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Assessment of drug-induced mitochondrial dysfunction via altered cellular respiration and acidification measured in a 96-well platform

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Abstract High-throughput applicable screens for identifying drug-induced mitochondrial impairment are necessary in the pharmaceutical industry. Hence, we evaluated the XF96 Extracellular Flux Analyzer, a 96-well platform that measures changes in the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of cells. The sensitivity of the platform was bench-marked with known modulators of oxidative phosphorylation and glycolysis. Sixteen therapeutic agents were screened in HepG2 cells for mitochondrial effects. Four of these compounds, thiazolidinediones, were also tested in primary feline cardiomyocytes for cell-type specific effects. We show that the XF96 platform is a robust, sensitive system for analyzing drug-induced mitochondrial impairment in whole cells. We identified changes in cellular respiration and acidification upon addition of therapeutic agents reported to have a mitochondrial effect. Furthermore, we show that respiration and acidification changes upon addition of the thiazoldinediones were cell-type specific, with the rank order of mitochondrial impairment in whole cells being in accord with the known adverse effects of these drugs.

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Abbreviations

2-DG	2-Deoxy-D-glucose
ECAR	extracellular acidification rate
FCCP	Carbonylcyanide-p-trifluoromethoxy-phenylhydrazone
OCR	oxygen consumption rate
OXPHOS	oxidative phosphorylation
R&D	research and development
SD	Standard Deviation
SEM	Standard Error of Mean

Introduction

Billions of dollars are incurred by the pharmaceutical industry as a result of late-stage compound attrition, with toxicity being a major reason for attrition (Kola and Landis 2004; Kramer et al. 2007). In an effort to reduce costs, there has been a gradual shift in the toxicity testing paradigm such that safety assessments are now encouraged early in the drug discovery process, well before a lead candidate has been identified. This has given rise to the need for a wide array of in vitro assays that can help predict toxicity. Recent evidence shows that drug-induced mitochondrial impairment is a contributing factor to toxicity (Wallace 2008; Dykens et al. 2007). For example, cerivastatin, troglitazone and nefazodone, drugs that were removed from the market because of safety concerns, and tolcapone, a drug that has been given a black box warning, have been shown to cause mitochondrial impairment (Dykens and Will 2007). As a result, in vitro

assays that predict mitochondrial impairment are essential in discovery toxicology.

Mitochondria are essential for making almost 95 % of the cell's energy supply in the form of ATP (adenosine triphosphate) by a process called oxidative phosphorylation. Oxidative phosphorylation is carried out by five mitochondrial protein complexes, Complexes I, II, III, IV, and V (Wojtczak and Zablocki 2008). Energy released during oxidation of substrates within mitochondria is used to reduce the electron carriers, nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD). NADH and $FADH_2$ donate electrons to Complex I (NADH-ubiquinone oxidoreductase) and Complex II (succinate-ubiquinone oxidoreductase), respectively, and electrons from these proteins are then transferred to Complex III (ubiquinol-cytochrome *c* oxidoreductase) via a mobile electron shuttler, ubiquinone. Subsequently, electrons are transferred from Complex III to Complex IV (cytochrome *c* oxidase), via cytochrome *c*. Complex IV donates electrons to oxygen, and with the addition of protons, water is formed. The energy that is released during electron transfer along the four protein complexes is used to build a gradient of protons across the inner mitochondrial membrane, the energy-rich gradient being used by Complex V (the ATP synthase) to make ATP from ADP and phosphate.

Since oxidative phosphorylation depends on the reduction of oxygen to water by Complex IV, one of the methods by which drug-induced mitochondrial impairment can be assessed is by measuring oxygen consumption of mitochondria. Oxygen consumption measurements have traditionally been done with a polarographic electrode known as the Clark electrode (Clark 1960) but the disadvantage of the Clark electrode is that throughput is limited to approximately 20 measurements per day. In recent years, the development of a conjugated platinum-porphyrin probe that is a soluble oxygen sensor has facilitated high-throughput screening of compounds in both a 96-well format and a 384-well format (Hynes et al. 2006). This oxygen sensor identifies acute inhibitors and uncouplers of oxidative phosphorylation and can be used to rank order compounds within a series for their mitochondrial toxicity effects (Hynes et al. 2006). This sensor has been used to assess the effects of many drugs including thiazolidinediones, statins (Nadanaciva et al. 2007), biguanides (Dykens et al. 2008a) and antidepressants (Dykens et al. 2008b) on mitochondria isolated from rat liver. However, one of the limitations of studying oxygen consumption of isolated mitochondria is that there may be either an over-estimation or underestimation of mitochondrial toxicity since isolated organelles lack the complexities of whole cells. For instance, in a recent phosphoproteomic analysis of mitochondria isolated from human skeletal muscle, it was discovered that many of the subunits of the oxidative phosphorylation (OXPHOS) complexes are phosphoproteins (Zhao et al. 2011). Bioinformatic analysis of the phosphorylaton sites suggested that the OXPHOS complexes and other mitochondrial phosphoproteins could be the substrates of protein kinase A, protein kinase C, casein kinase II and DNA-dependent protein kinase (Zhao et al. 2011). Thus, if a compound has an off-target effect on a kinase which regulates the activity of the OXPHOS complexes, this effect may not be identified in isolated mitochondria if the affected kinase resides outside mitochondria.

In light of this, we assessed a 96-well platform, the XF96 Extracellular Flux Analyzer (Seahorse Bioscience), to test the effects of a selection of compounds on both the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of whole cells. The OCR of cells is primarily a measure of mitochondrial respiration and the ECAR is primarily a measure of lactic acid release and thus a measure of glycolysis. The 96-well platform utilizes a disposable sensor cartridge that has 96 pairs of fluorescent sensors which measure the oxygen concentration and proton concentration of the medium surrounding the cells seeded in a 96-well plate. The fluorescence sensors are coupled to a fiber-optic wave guide that delivers light which excites the oxygen sensors and pH sensors at 530 nm and 470 nm, respectively. The resulting fluorescence signal from the oxygen and pH sensors is emitted at 650 nm and 530 nm, respectively, and is detected by photodetectors within the XF96 instrument. We tested the OCR and ECAR of a liver-derived carcinoma cell line, HepG2, and show that the measurements had ≤15 % intra- and inter-assay variation. The sensitivity of the platform was assessed with compounds that are modulators of either oxidative phosphorylation or glycolysis. The utility of the platform for screening drug-induced mitochondrial impairment in HepG2 cells was then assessed with 16 therapeutic agents, including four thiazolidinediones, troglitazone, ciglitazone, rosiglitazone and pioglitazone. Finally, we tested the effects of the four thiazolidinediones on the OCR and ECAR of primary cardiomyocytes to determine if there were cell-type specific differences with respect to mitochondrial impairment.

Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Axxora LLC (San Diego, CA) or Toronto Research Chemicals (Toronto, Canada) and were of the highest purity available. Cell culture media and supplements for HepG2 cells were purchased from Invitrogen (Carlsbad, CA). Low-buffered RPMI medium was purchased from Molecular Devices (Sunnyvale, CA). M199 medium was purchased from Invitrogen. BD Matrigel™ was purchased from BD Biosciences (Franklin Lakes, NJ). Dulbecco's modified Eagle's medium (DMEM) powder (catalog# D5030) for preparing low buffered medium to measure the OCR and ECAR of cardiomyocytes was purchased from Sigma-Aldrich. XF96 sensor cartridges, XF96-

well plates, calibration buffer and calibration plates were obtained from Seahorse Bioscience (Billerica, MA).

Cell culture conditions for HepG2 cells

HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen 11885–084) containing 5.5 mM glucose and 1 mM sodium pyruvate and supplemented with 10 % fetal bovine serum, 5 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and 100 units/mL penicillin-streptomycin in a 37 °C, 5 % CO_2 humidified atmosphere.

Cardiomyocyte isolation

Adult feline cardiomyocytes (AFCs) were kindly provided by Dr. Don Menick (MUSC, Charleston, SC) and isolated as reported previously (Kato et al. 1995). In brief, hearts were removed from adult cats of either sex (1.5–3.5 kg) under anesthesia with ketamine hydrochloride (25 mg/kg), meprer-idine (2.2 mg/kg), and acepromazine maleate (5 mg/kg, i.m.) in sterile conditions. The aorta was cannulated, and the coronary arteries were perfused with a Krebs solution containing 500 mol/L Ca^{2+} and then with Ca^{2+}-free Krebs buffer. All buffers and enzyme solutions were maintained at 37 °C and at pH 7.4 by equilibration with 95 % O_2–5 % CO_2. The hearts were perfused with 0.72 U/mL collagenase B until the heart was flaccid. The atria were removed, and the ventricle and septum tissue was minced in the collagenase buffer for 5 min at 37 °C in a shaking bath. The homogenate was sieved through a 210 m nylon mesh to isolate the cardiomyocytes and centrifuged at 100× *g* for 2 min. The cells were resuspended in Ca^{2+}-free Krebs buffer and allowed to settle. The pellet was resuspended in Krebs buffer with 250 mol/L Ca^{2+} and 1 % bovine serum albumin. The Ca^{2+}-tolerant cardiomyocytes were maintained in serum-free M199 medium with Earle's balance salts, 100 U/mL penicillin, 100 g/mL streptomycin, 100 mol/L ascorbic acid, 0.2 % (w/v) bovine serum albumin, 5 mmol/L creatine, 5 mmol/L taurine, 2 mmol/L carnitine, 10 mol/L cytosine arabinoside, and 100 nmol/L insulin.

