Published in final edited form as:

Anal Biochem. 2010 September 1; 404(1): 75–81. doi:10.1016/j.ab.2010.04.040.

A high throughput respirometric assay for mitochondrial biogenesis and toxicity

Craig C. Beeson, Gyda C. Beeson, and Rick G. Schnellmann

Department of Pharmaceutical and Biomedical Sciences Medical University of South Carolina, Charleston, SC 29425

Abstract

Mitochondria are a common target of toxicity for drugs and other chemicals, and results in decreased aerobic metabolism and cell death. In contrast, mitochondrial biogenesis restores cell vitality and there is a need for new agents to induce biogenesis. Current cell-based models of mitochondrial biogenesis or toxicity are inadequate because cultured cell lines are highly glycolytic with minimal aerobic metabolism and altered mitochondrial physiology. In addition, there are no high-throughput, real-time assays that assess mitochondrial function. We adapted primary cultures of renal proximal tubular cells (RPTC) that exhibit *in vivo* levels of aerobic metabolism, are not glycolytic, and retain higher levels of differentiated functions and used the Seahorse Biosciences analyzer to measure mitochondrial function in real time in multi-well plates. Using uncoupled respiration as a marker of electron transport chain (ETC) integrity, the nephrotoxics cisplatin, HgCl₂ and gentamicin exhibited mitochondrial toxicity prior to decreases in basal respiration and cell death. Conversely, using FCCP-uncoupled respiration as a marker of maximal ETC activity, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), SRT1720, resveratrol, daidzein, and metformin produced mitochondrial biogenesis in RPTC. The merger of the RPTC model and multi-well respirometry results in a single high throughput assay to measure mitochondrial biogenesis and toxicity, and nephrotoxic potential.

Keywords

biogenesis; nephrotoxic; oxygen consumption; proximal tubule

Introduction

Many drugs and industrial and environmental chemicals are toxic because they target mitochondria [1]. This is a particular problem in those cells/tissues that primarily rely on aerobic metabolism (e.g. kidney). The resulting mitochondrial damage leads to decreased aerobic metabolism and ATP, disrupted cellular functions, and cell injury and death. Thus, mitochondrial function is a superb proxy for cell and organ vitality.

© 2010 Elsevier Inc. All rights reserved.

Correspondence To: Rick G. Schnellmann Medical University of South Carolina 280 Calhoun Street, MSC 140 Charleston, SC 29425
schnell@muscc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

For example, the kidney is capable of transporting and accumulating many low molecular weight chemicals and possesses enzymes that metabolize xenobiotics to toxic reactive intermediates [1]. Most nephrotoxics damage the tubular epithelial cells and/or the glomeruli [2]. The tubular Na⁺-dependent uptake processes result in high ATP demand making them highly aerobic and particularly sensitive to such insults [3]. In light of the fact that many known nephrotoxics cause mitochondrial damage, the development of a high-throughput assay to measure the loss of mitochondrial respiratory capacity in RPTC would greatly enhance efforts to assess new chemicals and biological agents as potential nephrotoxics. Perhaps more importantly, we propose that RPTC are an ideal model to measure mitochondrial toxicity in general because they exhibit *in vivo* levels of mitochondrial function and express transporters and biotransformation enzymes to insure uptake and metabolism of toxicants.

The renal tubular epithelia are somewhat unique among differentiated tissue in that they have some capacity for repair and regeneration. RPTC mitochondrial loss following oxidant injury results in a signaling cascade that leads to mitochondrial biogenesis [4]. Furthermore, several compounds have been shown to produce mitochondrial biogenesis and that mitochondrial biogenesis following oxidant injury accelerates the return of mitochondrial and cellular functions [5,6,7]. In light of these and related observations, understanding the mechanisms of mitochondrial biogenesis and the development of agents to induce it are needed. Because the master regulator PGC-1 α is required for mitochondrial biogenesis, it has been the subject of many of these efforts. Unfortunately, assays of PGC-1 α activation, or the activation of upstream effectors, do not consistently predict the subsequent biogenic process. Thus, a functional assay of mitochondrial biogenesis is needed.

