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A high-throughput dual parameter assay for assessing drug-induced mitochondrial dysfunction provides additional predictivity over two established mitochondrial toxicity assays

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ABSTRACT

Mitochondrial toxicity is a major reason for safety-related compound attrition and post-market drug withdrawals, highlighting the necessity for higher-throughput screens that can identify this mechanism of toxicity during the early stages of drug discovery. Here, we present the validation of a 384-well dual parameter plate-based assay capable of measuring oxygen consumption and extracellular acidification in intact cells simultaneously. The assay showed good reproducibility and robustness and is suitable for use with both suspension cells and adherent cells. To determine if the assay provides additional value in detecting mitochondrial toxicity over existing platforms, 200 commercially available drugs were tested in the assay using HL60 suspension cells as well as in two conventional mitochondrial toxicity assays: an oxygen consumption assay that uses isolated mitochondria and a cell-based assay that uses HepG2 cells grown in glucose and galactose media. The combination of the dual parameter assay and the isolated mitochondrial oxygen consumption assay identified more compounds that caused mitochondrial impairment than any other combination of the three assays or each of the three assays on its own. Furthermore, novel information was obtained from the dual parameter assay on drugs not previously reported to cause mitochondrial impairment.

1. Introduction

Mitochondrial dysfunction has been implicated as a major contributor to drug-induced toxicity (Wallace, 2008). This is unsurprising considering the pivotal role played by these organelles, not only in ATP production but in the integration of various cell signalling pathways and in the maintenance of cellular homeostasis. In short, when mitochondria fail, the cell dies (Dykens, 2007), and this situation is exacerbated by the multitude of mechanisms by which mitochondrial functions can be perturbed. These perturbations are often mediated directly through inhibition of either the enzymes involved in electron transport or ATP synthesis or through the uncoupling of the ATP synthase from electron transport. Furthermore, indirect perturbation is also possible through the disruption of mitochondrial transcription/translation and/or the acceleration of free-radical production. Considerable advances have been made in understanding these mechanisms (Amacher, 2005; Wallace and Starkov, 2000; Zhou and Wallace, 1999) and drug-induced mitochondrial dysfunction has now been shown to contribute to toxicity in the liver, heart, kidney, muscle, and the central nervous system.

Of the fifty or so drugs removed from the market due to safety concerns between 1960 and 1996, at least five have subsequently been associated with mitochondrial toxicity (Dykens and Will, 2007). Examples include the biguanides, phenformin and buformin, which were withdrawn due to severe lactic acidosis, a hallmark of mitochondrial toxicity (Dykens et al., 2008a). The biguaninde, metformin, on the other hand, is still on the market and, in general, is well tolerated, a situation which suggests that appropriate screening strategies could reduce the risk of deleterious effects while maintaining drug efficacy. Cerivastatin was discontinued due to severe rhabdomyolysis (Evans and Rees, 2002; Kaufmann et al., 2006) a side effect reported for many statins and the reason for a recent label change for simvastatin. Troglitazone and nefazodone were discontinued due to idiosyncratic liver toxicity, with mitochondrial dysfunction being one of the contributing mechanisms (Dykens et al., 2008b; Nadanaciva et al., 2007). In addition to post-market drug withdrawals, mitochondrial liabilities have also been associated with many drugs carrying a black box label for hepatic or cardiac toxicity (Dykens et al., 2007), highlighting again the importance of mitochondrial toxicity when considering off-target effects. These observations point to a weakness

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in the approaches used for assessing drug safety. The animals used in regulatory toxicology studies are generally young and healthy and are thus unsuitable for identifying compounds that may cause sub-lethal reductions of mitochondrial capacity. The development of suitable *in vitro* assays is therefore of particular importance in the investigation and prediction of human mitochondrial toxicity.

Traditionally, *in vitro* analysis of oxygen consumption of both mitochondria and whole cells was conducted using a Clark electrode (Clark, 1959) but this lacks the throughput required in the application for large compound libraries and IC_{50} determination. This limitation was addressed through the use of an oxygen-sensitive fluorescent probe which formed the basis for a microtitre plate-based oxygen consumption assay capable of high throughput analysis of isolated mitochondria (Hynes et al., 2007; Will, 2007). Using this platform, we have identified mitochondrial liabilities for members of a wide variety of drug classes, including thiazolidonediones, statins, antidepressants and biguanides (Dykens et al., 2008a,b; Nadanaciva et al., 2007). However, an assay that uses an isolated organelle may possibly over-predict direct effects on respiration and/or under-predict indirect effects. This prompted the development of a cell-based approach based on the differential sensitivity of glucose- and galactose-grown cells to compounds that impair mitochondrial function (Marroquin et al., 2007). The resultant cellular ATP IC_{50} values of compounds in the two media conditions are compared and an IC_{50} Glucose:IC_{50} Galactose ratio of >3 is taken as being indicative of mitochondrial toxicity. Mitochondrial liability for members of the biguanide family as well as certain antidepressants (nefazodone) has been identified in this manner (Dykens et al., 2008a,b). The assay does, however, have the drawback in that when drugs cause multiple mechanisms of toxicity, it is not possible to identify whether mitochondrial toxicity occurs or not.