Measurement of the oxygen consumption rate and extracellular acidification rate of HepG2 cells

Oxygen consumption rates and the extracellular acidification rates of HepG2 cells were measured in real-time, simultaneously, in an XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA). The instrument measures the extracellular flux changes of oxygen and protons in the medium surrounding the cells seeded in XF96-well plates.

To test the effect of compounds on the OCR and ECAR of HepG2 cells, cells were seeded in XF96-well plates at 24,000 cells/80 μL culture medium/well and incubated in a 37 °C, 5 % CO_2 humidified atmosphere for 24 h. In addition, an XF96 sensor cartridge for each cell plate was placed in a 96-well calibration plate containing 200 μL/well calibration buffer and left to hydrate overnight at 37 °C. Each sensor cartridge contained 2 reagent delivery ports (ports A and B) per well for injecting test compounds.

The following day, the culture medium from the cell plates was aspirated and the cells rinsed three times in pre-warmed serum-free low-buffered RPMI medium. The cells were then maintained in 150 μL/well of serum-free low-buffered RPMI medium at 37 °C for 30 min to allow the temperature and pH of the medium to reach equilibrium before the first rate measurement.

All test compounds with the exception of 2-Deoxy-D-glucose, salicylic acid and acetylsalicylic acid were prepared as 700× stock solutions in DMSO, diluted to 7× in serum-free low-buffered RPMI medium and the pH adjusted to 7.4 with 1 M NaOH. 2-Deoxy-D-glucose, salicylic acid and acetylsalicylic acid were prepared as 7× stock solutions in serum-free low-buffered RPMI medium and their pH adjusted to 7.4 with 1 M NaOH. Compound plates were prepared by diluting the 7× test compounds either 2 fold (for rotenone, antimycin, oligomycin, FCCP, 2-Deoxy-D-glucose) or 3 fold (for the 16 therapeutic agents) over multiple concentrations in serum-free low-buffered RPMI medium supplemented with 1 % (v/v) DMSO. 25 μL of compound or vehicle (1 % (v/v) DMSO) in serum free low-buffered RPMI medium was then pre-loaded into reagent delivery port A of each well in the XF96 sensor cartridge. Since each well in the cell plate contained 150 μl of medium, the final concentration of DMSO in each well after reagent injection was 0.14 % (v/v).

The sensor cartridge and the calibration plate were loaded into the XF96 Extracellular Flux Analyzer so that the cartridge could be calibrated. When the calibration was complete, the calibration plate was replaced with the cell plate. Four baseline rate measurements of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of the HepG2 cells were made using a 2 min mix, 5 min measure cycle. The compounds were then injected pneumatically by the XF96 Analyzer into each well, mixed, and six OCR and ECAR measurements were made using the 2 min mix, 5 min measure cycle.

Measurement of the oxygen consumption rate and extracellular acidification rate of cardiomyocytes

Each well of the XF96-well plates was first coated with 5 μL of BD Matrigel™ (BD Biosciences) at a 1:50 dilution in M199 medium and incubated at 37 °C for 2 h. Adult feline cardiomyocytes were seeded onto XF96-well plates at 7,500 cells in 100 μL M199 medium per well. The cells were then

incubated in a 5 % CO_2 incubator at 37 °C for 48 h prior to experimentation. An additional 74 μL of medium was added to each well 24 h after plating.

For measurements of OCR and ECAR, the M199 medium was replaced with a low buffered medium pre-warmed to 37 °C. The low buffered medium contained DMEM (8.3 g/L), 2 mM GlutaMax, 1 mM sodium pyruvate, 25 mM glucose, 31.58 mM NaCl, and 15 mg of Phenol Red, and its pH was adjusted to 7.4 with NaOH. The test compounds were prepared as 700× stocks in DMSO and diluted to 7× in the low buffered medium. Compound plates were prepared by diluting the 7× test compound 2 fold in the low buffered medium over multiple concentrations. 25 μL of compound or vehicle control was then pre-loaded into reagent delivery port A of each well in the XF96 sensor cartridge. Five baseline OCR measurements were made using a 2 min mix, 2 min measure cycle. Compounds were then injected pneumatically into the wells, mixed, and six OCR and ECAR measurements were made using the same 2 min mix, 2 min measure cycle.

Data analysis

The values of OCR and ECAR reflect the metabolic activities of the cells as well as the number of cells being measured. The number of cells present within a well does not affect the analysis when the percentage change in OCR and ECAR is determined after compound addition since the same cell population is assayed pre- and post-compound addition.

In the HepG2 cell experiments, the oxygen consumption rate and extracellular acidification rate immediately prior to compound injection were used as the basal OCR and ECAR, respectively, and were defined as 100 % when generating concentration response curves. All OCR measurements *prior* to compound injection were similar with the exception of the first OCR measurement which was usually lower in value; this was also the case with the ECAR measurements. The second oxygen consumption rate measurement and the first extracellular acidification rate measurement obtained *after* compound injection were used to determine the percentage change in OCR and ECAR caused by the compound. All post-compound OCR measurements were similar with the exception of the first post-compound OCR measurement which was slightly less than that of the others. The first ECAR measurement obtained after compound injection was used for determining the percentage change in ECAR caused by the compound since there was a slight decline in the acidification over time.

In the cardiomyocyte experiments, the average of the two oxygen consumption rates prior to compound injection was used as the basal OCR; the average of the last two oxygen consumption rates after compound injection was used to determine the percentage change in OCR caused by the compound. The average of the two extracellular acidification rates prior to compound injection was used as the basal ECAR; the average of the last two ECAR measurements obtained after compound injection was used for determining the percentage change in ECAR.

Concentration response curves were generated in GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA).

Results

Determination of optimal cell seeding density for measuring the OCR and ECAR of HepG2 cells

We first determined the optimal cell seeding density of HepG2 cells for measuring their oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in the XF96 Analyzer. Cells were seeded at a range of densities from 12,000–32,000 cells/well in an XF96-well plate and left to attach overnight as described in Materials and Methods. The OCR and ECAR increased linearly with increasing cell number (Fig. 1a and b, respectively). We chose to use a seeding density of 24,000 cells/well for subsequent experiments so that optimal detection of changes in OCR and ECAR could be made. HepG2 cells grown as described in the Materials and Methods and seeded at 24,000 cells/well had a basal OCR of 196.8± 14.5 pmol/min ($8.2\times10^{-3}\pm6\times10^{-4}$ pmol/min/cell) (Fig. 1a) and a basal ECAR of 62.9±4.2 mpH units/min ($2.6\times10^{-3}\pm 1.8\times10^{-4}$ mpH units/min/cell) (Fig. 1b).

Intra- and inter-assay variation of the OCR and ECAR of HepG2 cells

We next determined the intra- and inter-assay variation of the OCR and ECAR of HepG2 cells. The intra-assay (within-plate) variation of the OCR was 7–15 % and the inter-assay (day-to-day) variation was 7–13 % on 5 separate days. The intra-assay variation of the ECAR was 9–15 % and the inter-assay variation was 8–14 %. The intra- and inter- assay variations for both OCR and ECAR were acceptable values for cell-based experiments.

Effect of compounds known to modulate oxidative phosphorylation or glycolysis

We next tested the acute effects of five compounds on the OCR and ECAR of HepG2 cells. These compounds are known modulators of either oxidative phosphorylation or glycolysis.

