

whereas mitochondrial toxicity results in decreased ATP production with little to no change in membrane integrity. The assay's efficiency is further enhanced via automation.

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 Invitrogen. 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 resuspended 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% CO₂/37°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

Per Figure 3, ATP Detection Assay data, ATP production, as a function of the detected luminescent signal, substantially decreases in the hepatocyte cells in the presence of antimycin. The lack of significant change in cytotoxicity data proves that toxicity from antimycin is due to the compound impeding mitochondrial function, and not a result of primary necrosis. ATP production and cytotoxicity data are similar in hepatocytes grown with and without glucose.

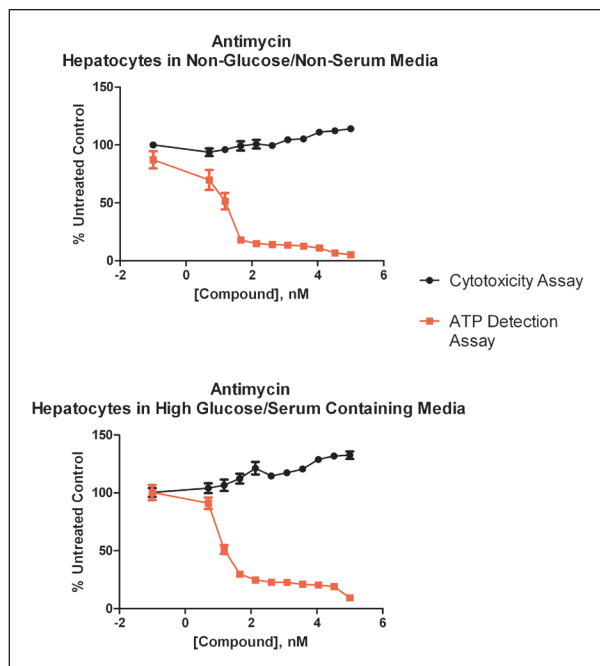


Figure 3. Primary hepatocytes assayed in either high glucose or no glucose in the presence of increasing concentrations of the mitotoxicant antimycin show significant decrease in ATP production as expected

ATP production in the HepG2 cells (Figure 4) reveals a sharp contrast between cells grown in glucose media and those in non-glucose media. Mainly, those HepG2 cells grown in non-glucose media were forced to revert to produce ATP via mitochondrial oxidative phosphorylation, and therefore behaved similarly to the hepatocyte cells, while HepG2 cells grown in glucose media produced ATP via glycolysis, therefore the known antimycin toxic properties on mitochondria were masked in a classic Crabtree Effect.

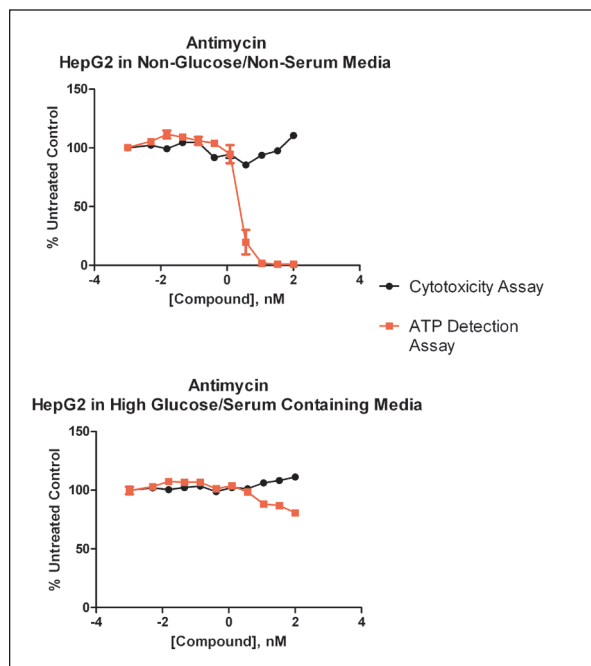


Figure 4. Cancerous hepatocytes (HepG2) grown in no glucose media display a similar ATP decrease, while those grown in high glucose media have very little ATP decrease, thus representative of the Crabtree Effect.

Summary

Because of their ease of use, and availability, highly proliferative tumor-derived immortalized cell lines have become the model of choice for mitochondrial toxicity studies. This has led to possible false conclusions regarding lead compound toxicity due to the cells propensity to derive energy from glycolysis rather than through oxidative phosphorylation in the mitochondria. In order to circumvent this phenomenon, and attain the correct data, it is necessary to force these cell lines to use mitochondrial generated ATP through the use of a non-glucose media. Here we have demonstrated how this effect can be monitored through the use of a multiplexed cytotoxicity and ATP detection assay. The homogeneous format allows for automation of the assay using simple, yet robust instrumentation. The results show how accurate titration data for cytotoxicity and ATP production can be attained, and the Crabtree Effect overcome, when using the correct media or cell model conditions.

Acknowledgements

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