Immortalized cell lines have been used extensively to study mechanisms of toxicity [2,8]. Two severe limitations of these cells are the loss of differentiated functions and high rates of glycolysis with limited respiration. A number of years ago we modified the culture conditions of primary cultures of RPTC to provide polarized cells with a greater retention of differentiated functions; and the cells exhibited respiration and gluconeogenesis rates comparable to the rates measured *in vivo* [9,10].

Methods for determining mitochondrial function by measuring cellular respiration have largely relied on Clark electrode chambers that lack the throughput needed in modern research. A multi-well plate-based assay platform, the Seahorse Biosciences extracellular flux (XF) analyzer, was recently introduced to address the need for higher throughput respirometric measurements. The XF instrument uses fluorescent optode detectors to measure oxygen consumption rates (OCR) from cells plated in custom 24-well plates. The XF instrument has shown potential to measure cell metabolism in primary cardiomyocytes and many cell lines [11,12,13]. Although the XF instrument has not been used to assess mitochondrial dysfunction or nephrotoxicity potential, it offers the possibility to be a moderate to high-throughput assay platform. To assess its potential, the primary culture of RPTC model was optimized for the XF-24 platform and tested with several nephrotoxics and mitochondrial biogenesis activators.

Materials and Methods

Materials

Female New Zealand White Rabbits (1.5 - 2.0 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). The basal medium is a 50:50 mixture of Dulbecco's modified Eagles' essential medium and Ham's F12 nutrient mix without phenol red, supplemented with 15 mM NaHCO₃ and 0.2 mM glycine and 6 mM sodium lactate. The medium is adjusted to pH 7.4 while gassing with 95% O₂-5% CO₂ and was diluted to 295 mosmol/kg H₂O before filter sterilization. The isolation media is the basal medium supplemented with 2 mM heptanoic acid,

penicillin G (100 U/mL), and 0.5 mM deferoxamine. The culture medium used for cell growth is the basal medium supplemented with human transferrin (5 µg/mL), selenium (5 ng/mL), hydrocortisone (50 nM) and bovine insulin (10 nM). In some experiments cultures were grown in the presence of 17 mM glucose for comparison to previous studies.

Isolation of proximal tubules and culture conditions

In brief, rabbit renal tubules were isolated using the iron oxide perfusion method as described previously [9,10]. The resulting proximal tubules were plated on 100-mm tissue culture grade plastic petri dishes or XF-24 V28 plates. Plates were constantly swirled on an orbital shaker at 80 rpm or held stationary. The optimal procedure was to culture RPTC on tissue culture dishes for 3 days, the cells were removed with trypsin, and then plated into XF-24 wells at 40,000 cells/well. The plates were shaken on an orbital shaker at 80 rpm for 4 days and then treated and assayed 24 h later.

Respirometry Assay

The OCR measurements were performed using a Seahorse Bioscience XF-24 instrument (Seahorse Bioscience, North Billerica, MA). On the day before the experiment, the sensor cartridge was placed into the calibration buffer supplied by Seahorse Biosciences to hydrate overnight. On the day of the experiment, the cell media was removed from the wells and each well washed with PBS with Mg^{2+} and Ca^{2+} two times. The running media (growth media) was then placed into the wells and warmed to 37°C in an incubator. The injection ports of the sensors were filled with 100 µL of treatment or vehicle in buffer. The sensor was then placed into the XF-24 instrument and calibrated. After calibration, the calibration fluid plate was replaced with the cell plate. The measurement cycle consisted of a 2 min mix, 1 min wait, and a 2 min measurement. Four basal rate measurements were followed with injections and each injection is followed by four measurement cycles. The consumption rates were calculated from the continuous average slope of the O_2 decreases using a compartmentalization model that accounts for O_2 partitioning between plastic, atmosphere, and cellular uptake [13]. For any one treatment, the rates from 3 - 4 wells were used. Rates for the wells were normalized for protein content. Average basal rates are the averages of the 3rd and 4th basal rates and average uncoupled rates were the averages of the 1st and 2nd rates after FCCP injection. All average rates were normalized to the vehicle control basal and t-tests between control and treatment were used to assess statistical significance.