Rotenone, an inhibitor of Complex I (NADH-ubiquinone oxidoreductase) (Horgan et al. 1968), decreased the OCR of HepG2 cells by 50 % at 263±43 nM (Fig. 2a) and increased the ECAR by 50 % at 371±58 nM (Fig. 2b). At the highest

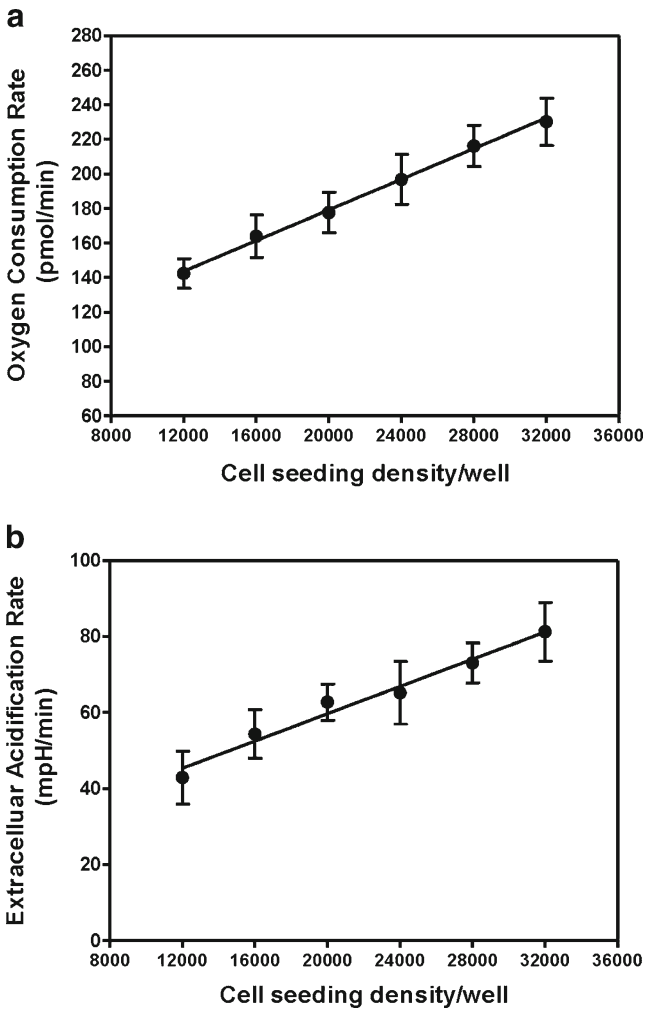


Fig. 1 Basal oxygen consumption rates (**a**) and basal extracellular acidification rates (**b**) of HepG2 cells seeded at densities between 12,000–32,000 cells/well. Each data point represents the mean ± SD, $n=12$ wells per seeding density

concentration of rotenone tested, 1 μM, the OCR decreased by 76±2.4 % and the ECAR increased by 59±1.2 % (Fig. 2a and b, respectively).

Antimycin, an inhibitor of Complex III (ubiquinol-cytochrome *c* oxidoreductase) (Schagger et al. 1995) decreased the OCR of HepG2 cells by 50 % at 4±0.2 nM (Fig. 2c) and increased the ECAR by 50 % at 100±20 nM (Fig. 2d). The maximum decrease in OCR with antimycin at the highest concentration tested was 86±1 % and the maximum increase in ECAR was 51±2 % (Fig. 2c and d, respectively).

Oligomycin, an inhibitor of the ATP synthase (Walker et al. 1995), decreased the OCR of HepG2 cells by 50 % at 0.92±0.18 μM and increased the ECAR by 50 % at 1.1±0.23 μM (data not shown). At the highest concentration of oligomycin tested, 3 μM, the OCR decreased by 65±2 % and the ECAR increased by 60±5 %.

Carbonylcyanide-p-trifluoromethoxy-phenylhydrazone (FCCP), an uncoupler of oxidative phosphorylation (Cunarro and Weiner 1975), increased the OCR of HepG2 cells by 50 % at 54±7 nM (Fig. 2e) and increased the ECAR by 50 % at 141±20 nM (Fig. 2f). At the highest concentration of FCCP tested, 1 μM, the OCR and the ECAR increased by 300±7 % and 153±6 % respectively.

2-Deoxy-D-glucose (2-DG) inhibits hexokinase, the first enzyme in the glycolytic pathway, by competing with the substrate, glucose (Bachelard et al. 1971). With increasing concentrations of 2-DG, the oxygen consumption rate increased while the extracellular acidification rate declined. At the highest concentration of 2-DG tested, 150 mM, the OCR of HepG2 cells increased by 20±3 % (Fig. 2g) while the ECAR decreased by 54±2 % (Fig. 2h).

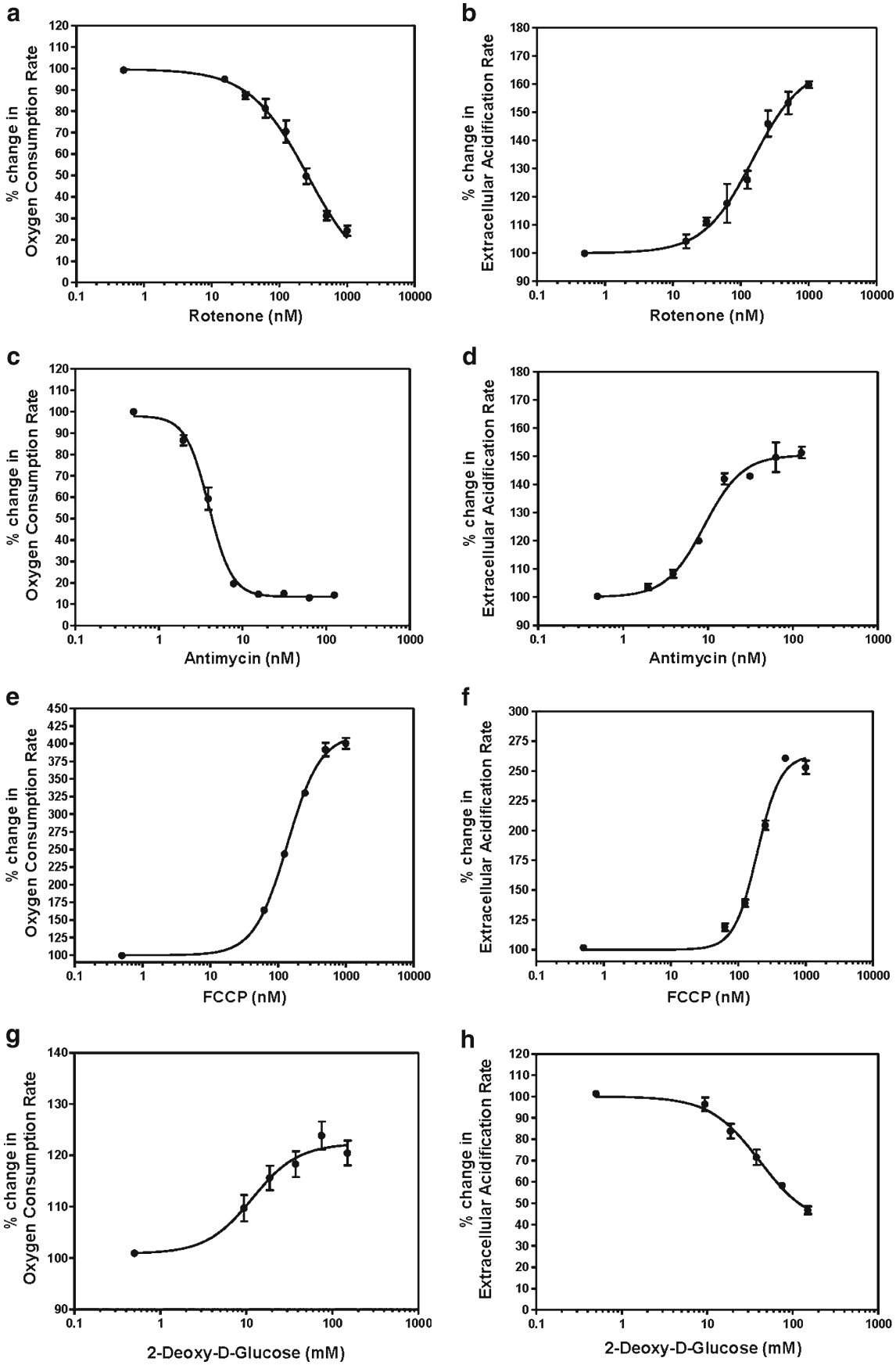
To summarize, rotenone, antimycin, and oligomycin which are known inhibitors of oxidative phosphorylation, inhibited the OCR of HepG2 cells, whereas the uncoupler, FCCP, increased the OCR; furthermore, the ECAR of HepG2 cells increased upon addition of these four compounds. In contrast, the glycolytic inhibitor, 2-DG, decreased the ECAR and increased the OCR of HepG2 cells.

Screening compounds for their acute effects on the OCR and ECAR of HepG2 cells

The main aim of our study was to detect drug-induced mitochondrial dysfunction in whole cells by measuring the effect of compounds on the OCR and ECAR of HepG2 cells. Hence, 16 compounds from a variety of therapeutic classes were tested for their acute effects as described in Materials and Methods. Table 1 lists the compounds tested, their therapeutic uses, their Cmax values if known, reported organ toxicities and known mitochondrial effects.