In light of this, we examined if a 384 well plate-based assay capable of measuring cellular oxygen consumption and extracellular acidification simultaneously in cells could further improve or refine our current mitochondrial toxicity assessments. This was made possible by the recent development of a microtitre plate-based extracellular acidification assay that is compatible with parallel oxygen consumption measurements (Hynes et al., 2009b). If a drug displays mitochondrial liabilities, oxygen consumption would be affected and extracellular acidification would increase concomitantly as cells try to circumvent the mitochondrial insult through increased glycolytic flux (Hynes et al., 2009a). This 384 well assay would therefore combine the specificity of the isolated mitochondrial oxygen consumption analysis with the cell component. We present the validation of the assay and also apply it to the analysis of a 200 compound library and compare the data generated from this assay with data generated from both the isolated mitochondrial oxygen consumption assay and the HepG2 glucose/galactose assay with the aim of determining the most appropriate assay or combination of assays to effectively detect drug-induced mitochondrial toxicity.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO), or Toronto Research Chemicals Inc. (North York, Ontario, Canada), and were of highest purity available. HL60 cells, HepG2 cells and THLE cells were purchased from the American Tissue Culture collection (ATCC). For the dual parameter assay, cell culture media and supplements were purchased from Sigma Aldrich (Ireland), tissue culture flasks and PS 96-well TC+ micro plates from Sarstedt (Germany). For the HepG2 assay in glucose and galactose media and the THLE cytotoxicity assay, cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA) and Lonza (Walkersville, MD), and tissue culture flasks (BD Biocoat), 96-well plates and 384-well plates (BD Biocoat) were purchased from VWR (Westchester, PA). CellTiter-Glo Luminescent Cell Viability Assay kits were purchased from Promega (Madison, WI). MitoXpress®-Xtra and pH-Xtra were purchased from Luxcel Biosciences (Cork, Ireland).

2.2. Methods

2.2.1. Cell culture

HL60 cells were cultured in RPMI containing 11 mM glucose, 24 mM sodium bicarbonate and 4 mM glutamine and supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in 175 cm^2 tissue culture flasks (83.1812.502 Sarstedt) and grown in a cell culture incubator at 37 °C in a 5% CO_2 atmosphere.

HepG2 cells were cultured in both glucose- and galactose-containing growth media (Marroquin et al., 2007). High-glucose media:high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen 11995–065) containing 25 mM glucose and 1 mM sodium pyruvate and supplemented with 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Galactose media: DMEM deprived of glucose (Invitrogen 11966–025) supplemented with 10 mM galactose, 2 mM glutamine (6 mM final), 5 mM HEPES, 10% fetal bovine serum, 1 mM sodium pyruvate, and pen-strep as above. Cells were maintained in collagen-coated 150 cm^2 flasks (356486, BD Biocoat) in a cell culture incubator at 37 °C in a 5% CO_2 atmosphere.

Transformed Human Liver Epithelial (THLE-2) cells were cultured in Bronchial Epithelial cell Basal Medium (Lonza, Walkersville, MD) supplemented with the individual components of a Bronchial Epithelial Growth Media bullet kit (with the exception of gentamicin and epinephrine) from Lonza (Walkersville, MD), 10% fetal bovine serum, 5 ng/ml epidermal growth factor, 70 ng/ml phosphoethanolamine, 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were grown in a cell culture incubator at 37 °C in a 5% CO_2 atmosphere.

2.3. Measurement of cellular ATP content

HepG2 cells were plated at 20,000 cells/well on collagen-coated, clear bottom 96-well plates. THLE cells were plated at 2500 cells/well on collagen-coated clear bottom 384-well plates. For drug treatments, compound stock solutions were prepared in DMSO and added to the wells to give the indicated final drug concentrations. Final DMSO concentration was 0.5%. Cellular ATP concentrations were assessed by using the CellTiter-Glo Cell Viability Assay as per the manufacturer's instructions 24 h after drug addition for the HepG2 glucose/galactose assay and 72 h after drug addition for the THLE cytotoxicity assay.

2.4. Isolation of liver mitochondria

Mitochondria were isolated from male Sprague–Dawley rats (150–180 g) essentially as described earlier (Hynes et al., 2012): the animals were euthanized with an overdose of carbon dioxide. The liver was rapidly excised and placed in ice-cold Buffer I (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, 1 mM EGTA, 0.5% BSA, pH 7.). Using a pair of scissors, approximately 6 g of liver tissue were finely minced and washed repeatedly in Buffer I until the homogenate was blood-free. Five volumes of Buffer I were added and the liver was homogenized using 6–8 passes of a smooth glass grinder with Teflon pestle driven by a power drill