A Multiplex, Automated Approach to Screen for Mitotoxicity in Human Hepatocytes and HepG2 Cells

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ABSTRACT

Many drugs and environmental chemicals have been implicated in mitochondrial toxicity resulting in myopathies, hepatotoxicities, peripheral neuropathies and cardiovascular disorders. The mechanisms of mitochondrial toxicities are complex due to multiple modes of action such as modulating mitochondrial replication and disruption of electron transport chain, and by organ-specific susceptibility. For example, the mitotoxin propionate preferentially targets the liver over muscle due to metabolic activation in hepatocytes. Therefore, multiple approaches are needed to assess a chemical's mitochondrial liabilities in specific cellular systems. Herein, we present one approach to utilize two markers of cellular viability in an automated multiplexed format for high-throughput screening to monitor hepatotoxicity that can discriminate primary mitochondrial toxicity from general cytotoxicity. Primary human hepatocytes and HepG2 cells were used in a 384-well suspension culture format in serum-free, glucose-free galacatose-containing medium for up to 6 hour exposure with cytotoxins in an 11-point response curve. ATP Detection Reagent and Cytotoxicity Reagent were used to measure ATP levels and cell membrane integrity, respectively. A mitochondrial toxic effect was defined as a reduction of ATP relative to untreated control, with no or minor changes in cell membrane integrity. CCCP and antimycin were used as model compounds to disrupt mitochondrial activity. Digitonin cytotoxicity was caused by solubilizing the cell membrane, resulting in decreased ATP and loss of cell membrane integrity. Staurosporine and tamoxifen were also used as cytotoxins to validate the system. Similar results were observed between HepG2 and human hepatocytes except for antimycin where a Crabtree effect was implicated for the difference in its potency. In summary, the multiplex system provided a robust and informative platform to screen drugs and chemicals for hepatic mitotoxicity by measuring ATP and cell membrane integrity.

INTRODUCTION

Mitochondrion, an organelle found in most eukaryotic cells, is responsible for the majority of the cellular energy through the production of adenosine triphosphate (ATP). The production of ATP is through oxidative metabolism of sugars, fatty acids and pyruvate that are converted into acetyl-CoA and further degraded in the citric cycle to drive the electron transport chain and the oxidative phosphorylation of ADP and P_i. ATP is then distributed throughout the cell to mediate signal transduction, metabolism, active transport, DNA and RNA synthesis, and other critical pathways. Dysfunction of the mitchondrion and the reduction of the ATP levels has severe consequences to the cell's function and health, as well as to the associated organ and to the whole body. ATP production may be directly reduced by inhibiting any of the numerous enzymes associated with ATP production. The electron transport chain, too, may be disrupted causing a decrease in ATP production and increase in oxidative stress. This may cause a loss of cellular function and cellular damage, respectively.

The source of the mitochondrial toxins (mitotoxins) may be from medications, environmental agents and occupational chemicals, as well as dietary substances and behavioral influences. Neither the FDA nor EPA have a guidance pertaining to testing for mitochondrial toxicity (mitotoxicity) potential; however, a growing body of evidence has implicated pharmaceutical and chemical sources as clinically relevant mitotoxins. For example, troglitazone (Rezulin) caused liver injury and liver failure in patients, and was removed from the market in 2000 for these events. Mitochondrial injury through reduction of mitochondrial transmembrane potential and depletion of ATP has been purported as one of the mechanisms of toxicity caused by troglitazone[1]. Therefore, it is important to determine the potential of compounds that may act as toxins to mitochondria as one potential mechanism of toxicity for new chemical entities.

Herein, we demonstrate the utility of an automated multiplexed assay to assess cell membrane integrity (cytotoxicity) and mitochondrial function (ATP levels) in primary hepatocytes and HepG2 cells. Cytotoxicity was determined using pro-fluorogenic peptide bis-AAF R110 that is impermeable to live cells, and cleaved by a protease released into the medium when the cell membrane becomes compromised. ATP levels were measured using luminescent signal from luciferin-luciferase reaction. Together, the readouts can distinguish between general cytotoxicity and mitochondrial function. Mitotoxicity was defined as a lower ATP with no changes to cytotoxicity.

MATERIALS & METHODS

Cell Preparations. HepG2 cells were propagated in a medium formulation consisting of high glucose DMEM, 10% FBS and 1% Pen/Strep. After removal from the growth flask, the cells were resuspended in a glucose-free medium formulation consisting of glucose-free DMEM, 5 mM HEPES, 10 mM galactose, 2 mM glutamine, 1 mM Na-pyruvate, and 1% Pen/Strep. LiverPool™ (BioreclamationIVT) cryopreserved human suspension hepatocytes were thawed and resuspended in *InVitroGro* HT Medium. After the cells were centrifuged for 5 minutes at 50 x g, they were resuspended in the glucose-free medium described above. Trypan Blue exclusion was used to count cells. Suspensions were dilute to 2 x 10^6 cells/mL.