Tolcapone, a drug given in the treatment of Parkinson's disease, increased the OCR of HepG2 cells (Fig. 3a) in a concentration-response manner up to 33.3 μM. A 50 % increase in both OCR and ECAR was seen with 3.7 μM tolcapone (Fig. 3a and b; Table 2). The maximum increase in OCR and ECAR with tolcapone was 452±15.5 and 183±8 % respectively and occurred at 33.3 μM (Fig. 3a and b, respectively). At concentrations higher than 33.3 μM, both the OCR and ECAR started to decline. Entacapone, another drug given in the treatment of Parkinson's disease, increased the OCR and ECAR of HepG2 cells by 50 % at 33 μM and 100 μM, respectively (Fig. 3c and d, respectively; Table 2). The maximum increase in OCR and ECAR with entacapone was 591±72 % and 57±2.7 %, respectively, and occurred at 300 μM (Fig. 3c and d, respectively).

Nilutamide, an anti-androgen used in the treatment of prostate cancer, decreased the OCR of HepG2 cells by 50 % at 66 μM (Table 2) and increased the ECAR by 50 % at 79 μM. Nilutamide caused a maximal decrease in OCR of 63±2 % and a maximum increase in ECAR of 53.9±2.6 % at 100 μM (Fig. 3e and f, respectively). Flutamide, another anti-


a
% change in Oxygen Consumption Rate
Rotenone (nM)
b
% change in Extracellular Acidification Rate
Rotenone (nM)
c
% change in Oxygen Consumption Rate
Antimycin (nM)
d
% change in Extracellular Acidification Rate
Antimycin (nM)
e
% change in Oxygen Consumption Rate
FCCP (nM)
f
% change in Extracellular Acidification Rate
FCCP (nM)
g
% change in Oxygen Consumption Rate
2-Deoxy-D-Glucose (mM)
h
% change in Extracellular Acidification Rate
2-Deoxy-D-Glucose (mM)

◀ **Fig. 2** The percentage change in the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of HepG2 cells on addition of rotenone (**a** and **b**, respectively), antimycin (**c** and **d**, respectively), FCCP (**e** and **f**, respectively), and 2-Deoxy-D-glucose (**g** and **h**, respectively). Each data point represents the mean ± SEM, $n=5$ separate experiments

androgen, caused a 26±4 % decrease in OCR (Fig. 3g) and a 48±2 % increase in ECAR (Fig. 3h) at 100 μM, the highest concentration tested.

Chlorpromazine, an antipsychotic, increased the OCR and ECAR of HepG2 cells by 41±6 % and 35±4.5 %, respectively, at 300 μM (data not shown). No change in OCR or ECAR was seen with the antipsychotic, clozapine (data not shown), even at 300 μM, the highest concentration tested.

Two anesthetics, quinisocaine and lidocaine, had no effect on the OCR and ECAR of HepG2 cells (data not shown) even at 300 μM, the highest concentration tested.

Diflunisal, a nonsteroidal anti-inflammatory drug (NSAID), increased the OCR and ECAR of HepG2 cells by 50 % at 15 μM and 30 μM respectively (Table 2). The maximum increase in OCR and ECAR with diflunisal were 175±11 % and 119±9 %, respectively (Fig. 4a and b) and occurred at 100 μM. Two other NSAIDs, salicylic acid and acetylsalicylic acid caused no impairment on the OCR and ECAR (data not shown) even at 1 mM, the highest concentration tested. The NSAID, benoxaprofen, inhibited the OCR of HepG2 cells by 50 % at 30 μM (Table 2). The maximum decrease in OCR, 65 ±2 %, occurred at 100 μM (Fig. 4c) while the increase in ECAR at this concentration was 39±4 % (Fig. 4d).

Four thiazolidinediones, drugs used in the treatment of Type 2 diabetes, were tested for their effects on the OCR and ECAR of HepG2 cells. Troglitazone decreased the OCR of HepG2 cells by 30±1 % (Fig. 5a) and increased the ECAR by 36±4.5 % (Fig. 5b) at 100 μM. Ciglitazone decreased the OCR of HepG2 cells by 42±4.7 % (Fig. 5c) and increased the ECAR by 34.1±3.3 % (Fig. 5d) at 100 μM. In contrast, rosiglitazone and pioglitazone had no effect on either the OCR or ECAR of HepG2 cells (data not shown) even at the highest concentration (100 μM) tested.

Effects of thiazolidinediones on the ATP-coupled respiration rate and maximal uncoupled rate of HepG2 cells

We next tested the effect of the four thiazolidinediones on the ATP-coupled respiration rate of HepG2 cells. HepG2 cells

Table 1 Compounds tested in this study, their Cmax values, their therapeutic use, reported organ toxicities and reported mitochondrial impairment

Compound	Therapeutic Use	Cmax (μM)	Reported Organ Toxicities	Reported Mitochondrial Impairment	Reference
Tolcapone	For Parkinson's Disease	20.8	Idiosyncratic liver injury	Uncouples oxidative phosphorylation	(Haasio et al. 2002)
Entacapone	For Parkinson's Disease	4	–	Uncouples oxidative phosphorylation	(Haasio et al. 2002)
Nilutamide	For prostate cancer	18.9	Idiosyncratic liver injury	Inhibits Complex I activity	(Boelsterli et al. 2006; Berson et al. 1994)
Flutamide	For prostate cancer	6	Idiosyncratic liver injury	Inhibits Complex I activity	(Boelsterli et al. 2006; Fau et al. 1994)
Chlorpromazine	Antipsychotic	0.94	Tardive dyskinesia	Inhibits Complex I; Uncouples oxidative phosphorylation	(Maurer and Moller 1997)
Clozapine	Antipsychotic	0.95	Tardive dyskinesia	Inhibits Complex I activity	(Maurer and Moller 1997)
Quinisocaine	Anesthetic	–	–	Inhibits Complex IV activity	(Casanovas et al. 1983)
Lidocaine	Anesthetic	36	–	Inhibits Complex IV activity	(Casanovas et al. 1983)
Diflunisal	Nonsteroidal anti-inflammatory drug (NSAID)	495	–	Uncouples oxidative phosphorylation	(McDougall et al. 1983)
Salicylic acid	NSAID	–	–	–	–
Acetylsalicylic acid	NSAID	–	–	–	–
Benoxaprofen	NSAID	155	Idiosyncratic liver injury	–	(Duthie et al. 1982)
Troglitazone	Antidiabetic agent	6.4	Idiosyncratic liver injury	Inhibits oxidative phosphorylation; Induces the MPTP	(Masubuchi et al. 2006; Nadanaciva et al. 2007; Isley 2003)
Ciglitazone	Antidiabetic agent; discontinued during development	–	Cataractogenic potential in rats	Inhibits oxidative phosphorylation; Induces the MPTP	(Aleo et al. 2005; Nadanaciva et al. 2007; Masubuchi et al. 2006)
Rosiglitazone	Antidiabetic agent	1.04	Cardiac failure	–	(Nissen and Wolski 2007)
Pioglitazone	Antidiabetic agent	1	–	–	–

Table 2 The concentration of compound required to change the OCR and ECAR of HepG2 cells by 50 %

Compound	Concentration (μM) required to change the OCR of HepG2 cells by 50 %	Concentration (μM) required to change the ECAR of HepG2 cells by 50 %	Effect of compound on the OCR and ECAR of HepG2 cells
Tolcapone	3.7	3.7	Increased both OCR and ECAR at low concentrations and decreased them at higher concentrations
Entacapone	33	100	Increased both OCR and ECAR
Nilutamide	66	79	Decreased OCR and increased ECAR
Flutamide	> 100	> 100	Decreased OCR and increased ECAR
Chlorpromazine	> 300	> 300	Increased both OCR and ECAR
Clozapine			No effect between 0.4 and 300 μM
Quinisocaine			No effect between 0.4 and 300 μM
Lidocaine			No effect between 0.4 and 300 μM
Diflunisal	15	30	Increased both OCR and ECAR
Salicylic acid			No effect between 1.3 μM–1 mM
Acetylsalicylic acid			No effect between 1.3 μM–1 mM
Benoxaprofen	30	> 100	Decreased OCR and increased ECAR
Troglitazone	> 100	> 100	Decreased OCR and increased ECAR
Ciglitazone	> 100	> 100	Decreased OCR and increased ECAR
Rosiglitazone			No effect between 0.13 and 100 μM
Pioglitazone			No effect between 0.13 and 100 μM

were first incubated for 20 h with each of the thiazolidinediones or with the vehicle in serum-free DMEM containing 5.5 mM glucose and 1 mM sodium pyruvate and supplemented with 5 mM HEPES. The following day, the medium was replaced with pre-warmed serum-free low-buffered RPMI medium and the cells were then tested for their ATP-coupled respiration rate by injecting oligomycin, the ATP synthase inhibitor, at a final concentration of 3 μM into port A. The decrease in OCR seen upon addition of oligomycin represents the ATP-coupled respiration of cells (Hill et al. 2009). The percentage decrease in OCR upon injection of oligomycin to vehicle-treated HepG2 cells was 66±2 % (Fig. 6). The percentage decrease in OCR upon injection of oligomycin to cells incubated overnight with 33 μM troglitazone, 33 μM ciglitazone, 33 μM rosiglitazone, and 33 μM pioglitazone was 44±2 %, 22±1 %, 56±3 %, and 54±3 % respectively (Fig. 6). Hence, the thiazolidinediones impaired the ATP-coupled respiration rate of HepG2 cells with the rank order, ciglitazone > troglitazone > rosiglitazone, pioglitazone.