Fluorescence Viability Assay

RPTC were plated at 50,000 cells per well in black-walled 96-well plates with optically clear bottom surface, and were then treated with the toxicants. After 24 h, the medium were aspirated, and the cells were stained for 60 min in the dark with Hoechst 33342 (20 µM) and propidium iodide (7.5 µM) in phosphate buffered saline containing 6 mM lactate. Bright field and fluorescence images from each well were taken on the IN-Cell 1000 (GE Healthcare, Chalfont St. Giles, United Kingdom) to give the number of dead (red) cells and the total number of cells (blue) in each well as a function of toxicant concentration. The number of dead cells was subtracted from the total number to obtain the number of viable cells in each well, which is represented as a percentage of the total number (% viability). Control wells receiving no treatment vehicle were also included for comparison.

Bicinchoninic Acid Assay for Protein Content

To assess protein content, the medium was first aspirated from the XF-24 wells and the cells were washed twice with phosphate buffered saline containing Ca^{2+}/Mg^{2+} . The cells were dissolved for a minimum of 4 h at room temperature in 500 µL of Triton buffer, which contained tris-base (100 mM), NaCl (150 mM), and Triton X-100 (0.05 %), adjusted to pH 7.5. The

solutions were transferred to microcentrifuge tubes, vortexed, and ultrasonicated for 30 s. Aliquots were assayed for protein concentration using a bicinchoninic acid kit (Sigma).

Results

RPTC cultured under standard conditions (stationary with 17 mM glucose), had basal OCR that were > 100-fold lower than for RPTC cultured under optimized conditions (shaking, with lactate and no glucose) (Fig. 1A). Inhibition of the F_1F_0 -ATPase with oligomycin is a measure of the fraction of the OCR that is coupled to ATP production. In “healthy” cells, in tissue, and in humans, the oligomycin-induced decrease in OCR is typically about 70% of basal [14,15]. RPTC OCR decreased by approximately 30% in standard conditions and by approximately 80% in optimized conditions following 1 μ M oligomycin addition (Fig. 1A). As a further test of their differentiation and respiratory function, the RPTC were treated with ouabain, an inhibitor of the plasma membrane Na^+/K^+ -ATPase, to determine the fraction of OCR that is coupled to maintenance of plasma membrane potential. Ouabain decreased OCR approximately 60% in RPTC cultured in optimized conditions while it had a minimal effect in RPTC cultured under standard conditions (Fig. 1B). Approximately 60% of the respiration is typically inhibited by ouabain in these cells, and in isolated tubules [16,17].

Treatment of RPTC with FCCP uncouples the mitochondrial membrane potential to cause an increase in the OCR. For isolated mitochondria, the increase in OCR relative to the oligomycin treated rate is often referred to as the maximum oxidative phosphorylation capacity and it is an approximate measure of the V_{max} for the electron transport chain [14,17]. Preliminary experiments determined that 5 μ M FCCP produced the maximal OCR in RPTC cultured under optimized conditions. The uncoupled rates were not increased with triple the lactate concentration (18 mM), added glucose (5.5 mM), or added palmitate (0.25 mM, BSA carrier). As shown in Fig. 1A, FCCP produced an approximately 10-fold increase in OCR in RPTC cultured under optimized conditions compared to oligomycin-OCR. The increase in OCR produced by FCCP in RPTC cultured under standard conditions was minimal.

To assess reproducibility, the experiment shown in Fig. 1 was repeated for multiple primary cell preparations. The basal rates were measured for 90 s followed by a 2 min mix period, a 2 min wait period, and the cycle was repeated four times before the injection of a pharmacological agent. For any given plate, the well-to-well variation over 20 wells at the 4th basal rate measurement was typically 12-15% and the standard deviation for the average of the last three basal rates for any one well was about 7%. The greatest source of variance was found to be among different RPTC preparations as would be anticipated for primary cell cultures.

