepatocytes (Figure 2a – 2b), toxicity is due to antimycin impeding mitochondrial function, and not the result of primary necrosis. This is also independent of the presence or absence of glucose. HepG2 cells grown and assayed in glucose-based media (Figure 2d) appear to have no toxic effects from the known mitotoxicant, therefore producing a false negative profile. When HepG2 cells, grown in glucose-based media are assayed in glucose-free media (Figure 2c), the mitotoxic effects of antimycin are similar, but not identical, to effects seen in primary hepatocytes.

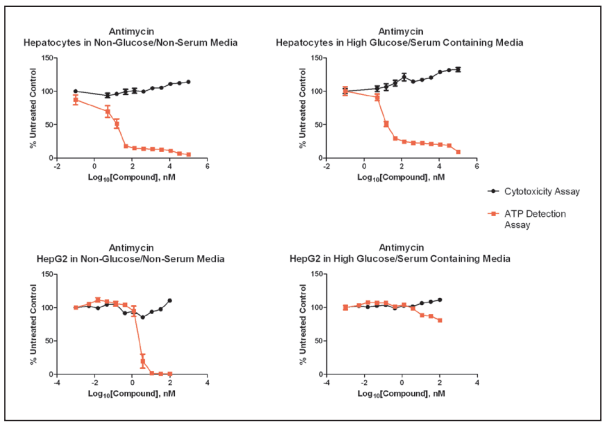

Figure 2. The effect of glucose-based media and non-glucose media on HepG2 cells and primary hepatocytes. Results confirm that toxic compound effects are masked when growing cancer cells (HepG2) in glucose-based media.

Mitotoxicant Analysis

Mitotoxicant analysis was performed using the known toxicants antimycin, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and tamoxifen. Digitonin, an inducer of cellular necrosis, was included as a cytotoxicity control; and staurosporine, an apoptosis inducer, was included as a negative control. The compounds were each incubated for two hours with HepG2 cells and primary hepatocytes that were grown in non-serum/non-glucose medium prior to addition of detection reagents. Per Figure 3, decreased cellular ATP concentrations were seen with increased mitotoxicant concentrations. Tamoxifen demonstrated a cellular ATP decrease only at the highest concentration tested. This may be an indication that the incubation time was insufficient for the compound to fully affect the cells. Digitonin demonstrates an ATP concentration decrease, and signal increase from the cytotoxicity assay, thus confirming its necrosis inducing characteristics. Finally, staurosporine did not affect any signal change, thus confirming its apoptosis inducing properties.

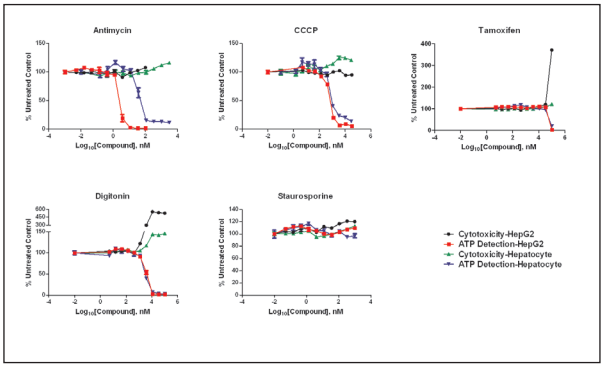

Figure 3. As expected, decreased cellular ATP concentrations were observed with each increased mitotoxicant concentration using a two-hour incubation. Cytotoxicity and negative controls also behaved as expected.

Variable Compound Incubation Time Analysis

The automated assay was also used to test tamoxifen-cell incubation times from one to six hours. Longer incubation times allow generation of more complete data for compounds that do not exhibit rapid toxic effects, or are less potent at lower concentrations. Conversely, shorter incubation times may allow for increased throughput. As seen in Figure 4, tamoxifen becomes more toxic to both cell models with increased exposure, although the results are variable among each cell model and incubation times. This reinforces the need to test multiple compound incubation rates when creating a toxicity profile. In addition to the variable results, HepG2 cells saw a change in cytotoxicity assay signal, possibly indicating a higher cytotoxic effect from tamoxifen in the cancer cell model.

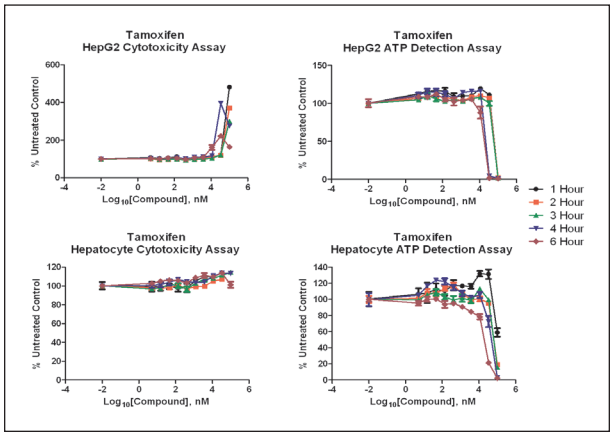

Figure 4. Cytotoxicity and ATP detection tests using tamoxifen, incubated at one to six hours, with both cell models, shows variable results.

Cell Model Analysis

Finally, data from HepG2 and primary hepatocyte cells treated with antimycin were compared to demonstrate the variability found using identical conditions and compounds on these different cells. Figure 5 clearly shows variability in cytotoxicity and ATP detection results among the two cell models in spite of their identical treatment, thus illustrating the need to use the most relevant cell model during mitochondrial toxicity testing.

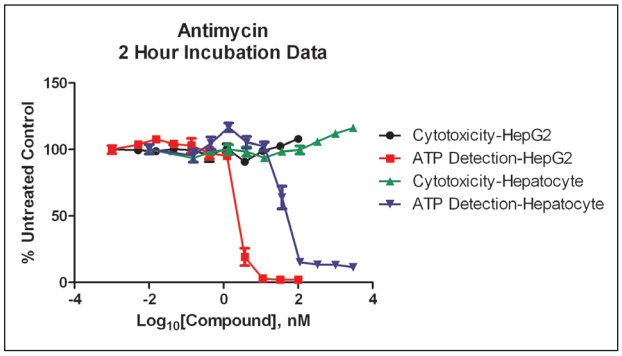

Figure 5. Variability between HepG2 and primary hepatocytes in the cytotoxicity and ATP detection tests illustrates the need to use the most relevant cell for toxicity testing. Variability among cell model data is also illustrated in Figure 4.

Summary

Multiplexed cell-based mitochondrial assays increase sample throughput and decrease variability, costs and overall time for project completion. In this example, it provides a simple way to distinguish between compounds that negatively affect mitochondrial function versus those that negatively affect the cell by other means such as primary necrosis. Clear distinctions are demonstrated between cancer cell lines and primary cells, and toxicity models should use the most relevant cell model in order to obtain a true in vivo profile. Automating the assay procedure with robotic instrumentation yields an efficient and robust method to increase throughput and repeatability, and decrease overall project time. Finally, the combination of instrumentation, assay chemistry and primary hepatocytes are an ideal solution to accurately predict potential mitochondrial toxicity in lead compounds.