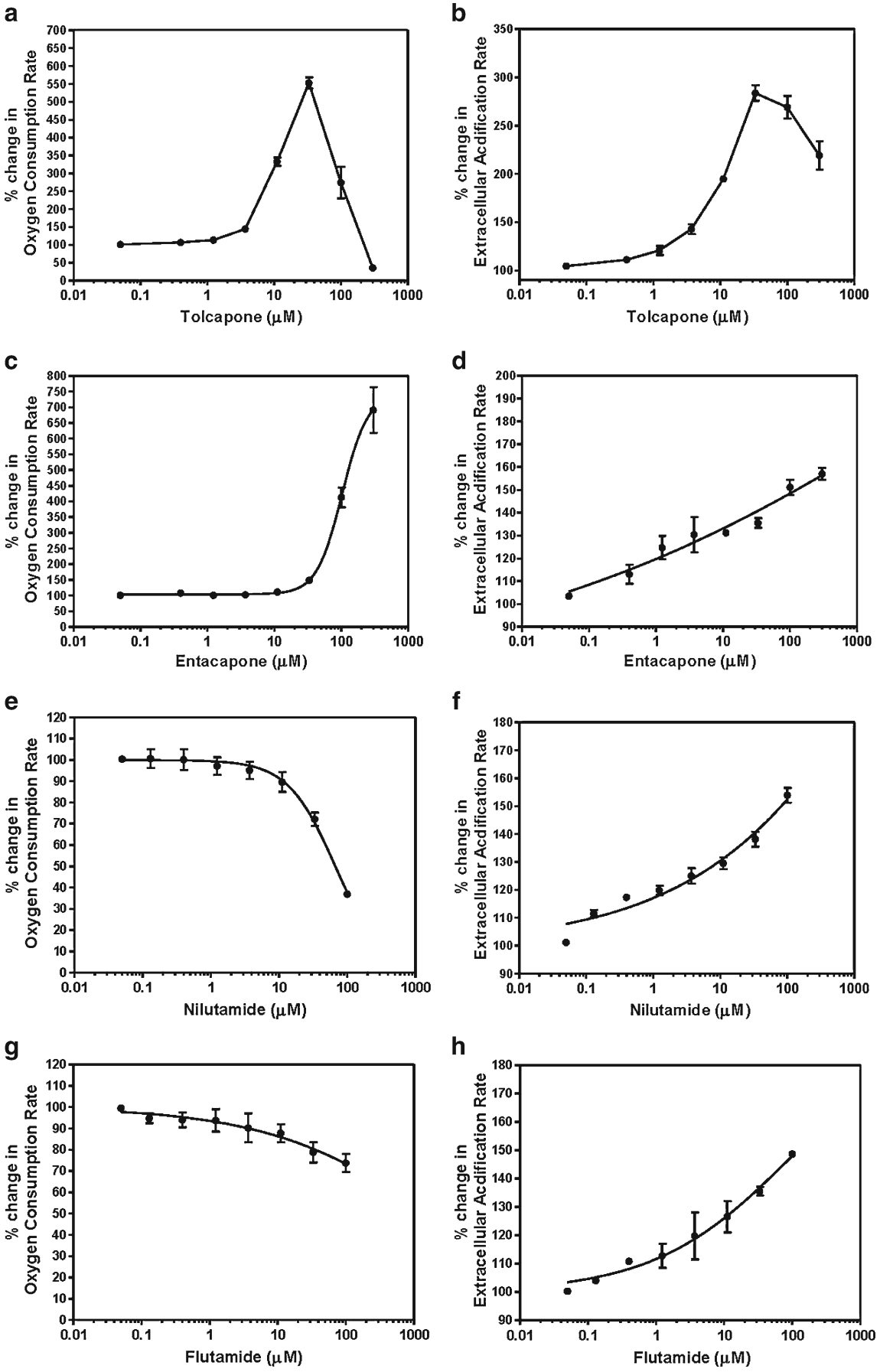
We also tested the effect of the four thiazolidinediones on the maximal uncoupled rate of HepG2 cells. The maximal uncoupled rate of cells is seen when oligomycin and FCCP are sequentially added to the cells (Hill et al. 2009). The increase in OCR that we observed with vehicle-treated cells upon sequential addition of oligomycin and FCCP (in ports A and B respectively) was 325±28 % (Fig. 6). The increase in OCR following sequential addition of oligomycin and FCCP to cells incubated overnight with 33 μM troglitazone, 33 μM ciglitazone, 33 μM rosiglitazone, and 33 μM pioglitazone was 211±4 %, 16±1 %, 298±17 % and 290±34 %, respectively (Fig. 6). Hence, the rank order of impairment on maximal uncoupling was ciglitazone > troglitazone > rosiglitazone, pioglitazone.

The acute effects of thiazolidinediones on the OCR and ECAR of cardiomyocytes

Since the effects of compounds on the OCR and ECAR of cells may be cell type-specific, we investigated the acute effects of the thiazolidinediones on freshly isolated cardiomyocytes. Adult feline cardiomyocytes were used for this purpose since they have been extensively characterized (Kato et al. 1995; Sridharan et al. 2008) and, moreover, can be isolated in greater abundance than rat/murine cardiomyocytes. Basal OCR of the feline cardiomyocytes ranged from 0.026 to 0.04 pmol/min/cell and the basal ECAR was $1.6–2.9\times10^{-3}$ mpH units/min/cell.

Troglitazone increased the OCR of cardiomyocytes by 50 % at 10 μM, with a maximum increase of 110±5 % occurring at 25 μM (Fig. 7a, Table 3). Thus, troglitazone had a more potent acute effect on the OCR of cardiomyocytes than it did on the OCR of HepG2 cells (Fig. 5a) and, moreover, caused stimulation of respiration rather than inhibition.

Fig. 3 The percentage change in OCR and ECAR of HepG2 cells on addition of tolcapone (**a** and **b**, respectively), entacapone (**c** and **d**, respectively), nilutamide (**e** and **f**, respectively), and flutamide (**g** and **h**, respectively). Each data point represents the mean ± SEM, $n=3$ separate experiments


a
% change in Oxygen Consumption Rate
Tolcapone (μM)
b
% change in Extracellular Acdification Rate
Tolcapone (μM)
c
% change in Oxygen Consumption Rate
Entacapone (μM)
d
% change in Extracellular Acdification Rate
Entacapone (μM)
e
% change in Oxygen Consumption Rate
Nilutamide (μM)
f
% change in Extracellular Acdification Rate
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% change in Extracellular Acdification Rate
Flutamide (μM)