Liquid-handling Equipment. The Precision™ Microplate Pipetting System (BioTek), consisting of an 8-channel pipetting head and 8-channel bulk reagent dispenser was used to dispense cells, serially titrate compounds across a 96-well PP plate, transfer compounds to the 384-well cell plates, as well as for reagent dispensing.

Compound Preparation. All compounds were purchased from Sigma-Aldrich. Compounds were diluted from the 100% DMSO stocks in non-serum/non-glucose medium to the final 2X concentration before addition to the assay plates. Final concentrations were 10 pM to 30 µM for antimycin and CCCP, and 100 pM to 100 µM for digitonin, diclofenac, staurosporine and tamoxifen .

Mitochondrial ToxGlo™ Assay. Assay kit consists of Cytotoxicity Reagent and ATP Detection Reagent prepared as described by the manufacturer (Promega). 5X Cytotoxicity Reagent was used to determine cytotoxicity (compromised membrane integrity) and ATP Detection Reagent was used to measure ATP content (mitochondrial function).

Assay Protocol. A 10 µL volume of cell suspension was transferred to each well of a 384-well microtiter plate. A 10 µL volume of titrated compounds was added to the wells with cells. The plates were incubated for 1 to 6 hours at 37°C, humidified 5% CO_2. At appropriate times, 5 µL of 5X cytotoxicity reagent was added to each well and incubated for 30 minutes at 37°C, humidified 5% CO_2. The fluorescence was measured at 485nm/528nm Ex/Em using a Synergy™ H4 (BioTek) plate reader. A 25 µL volume of ATP detection reagent was added to each well and incubated at ambient temperature for 5 minutes. The luminescence was measured using 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:

% Unstimulated Control = (Value(T) / 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. GraphPad Prism® 5 was used to graph the results.

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Assay Development

Crabtree Effect on Cellular ATP Levels

Studies, including those described by Marroquin[2], have shown that differences exist between cancer cell models and normal primary cells in how ATP is derived within the cell. Primary cells rely on mitochondrial oxidative phosphorylation to generate ATP. Cancer cells, in contrast, rely instead upon glycolysis when grown using typical high glucose media. Cancer cells will revert back to using oxidative phosphorylation when glucose is substituted with galactose. This phenomenon, known as the Crabtree Effect, can cause compounds that would normally induce mitochondrial toxicity in an *in vivo* setting to appear as having no toxicological effect when tested using a cancer cell line in combination with high glucose medium.

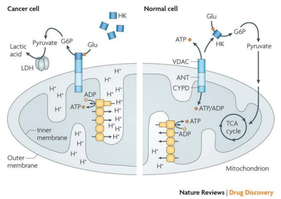
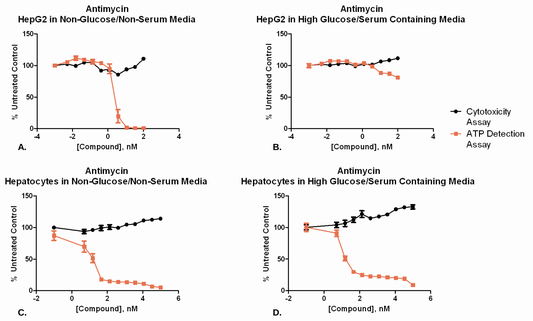


Fig 1. ATP production in cancer and normal primary cell models.[3]

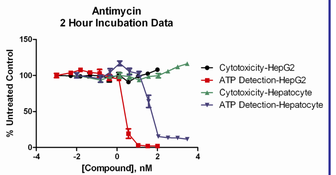
The ability to detect this effect was tested using the HepG2 cancer cell line and primary hepatocytes. Each cell type was resuspended and plated in either high or non-glucose medium, and then exposed to varying concentrations of antimycin, a known mitochondrial toxicant, for two hours. HepG2 cells demonstrated a significant difference in ATP levels between media types, while no difference was detected in the human hepatocytes. This confirmed the expected result of the Crabtree effect.



Graph 1. Cytotoxicity and ATP Detection graphed for response to antimycin (2 hour exposure) for (A.) HepG2 in non-glucose medium with no serum, (B.) HepG2 in high-glucose medium with serum, (C.) human hepatocytes in non-glucose medium with no serum, and (D.) human hepatocytes in high-glucose medium with serum.

Cell Dependent Response

As previous experiments demonstrating the Crabtree Effect have shown, forcing cancer cell lines to generate ATP by mitochondrial oxidative phosphorylation can lead to a more *in vivo*-like response. However this change may still not yield the same results that would be generated by using a true primary cell model. This was tested by comparing the results from HepG2 cells and hepatocytes treated with the various mitotoxicants in non-serum/non-glucose medium to ensure that all cells are relying on mitochondrial generated ATP. The results for antimycin demonstrate that even when the HepG2 cells and hepatocytes are tested using the same media conditions, there are still differences in potency of the compound between the cell models and mode of action. The other compounds showed no cell-dependent differences.