To evaluate preparation-dependent variances and to compare respiration rates to historical values, RPTC in each well were lysed and the protein concentration was measured with the bicinchoninic acid assay. The mean $\pm$ standard deviation of the basal and uncoupled OCR among 15 preparations were 14.4 ± 3.5 and 34.2 ± 7.2 , respectively, and the protein-normalized rates are comparable to previously reported values [9].

To examine the ability of the XF-24 instrument to measure RPTC mitochondrial dysfunction, RPTC were cultured under optimized conditions and treated with nephrotoxics or vehicle control (0.1% DMSO) for 24 h prior to assessment of OCR. After measuring basal rates, the cells were treated with FCCP to uncouple the mitochondria. To account for variations in OCR rates from different preparations, the basal rates for treated wells and the uncoupled rates (in nmol/min mg protein) were normalized to the basal rates of vehicle control wells. Separate experiments confirmed that 24 h pretreatment with 0.1% DMSO vehicle had no measurable effect on basal or uncoupled rates. It was found that the uncoupled rates, but not basal rates showed concentration-dependent decreases in RPTC treated with cisplatin, gentamicin, and

HgCl₂ (Fig. 2), nephrotoxics known to cause mitochondrial damage [18,19,20]. The uncoupled rates were decreased by as much as 40% with no significant decrease in basal. The FCCP uncoupled rates can be used as a stress response to uncover disrupted electron transport chain activity by toxicants, even though basal metabolism is not impaired [21]. As a control, RPTC were treated with mitomycin C, an antineoplastic agent that does not cause measurable tubular damage in rats [20]. RPTC exposed to 10 μ M mitomycin C did not exhibit changes in either basal or uncoupled OCR (data not shown).

The viability of the toxicant-treated RPTC were assessed via plasma membrane integrity as measured from automated imaging of cells stained with propidium iodide to measure loss of plasma membrane integrity. Staining with Hoechst 33342 was used to identify all cell nuclei and pink nuclei were counted as dead and divided by all nuclei. The proportion of live cells was ~95% for untreated and vehicle control cells, and the cells treated with the nephrotoxics, mitomycin C, and metformin also exhibited ~95% live cells. The integrity of the treated cells was also evident from the intact morphology of the monolayer as seen in the corresponding bright field images (Fig. 3). Thus, the XF-24 assay provides a sensitive measure of nephrotoxic damage of mitochondrial respiratory capacity that is evident before impairment of basal metabolism or cell death.

We recently demonstrated that several classes of compounds produce mitochondrial biogenesis in RPTC and that FCCP-uncoupled OCR is a marker of mitochondrial biogenesis [5,6,7]. To determine the ability of the XF-24 instrument to measure mitochondrial biogenesis, RPTC were cultured under optimized conditions, treated with agents known to produce mitochondrial biogenesis for 24 hr and basal and FCCP-uncoupled OCR determined. The AMP-kinase activator aminoimidazole carboxamide ribonucleotide (AICAR) has been reported to induce mitochondrial biogenesis [22] and increased FCCP OCR at 300 and 500 μ M and basal OCR increased at 500 μ M with a maximal increase of approximately 40% (Fig. 4). Metformin, a biguanide anti-diabetic drug, has also been reported to induce mitochondrial biogenesis [22] and it also increased basal and uncoupled rates to a similar extent as AICAR. The piperazine-thiazole SIRT1720 was originally reported as a SIRT-1 activator and has been shown to induce mitochondrial biogenesis by Milne, *et al.* [23] and by our laboratory [7]. Treatment with SIRT1720 also revealed concentration-dependent increases in basal and uncoupled rates with maximums of ~50%. The 5-hydroxytryptamine (5-HT) receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) has been extensively studied in the primary RPTC where it has been shown to induce mitochondrial biogenesis via activation of PGC-1 α [24]. In the respirometric assay, treatment with 10 and 20 μ M DOI produced up to 40% increases in both basal and uncoupled OCR. Similarly, treatment of RPTC with resveratrol or the isoflavone daidzein (10 μ M), characterized previously as biogenesis agents in RPTC [6], also increased basal and uncoupled respiration rates. These data confirm that the XF measurement of RPTC respiration is a sensitive, functional assay of mitochondrial biogenesis.