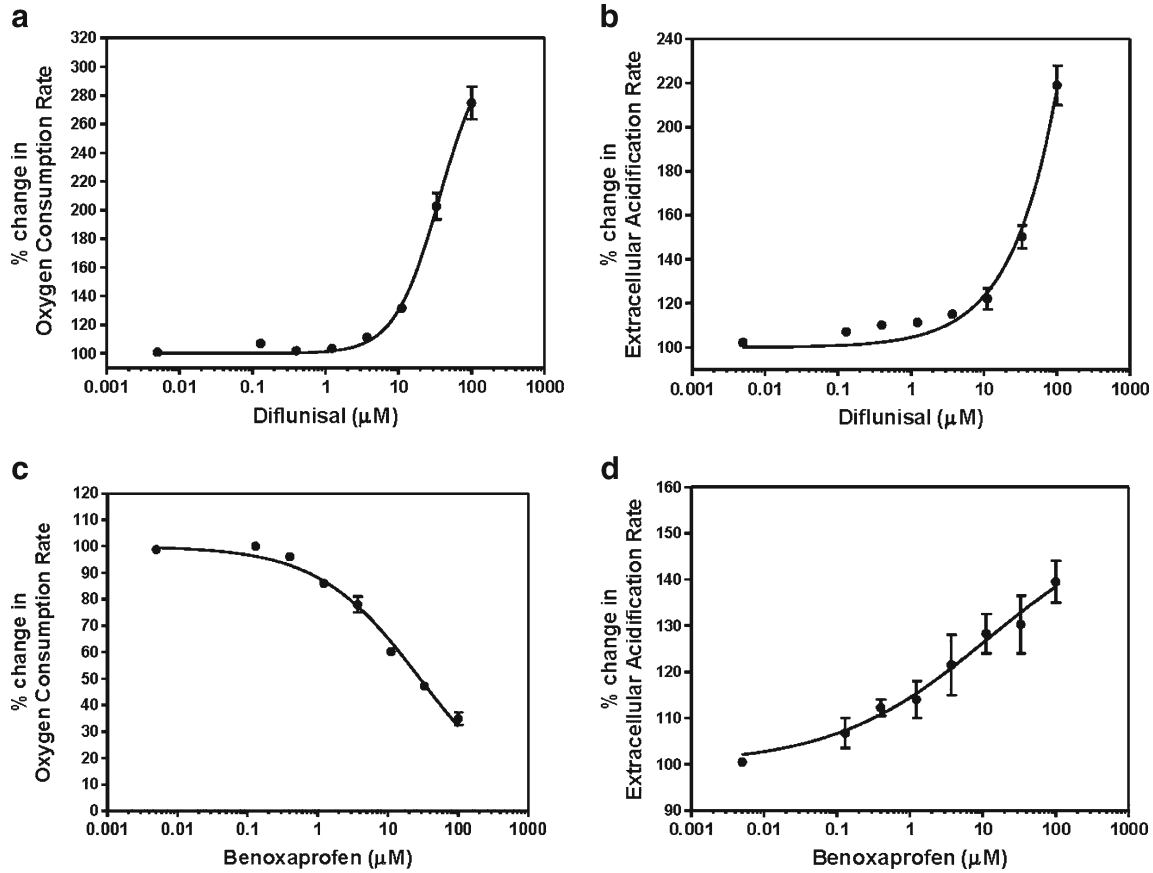

Fig. 4 The percentage change in OCR and ECAR of HepG2 cells on addition of diflunisal (**a** and **b**, respectively) and benoxaprofen (**c** and **d**, respectively). Each data point represents the mean ± SEM, n=3 separate experiments

The ECAR of the cardiomyocytes increased by 50 % with 10 μM troglitazone, with a maximum increase of 112±13 % occurring at 25 μM (Fig. 7b, Table 3).

Ciglitazone caused a concentration-dependent increase in the OCR of cardiomyocytes up to 25 μM, with the OCR being 40±4 % above basal respiration at this concentration (Fig. 7c). At concentrations higher than 25 μM ciglitazone, there was a decline in the OCR. Hence, ciglitazone had a stimulatory effect on the OCR of cardiomyocytes at low (≤25 μM) concentrations whereas it had a mild inhibitory effect on HepG2 cell respiration (Fig. 5c). In addition, ciglitazone caused an increase in the ECAR of cardiomyocytes, with a 50 % increase occurring at 10 μM and a maximum increase of 229± 24 % occurring at 100 μM (Fig. 7d, Table 3).

Rosiglitazone increased the OCR of cardiomyocytes by 21± 2 % at 100 μM (Fig. 7e, Table 3) and increased the ECAR by 44±7 % at 25 μM (Fig. 7f). Pioglitazone had no effect on either the OCR or ECAR of cardiomyocytes (data not shown).

Effects of thiazolidinediones on the ATP-coupled respiration rate and maximal uncoupled rate of cardiomyocytes

As in the case with HepG2 cells, we tested the effect of the four thiazolidinediones on the ATP-coupled respiration rate and maximum uncoupled rate of cardiomyocytes. The percentage decrease in OCR upon injection of 3 μM oligomycin to vehicle-treated cardiomyocytes was 64±4 % (Fig. 8). The largest percentage decrease in OCR upon injection of oligomycin to cardiomyocytes incubated overnight with 33 μM troglitazone, 33 μM ciglitazone, 33 μM rosiglitazone, and 33 μM pioglitazone was 62±4 %, 36±10 %, 57±7 %, and 59±4 % respectively (Fig. 8). Hence, of the four thiazolidinediones, only ciglitazone impaired the ATP-coupled respiration of the cardiomyocytes.

The maximum uncoupled rate of cardiomyocytes measured upon sequential addition of oligomycin and FCCP was 266±25 % (Fig. 8). The increase in OCR following sequential addition of oligomycin and FCCP to cardiomyocytes incubated overnight with 33 μM troglitazone, 33 μM ciglitazone, 33 μM rosiglitazone, and 33 μM pioglitazone was 226±15 %, 206±22 %, 246 ±18 % and 272±20 %, respectively (Fig. 8). Hence, the rank order of impairment on maximal uncoupling of cardiomyocytes was ciglitazone > troglitazone > rosiglitazone > pioglitazone. Thus, as in the case with HepG2 cells (Fig. 6), rosiglitazone and pioglitazone had no impairment on the maximum respiratory capacity of cardiomyocytes (Fig. 8). Troglitazone had a moderate impairment on the maximum respiratory capacity of both cell-types. In contrast to HepG2

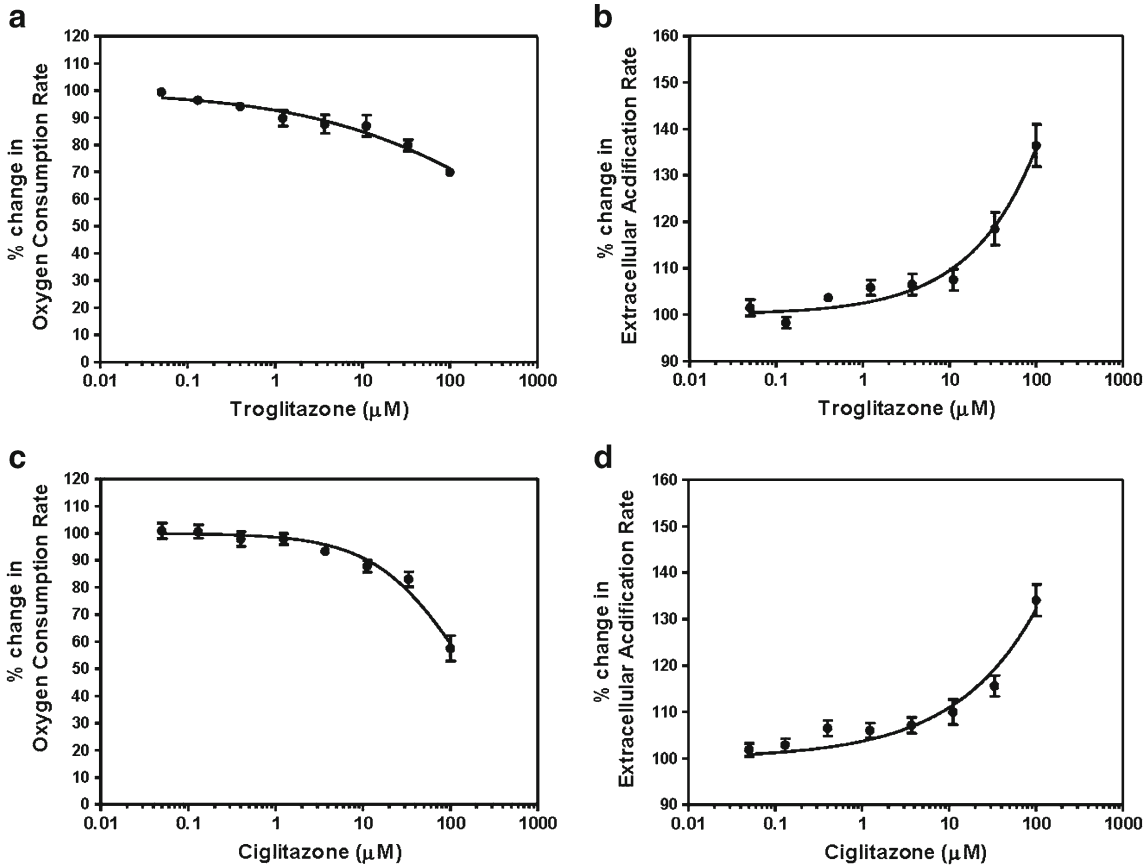

Fig. 5 The percentage change in OCR and ECAR of HepG2 cells on addition of troglitazone (**a** and **b**, respectively) and ciglitazone (**c** and **d**, respectively). Each data point represents the mean ± SEM, n=3 separate experiments

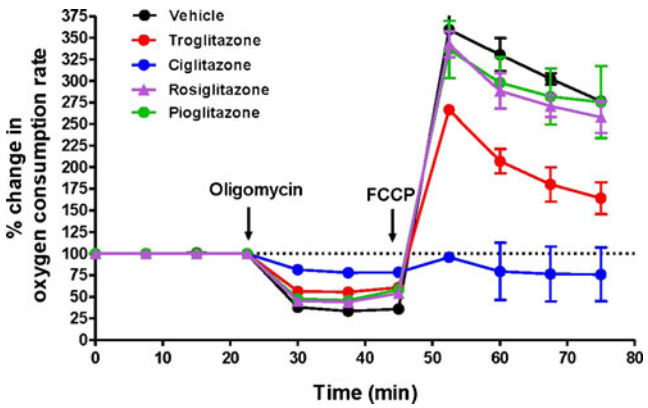

Fig. 6 The percentage change in OCR on injection of oligomycin (at a final concentration of 3 μM) and FCCP (at a final concentration of 0.5 μM) to HepG2 cells incubated for 20 h in vehicle (*black circles*), troglitazone (*red circles*), ciglitazone (*blue circles*), rosiglitazone (*purple triangles*), and pioglitazone (*green circles*). The final concentration of the thiazolidinediones during the 20 h incubation was 33 μM. The decrease in OCR upon injection of oligomycin represents the ATP-linked respiration of cells. The increase in OCR upon sequential injection of oligomycin and FCCP represents the maximal uncoupling of the cells. Each data point represents the mean ± SEM, n=3 separate experiments

cells (Fig. 6), the maximum respiratory capacity of cardiomyocytes was not significantly impaired by ciglitazone (Fig. 8).