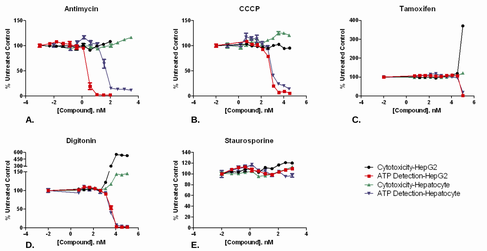


Graph 2. Cell dependent response to cytotoxicity (Black and Green) and ATP content (Red and Blue) between HepG2 (Black and Red) and human hepatocytes (Green and Blue) to antimycin concentration response curve at 2 hour exposure.

Mitotoxicity Screening Assay

Concentration Response Curve

Concentration dependent response to cytotoxins were investigated. Three known toxicants, antimycin, CCCP, and tamoxifen were tested using HepG2 and primary hepatocyte models. A known inducer of cellular necrosis, digitonin, was included as a cytotoxicity control. Staurosporine, a long-term apoptosis inducer was included as a negative control. The compounds were tested using cells resuspended and dispensed in non-serum/non-glucose medium. A two hour incubation period of compound with cells before addition of the detection reagents was incorporated for this test. A decrease in cellular ATP concentration was seen with increased concentrations of each of the three mitotoxicants tested with a two hour incubation. This was consistent with previously published literature references for antimycin[4], CCCP[5], and tamoxifen[6]. Tamoxifen demonstrated a decrease in cellular ATP only at the highest concentration tested, which may be indicative that the incubation time used here is not sufficient to see the complete effect of the compound. Digitonin demonstrated an increase in signal from the cytotoxicity assay, in cooperation with a decrease in ATP concentration, indicative of it's necrosis inducing characteristics. Finally, staurosporine did not cause any change in signal with either assay, which also agrees with the known effects of the compound using this particular incubation time.



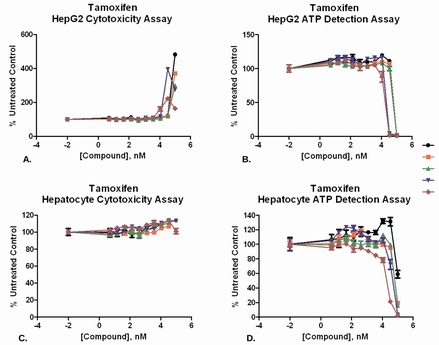
Graph 3. Dose response curves of (A.) antimycin, (B.) CCCP, (C.) tamoxifen, (D.) digitonin and (E.) staurosporine as measured for cytotoxicity (Black and Green) and ATP content (Red and Blue) in HepG2 (Black and Red) and human hepatocytes (Green and Blue) .

Time Response Curve

The automated assay was further tested using compound-cell incubation times from one to six hours. A longer incubation time can allow for a more complete set of data to be generated for compounds that do not exhibit rapid toxic effects, or are less potent at lower concentrations. Cells were dispensed as previously described. Compounds were then added to the cells at the appropriate time to create the proper incubation period. The data shown for tamoxifen illustrated how results can alter using variable incubation times, and thus the need to test multiple exposures of compound with cells. The compound became more potent with increased exposure to both cell models. Changes in the signal from the cytotoxicity assay were also seen using HepG2 cells. This phenomenon, which was not seen in hepatocytes, may indicate a higher susceptibility to cytotoxicity from tamoxifen in the cancer cell model. The other compounds exhibited varied responses (data not shown).

For human hepatocytes, antimycin showed minor time-dependent response for increased cytotoxocity and decreased ATP content. CCCP and staurosporine showed no time-dependent response for cytotoxicity, however did show a time dependent response of lower ATP levels at higher concentrations. Time had no influence on digitonin or diclofenac cytotoxicity or ATP levels.

For HepG2 cells, there were no time-dependent changes as measured by ATP content. For cytotoxicity, antimycin produced a time-dependent increase in signal at higher concentrations while digitonin showed a time-dependent decrease in signal at higher concentrations which may indicate protease activity is sensitive to digitonin over time more than a loss of membrane integrity. CCCP, diclofenac and staurosporine showed more time dependent difference.



Graph 4. Time response curves of tamoxifen as graphed by cell type and assay type (A.) HepG2 cytotoxicity assay, (B.) HepG2 ATP detection assay, (C.) human hepatocytes cytotoxicity assay, and (D.) human hepatocytes ATP detection assay.

CONCLUSIONS

1. The multiplexed assay provides an easy way to distinguish between compounds which negatively effect mitochondrial function, and those that effect the cell using other means such as primary necrosis.
2. The automated 384-well assay procedure, incorporating suspension cells, yields an efficient, yet robust way to perform the Mitochondrial ToxGlo™ assay in a high-throughput format.
3. Cryopreserved suspension hepatocytes offer a reproducible and convenient cell model, and may generate the most *in vivo*-like results due to retained physiologically relevant cellular activities.
4. The combination of instrumentation, assay chemistry, and hepatocytes create an ideal solution to help make accurate predictions about the potential mitochondrial toxicity liabilities of lead compounds.