Discussion

Scientists within the pharmaceutical industry are highly cognizant of the potential for adverse drug effects due to agents with mitochondrial liabilities. Indeed, about half of the drugs with FDA Black Box Warnings for cardiotoxicity or hepatotoxicity (*circa* 2007), 15 of the drugs have documented mitochondrial liabilities [25]. Unfortunately, nephrotoxicity has not received the same level of attention despite the frequency of loss in renal function due to adverse drug effects and xenobiotic exposure. In light of the fact that many known nephrotoxics target the mitochondria, the development of an HTS method to measure the loss of respiratory capacity in RPTC would greatly enhance efforts to assess nephrotoxic liability of new chemicals, environmental agents, and consumer products. The results presented here

demonstrate that respirometric measurements of primary rabbit RPTC that involve a “stress test” such as uncoupling with FCCP provides a sensitive measure of mitochondrial damage.

A report from the National Research Council advocates the use of *in vitro* toxicological screening, along with advanced bioinformatics modeling, for potential initial risk assessment of drugs, environmental agents, and household chemicals [26,27,28]. We show here that known toxicants produced concentration-dependent changes in respiratory capacity that could be measured *prior* to onset of any measurable cell death. The mitochondrial damage is a phenotypic stress response and is likely more physiological than a single pathway readout given that these cells retain function that is metabolically coupled. Indeed, the observation that measurable damage is detected prior to death suggests that the RPTC model could effectively capture the potential for chronic toxicity.

At this time there is not a direct HTS assay for mitochondrial biogenesis. For example, HTS assays have used changes in mitochondrial gene expression as markers of mitochondrial biogenesis. This approach suffers from the lack of correlation of mRNA levels and mitochondrial biogenesis and leads to false negatives and false positives. Furthermore, there is not a single commonly accepted assay for mitochondrial biogenesis. Yet interest in mitochondrial biogenesis in general and specifically in the treatment of disease has increased dramatically. For example, resveratrol and SIRT1720 produce mitochondrial biogenesis and they are being studied for the treatment of type 2 diabetes and aging [23]. In addition, we have proposed that recovery of organ and cellular injury following an insult may be limited by the remaining mitochondrial function and ATP levels and that the stimulation mitochondrial biogenesis may promote recovery of organ and cellular function in the short and long term.

In the primary cultures of RPTC we used multiple mitochondrial endpoints such as basal and uncoupled respiration, ATP levels, and mitochondrial protein levels to demonstrate that daidzein, SIRT1720, and the 5-HT agonist DOI produce mitochondrial biogenesis and that stimulation of mitochondrial biogenesis following oxidant injury accelerates the recovery of cellular functions. Using these validated compounds, we demonstrate that these compounds, and other compounds known to produce mitochondrial biogenesis (AICAR, metformin), increase FCCP-uncoupled respiration confirming that it is a marker of mitochondrial biogenesis. This approach represents the first HTS assay to measure phenotypic mitochondrial biogenesis and is an improved approach to measure functional mitochondrial biogenesis.

Acknowledgments

This study was supported by National Institutes of Health/National Institute of General Medical Sciences Grant [GM 084147]. Dr. Nathan Perron provided the IN-Cell images.

References

1. Dekant W. Biotransformation and renal processing of nephrotoxic agents. Arch. Toxicol. Suppl 1996;18:163–72. [PubMed: 8678792]
2. Pfaller W, Gstraunthaler G. Nephrotoxicity testing in vitro - what we know and what we need to know. Environ. Health. Perspect 1998;106(Suppl 2):559–69. [PubMed: 9599703]
3. Hall AM, Unwin RJ. The not so ‘mighty chondrion’: emergence of renal diseases due to mitochondrial dysfunction. Nephron. Physiol 2007;105:1–10.
4. Rasbach KA, Schnellmann RG. Signaling of mitochondrial biogenesis following oxidant injury. J. Biol. Chem 2007;282:2355–62. [PubMed: 17116659]
5. Rasbach KA, Schnellmann RG. PGC-1 α over-expression promotes recovery from mitochondrial dysfunction and cell injury. Biochem. Biophys. Res. Commun 2007;13:734–9. [PubMed: 17307137]
6. Rasbach KA, Schnellmann RG. Isoflavones promote mitochondrial biogenesis. J. Pharmacol. Exp. Ther 2008;325:536–43. [PubMed: 18267976]