Discussion

It is estimated that at least 30 % of compound attrition at the clinical stage of drug development is a result of toxicity (Kola and Landis 2004). In the current environment of high R&D costs and a low approval rate of investigational new drugs by the Food and Drug Administration, reducing compound attrition has become a pressing fiscal concern for pharmaceutical companies. As a result, there has recently arisen an increased urgency to develop in vitro assays to predict toxicity at the early stages of drug discovery.

Although the toxicity of an agent is dependent on many variables that cannot be mimicked in cell culture, the evaluation of known or potential toxicants using relevant cellular systems offers many potential advantages. Perhaps most important is the fact that screening in cells enables the identification of specific molecular pathways for a given toxicant. Identification of a pathway can be used to predict

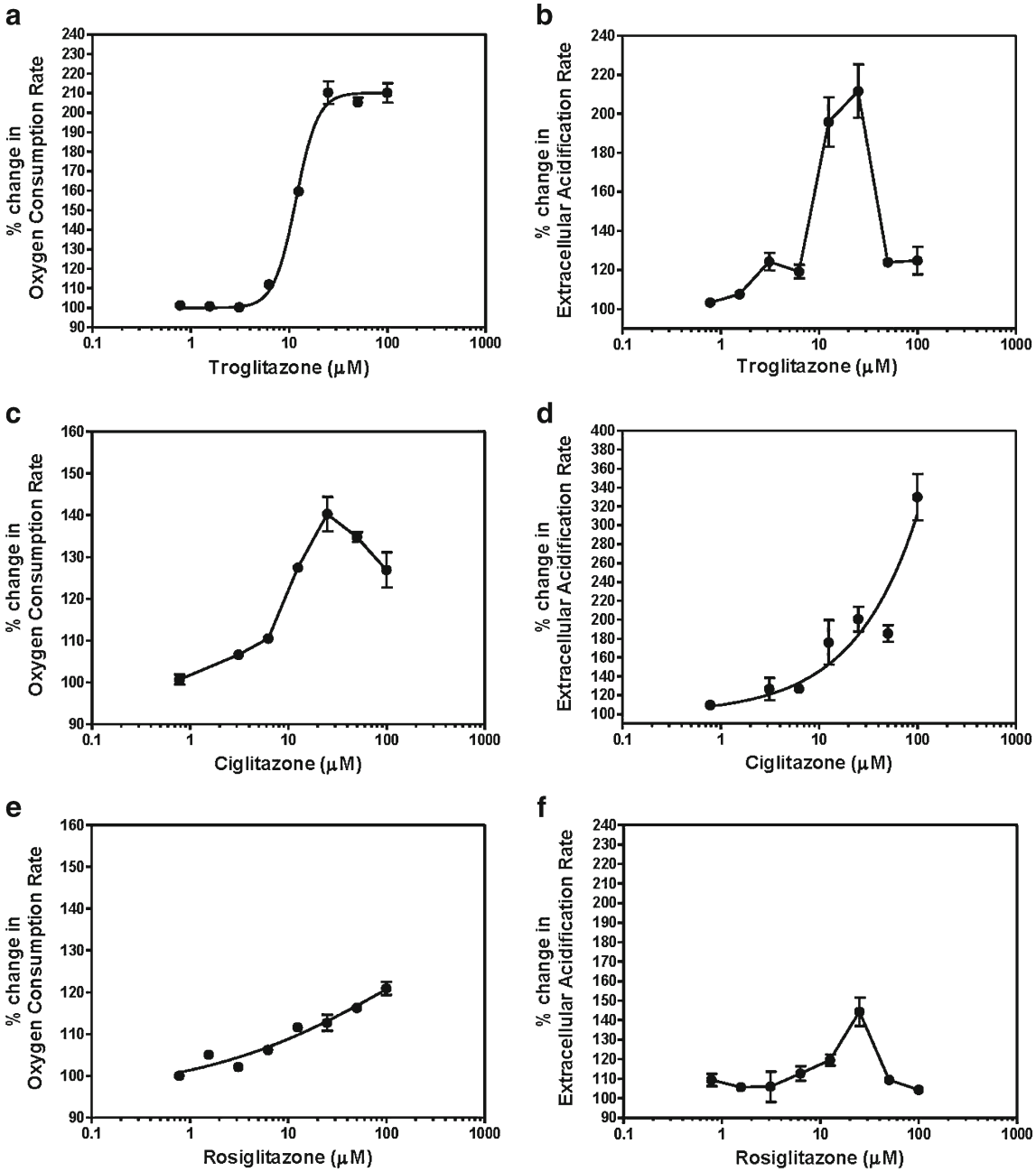

Fig. 7 The percentage change in OCR and ECAR of adult feline cardiomyocytes on addition of troglitazone (**a** and **b**, respectively), ciglitazone (**c** and **d**, respectively), and rosiglitazone (**e** and **f**, respectively). Each data point represents the mean ± SEM, $n=3$ separate experiments

potential clinical toxicity based on precedents observed with other agents that have the same target. Identification of pathways also aids in the development of clinical biomarkers and regulatory policies and is the stated vision of the recent TOX21 National Research Council report (Council et al. 2007).

The aim of our study was to evaluate a 96-well platform, the XF96 Extracellular Flux Analyzer, as a means to screen compounds for mitochondrial impairment in whole cells. The advantage of measuring the oxygen consumption rate as well as the extracellular acidification rate of cells is that one gets an insight into how well the cell type under investigation can withstand mitochondrial impairment. Cells which are able to increase their glycolytic rate dramatically in response to drugs that impair mitochondria are more likely to be able to cope with cellular ATP demands and are therefore more likely to tolerate adverse effects on mitochondria. In contrast, cells which do not show a large increase in glycolysis when their mitochondria are impaired are more likely to fail to keep up with ATP requirements and are therefore more likely to be susceptible to the adverse effects of drug-induced mitochondrial impairment.

Table 3 The concentration of drug required to change the OCR and ECAR of adult feline cardiomyocytes by 50 %

Drug	Concentration (μM) required to change the OCR of cardiomyocytes by 50 %	Concentration (μM) required to change the ECAR of cardiomyocytes by 50 %	Effect of compound on OCR and ECAR of cardiomyocytes
Troglitazone	10	10	Increased OCR. Increased ECAR at low concentrations and decreased it at higher concentrations
Ciglitazone	> 100	10	Increased OCR at low concentrations and decreased it at higher concentrations. Increased ECAR
Rosiglitazone	> 100	> 100	Increased OCR. Increased ECAR at low concentrations and decreased it at higher concentrations
Pioglitazone			No effect between 1.56 and 100 μM

Although a 24-well platform, the XF24 Extracellular Flux Analyzer, has been used before for mitochondrial toxicity assessments in whole cells (Dykens et al. 2008a, b; Beeson et al. 2010), the 96-well platform allows for greater throughput and enables one to test compounds over multiple concentrations within the same plate. One limitation of the XF96 Extracellular Flux analyzer is that real-time monitoring of OCR and ECAR over prolonged periods (>2 h) is not possible since measurements cannot be carried out in a humid, 5 % CO_2 environment conducive to cell maintenance. Hence, adaptation of cells to an initial toxic insult or, conversely, the effect of slow-acting toxic compounds may be missed.