7. Funk JA, Odejinmi S, Schnellmann RG. SRT1720 Induces Mitochondrial Biogenesis and Rescues Mitochondrial Function After Oxidant Injury in Renal Proximal Tubule Cells. *J. Pharmacol. Exp. Ther.* Jan 26;2010 [Epub ahead of print] PMID: 20103585.
8. Houck KA, Kavlock RJ. Understanding mechanisms of toxicity: insights from drug discovery research. *Toxicol. Appl. Pharmacol* 2008;227:163–78. [PubMed: 18063003]
9. Nowak G, Schnellmann RG. Improved culture conditions stimulate gluconeogenesis in primary cultures of renal proximal tubule cells. *Am. J. Physiol* 1995;268:C1053–61. [PubMed: 7733227]
10. Nowak G, Schnellmann RG. L-ascorbic acid regulates growth and metabolism of renal cells: improvements in cell culture. *Am. J. Physiol* 1996;271:C2072–80. [PubMed: 8997210]
11. Ferrick DA, Neilson A, Beeson C. Advances in measuring cellular bioenergetics using extracellular flux. *Drug. Discov. Today* 2008;13:268–74. [PubMed: 18342804]
12. Sridharan V, Guichard J, Li C-Y, Muise-Helmrichs R, Beeson C, Wright GL. The oxygen-sensing signal cascade: clamping of O₂ respiration, reduced ATP utilization and inducible fumarate respiration. *Am. J. Phys* 2008;295:C29–37.
13. Gerencser AA, Neilson A, Choi SW, Edman U, Yadava N, Oh RJ, Ferrick DA, Nicholls DG, Brand MD. Quantitative Microplate-Based Respirometry with Correction for Oxygen Diffusion. *Anal. Chem* 2009;81:6868–6878. [PubMed: 19555051]
14. Affourtit C, Brand MD. Measuring mitochondrial bioenergetics in INS-1E insulinoma cells. *Methods Enzymol* 2009;457:405–24. [PubMed: 19426881]
15. Hunter GR, Bamman MM, Larson-Meyer DE, Joannisse DR, McCarthy JP, Blaudeau TE, Newcomer BR. Inverse relationship between exercise economy and oxidative capacity in muscle. *Eur. J. Appl. Physiol* 2005;94:558–68. [PubMed: 15959800]
16. Soltoff SP, Mandel LJ. Active ion transport in the renal proximal tubule. I. Transport and metabolic studies. *J. Gen. Physiol* 1984;84:601–22. [PubMed: 6502133]
17. Gnaiger E. Oxygen conformance of cellular respiration - a perspective of mitochondrial physiology. *Adv. Exp. Med. Biol* 2003;543:39–55. [PubMed: 14713113]
18. Kramer JA, Pettit SD, Amin RP, Bertram TA, Car B, Cunningham M, Curtiss SW, Davis JW, Kind C, Lawton M, Naciff JM, Oreffo V, Roman RJ, Sistare FD, Stevens J, Thompson K, Vickers AE, Wild S, Afshari CA. Overview on the application of transcription profiling using selected nephrotoxics for toxicology assessment. *Environ. Health Perspect* 2004;112:460–4. [PubMed: 15033596]
19. Thukral SK, Nordone PJ, Hu R, Sullivan L, Galambos E, Fitzpatrick VD, Healy L, Bass MB, Cosenza ME, Afshari CA. Prediction of nephrotoxicant action and identification of candidate toxicity-related biomarkers. *Toxicol. Pathol* 2005;33:343–55. [PubMed: 15805072]
20. Wang EJ, Snyder SD, Fielden MR, Smith RJ, Gu YZ. Validation of putative genomic biomarkers of nephrotoxicity in rats. *Toxicology* 2008;246:91–100. [PubMed: 18289764]
21. Schnellmann, RG. Measurement of oxygen consumption. In: Tyson, CA.; Frazier, JM., editors. *Methods of Toxicology: In Vitro Toxicity Indicators*. Vol. 1B. Academic Press; San Diego: 1994. p. 128-139.
22. Kukidome D, Nishikawa T, Sonoda K, Imoto K, Fujisawa K, Yano M, Motoshima H, Taguchi T, Matsumura T, Araki E. Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes* 2006;55:120–7. [PubMed: 16380484]
23. Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ, Jin L, Boss O, Perni RB, Vu CB, Bemis JE, Xie R, Disch JS, Ng PY, Nunes JJ, Lynch AV, Yang H, Galonek H, Israelian K, Choy W, Iffland A, Lavu S, Medvedik O, Sinclair DA, Olefsky JM, Jirousek MR, Elliott PJ, Westphal CH. Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* 2007;29:712–6. [PubMed: 18046409]
24. Rasbach KA, Funk JA, Jayavelu T, Green PT, Schnellmann RG. 5-hydroxytryptamine receptor stimulation of mitochondrial biogenesis. *J. Pharmacol. Exp. Ther* 2010;332:632–9. [PubMed: 19875674]
25. Dykens JA, Will Y. The significance of mitochondrial toxicity testing in drug development. *Drug Discov. Today* 2007;12:777–85. [PubMed: 17826691]

26. National Research Council (NRC). Committee on Toxicity Testing and Assessment of Environmental Agents, Toxicity testing in the 21st century: a vision and a strategy. The National Academies Press; Washington, D.C.: 2007.
27. Inglese J, Auld DS, Jadhav A, Johnson RL, Simeonov A, Yasgar A, Zheng W, Austin CP. Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc. Natl. Acad. Sci. USA* 2006;103:11473–8. [PubMed: 16864780]
28. Xia M, Huang R, Witt KL, Southall N, Foster J, Cho MH, Jadhav A, Smith CS, Inglese J, Portier CJ, Tice RR, Austin CP. Compound cytotoxicity profiling using quantitative high-throughput screening, *Environ. Health Perspect* 2008;116:284–91.

Fig. 1. Assessment of RPTC mitochondrial metabolism using the Seahorse Biosciences XF instrument

Primary rabbit RPTC were plated and differentiated using either the traditional glucose containing media with no shaking (standard) or with optimized media with lactate and shaking and their basal OCR rates and OCR rates in the presence of oligomycin (5 μ M) followed by FCCP 1 μ M) (panel A), or with ouabain (0.1 mM) (panel B). Data are means $\pm$ standard deviations for 4-5 wells measured in a single experiment.

Fig. 2. Assessment of toxicity in RPTC using the Seahorse Biosciences XF instrument
Shown are basal and uncoupled (1 μM FCCP) OCR measured for RPTC treated for 24 h with cisplatin, gentamicin, and HgCl₂. The basal rates for treated wells, and the uncoupled rates for all wells, were normalized to the basal rates of vehicle control wells (Control). Bars are the mean ± SEM for n = 6 and the asterisk represent statistical difference from control basal or FCCP uncoupled at p<0.05.

Fig. 3. Viability of RPTC 24 h post-treatment

Cells treated with toxicants for 24 h were analyzed on the XF instrument, washed with PBS, and then stained with ethidium bromide and acridine orange and visualized via epifluorescence microscopy. Shown are the representative images for RPTC treated with (A) vehicle control, (B) 10 μ M gentamicin, (C) 10 μ M cisplatin, (D) 3 μ M HgCl₂.

Fig. 4. Respirometric measurement of mitochondrial biogenesis

Shown are the basal and uncoupled (1 μ M FCCP) OCR for primary rabbit RPTC treated with agents known to induce mitochondrial biogenesis. The OCR rates in nmol/min mg protein for treated cells were normalized to the rates of the basal, untreated wells for the given experiment. Bars are the mean $\pm$ SEM for n = 6 and the asterisk represents statistically significant difference from control basal or FCCP uncoupled at p < 0.05.