We used HepG2 cells, a human hepatocellular carcinoma-derived cell line, for most of the experiments described in our study since this cell line is easy to handle and is often used in routine in vitro toxicity assays. We found that both the OCR and ECAR measurements of HepG2 cells in the XF96

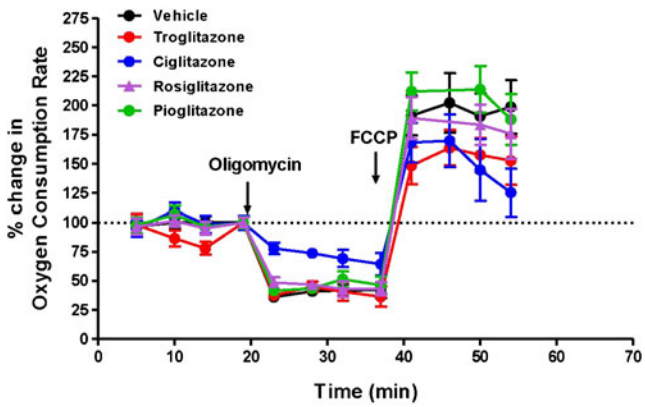

Fig. 8 The percentage change in OCR on injection of oligomycin (at a final concentration of 3 μM) and FCCP (at a final concentration of 0.5 μM) to adult feline cardiomyocytes incubated for 20 h in vehicle (*black circles*), troglitazone (*red circles*), ciglitazone (*blue circles*), rosiglitazone (*purple triangles*), and pioglitazone (*green circles*). The final concentration of the thiazolidinediones during the 20 h incubation was 33 μM. Each data point represents the mean ± SEM, $n=3$ separate experiments

platform had good reproducibility, with intra- and inter-assay variations of ≤15 %. It is possible that the inter-assay variation of OCR and ECAR with primary cells may be higher due to variability in isolating functional primary cells.

Our measurements in the XF96 analyzer indicated that the sensitivity of the HepG2 cells to classical mitochondrial modulators was in agreement with other reports where cell lines such as H460 and A549 have been characterized in the XF24 analyzer (Wu et al. 2007). Our results showed that the proportion of total respiration of HepG2 cells that was due to mitochondrial respiration (the respiration that was sensitive to antimycin) was 85–87 %. Thus, the proportion of total respiration that was due to non-mitochondrial respiration was 13–15 %. Non-mitochondrial respiration has been attributed to cell surface oxygen consumption (Herst and Berridge 2007) and cytosolic oxidase enzymes (Hill et al. 2009). The proportion of total respiration that was ATP-linked (the respiration that was sensitive to oligomycin) was 63–67 %, while the proportion of total respiration that resulted in proton leak (the mitochondrial respiration that was insensitive to oligomycin) was 18–24 %.

After establishing the sensitivity of the XF96 analyzer to well-known mitochondrial modulators, we tested the platform with 16 therapeutic agents, some of which have been reported to cause mitochondrial impairment. Tolcapone, a catechol-O-methyltransferase inhibitor (COMT) used in the treatment of Parkinson's disease, has been associated with idiosyncratic hepatotoxicity and is restricted in use. Mitochondrial toxicity has been implicated in tolcapone-associated hepatotoxicity (Haasio et al. 2002). In contrast, another COMT inhibitor, entacapone, has not been associated with hepatotoxicity. Our results show that tolcapone potently stimulated respiration of HepG2 cells (Fig. 3a). Although an increase in respiration could be due to either increased ATP utilization or uncoupling of oxidative phosphorylation, we interpret the increase in respiration as an effect of uncoupling because of the corresponding compensatory increase that we saw in the extracellular acidification rate (Fig. 3b). This is in agreement

with tolcapone's reported effects of uncoupling oxidative phosphorylation in isolated rat liver mitochondria (Nissinen et al. 1997; Haasio et al. 2002) and stimulating respiration in a murine lymphoma cell line (Nissinen et al. 1997). In contrast, entacapone stimulated respiration of HepG2 cells at a 10-fold higher concentration than tolcapone (Fig. 3c), in accord with reported information indicating that it is a less potent uncoupler than tolcapone (Haasio et al. 2002).

The antiandrogen drugs, nilutamide and flutamide, have been associated with rare cases of liver injury (Boelsterli et al. 2006). Mitochondrial impairment has been implicated in the adverse reactions associated with these two drugs and both have been found to inhibit Complex I activity in isolated rat liver mitochondria (Berson et al. 1994; Fau et al. 1994). Our results show that both drugs impaired oxygen consumption rates of HepG2 cells (Fig. 3e and g) and caused a compensatory increase in glycolytic rates (Fig. 3f and h).

Chlorpromazine and clozapine are dopamine receptor antagonists used in the treatment of bipolar disorders and schizophrenia. Maurer and Möller (1997) have shown that both drugs inhibit Complex I activity in human brain tissue, with chlorpromazine being more potent (IC_{50}~0.4 mM) than clozapine (IC_{50}~2.5 mM). We saw an increase in OCR with chlorpromazine but only at 300 μM and no change in OCR with clozapine possibly because we did not test either drug at the high concentrations that Maurer and Möller used. Our results are in agreement with those of Modica-Napolitano et al. (2003) who saw only modest uncoupling of oxidative phosphorylation in isolated rat liver mitochondria with chlorpromazine but not with clozapine. It has been suggested that the relative potencies of these two drugs on mitochondrial respiration is similar to their relative potencies in causing the adverse effect, tardive dyskinesia (Modica-Napolitano et al. 2003).

The local anesthetics, quinosocaine and lidocaine, have been reported to inhibit cytochrome *c* oxidase (Casanovas et al. 1983). However, we did not see any change in the OCR and ECAR of HepG2 cells with either of these drugs even at 300 μM. The inhibitory effect of these drugs seen by Casonovas et al. was with pure cytochrome *c* oxidase and it is possible that the concentrations that we used were not high enough to cause impairment of respiration in cells.

Nonsteroidal anti-inflammatory drugs (NSAID) have been associated with gastrointestinal and liver injury (Aithal and Day 2007) and mitochondrial impairment has been implicated as a contributing factor (Somasundaram et al. 1997; Somasundaram et al. 2000). Diflunisal has been reported to show strong uncoupling effects in mitochondria isolated from livers of rat, mice, rabbits and guinea-pigs (McDougall et al. 1983). This supports our results showing that diflunisal strongly accelerated oxygen consumption in HepG2 cells (Fig. 4a) with a compensatory increase in glycolysis (Fig. b). The NSAID, benoxaprofen, was removed from the market in 1982 (Duthie et al. 1982) due to reports of acute liver failure. Our results show that benoxaprofen caused a decrease in the OCR of HepG2 cells (Fig. 4c) with a concomitant increase in ECAR (Fig. 4d), suggesting that it inhibited oxidative phosphorylation. These results suggest that mitochondrial impairment may have been a contributing factor to liver injury caused by benoxaprofen.

The thiazolidinediones (TZDs) are therapeutic agents developed for the treatment of type II diabetes. They improve insulin sensitivity and lower blood glucose levels by activating the γ isoform of peroxisome proliferator-activated receptors (PPARs). Despite their beneficial effects, the TZDs, as a class, have been fraught with controversy due to adverse effects. Troglitazone was removed from the market in the US in 2000 because of several incidents of hepatotoxicity (Isley 2003). Ciglitazone was discontinued in development because of cataractogenic potential in rats (Aleo et al. 2005) while muraglitazar, the first TZD to activate both the α and γ isoforms of PPARs, was stopped in development because of increased cardiotoxicity (Nissen et al. 2005). After a meta-analysis of data suggesting increased myocardial infarction associated with rosiglitazone (Nissen and Wolski 2007), the drug was recently withdrawn from the market in Europe while, in the US, the FDA has requested a restricted access and dispensing program for this TZD. Hence, pioglitazone is currently the only TZD available to most type II diabetic patients. Our results with the TZDs showed that there was a rank order of potency with respect to changes in respiration both in HepG2 cells and cardiomyocytes, with ciglitazone being the most potent and pioglitazone causing no impairment. Moreover, our results showed that there were cell type-specific responses with respect to respiration. Some of these differences may be due to the fact that HepG2 cells are immortalized cells whereas cardiomyocytes are primary cells. Ciglitazone caused an immediate decrease in respiration in HepG2 cells whereas an increase in respiration was seen in cardiomyocytes. In both cell types, the change in OCR was accompanied by an increase in ECAR indicating that ciglitazone impaired mitochondrial function with an accompanying increase in the glycolytic rate of the cells. Overnight incubation with 33 μM ciglitazone impaired the ATP-linked respiration of HepG2 cells and cardiomyocytes by 67 % (Fig. 6) and 44 % respectively (Fig. 8). Furthermore, ciglitazone impaired the maximum respiratory capacity of HepG2 cells and cardiomyocytes by 95 % (Fig. 6) and 23 % (Fig. 8), respectively. Our data agree with previous reports describing ciglitazone-induced impairment of mitochondrial function. For instance, ciglitazone was shown to be a potent stimulator of state 2 respiration as well as an inhibitor of ADP-driven respiration in rat liver mitochondria (Nadanaciva et al. 2007) and was reported to cause irreversible opening of the mitochondrial permeability transition pore (MPTP) in mouse liver mitochondria (Masubuchi et

al. 2006). Ciglitazone was also shown to cause reactive oxygen species formation in astrocytes and glioma cells (Perez-Ortiz et al. 2004).

Like ciglitazone, troglitazone had different acute effects in HepG2 cells and cardiomyocytes: it caused an immediate inhibition of respiration in HepG2 cells (Fig. 6) but had a stimulatory effect on the respiration of cardiomyocytes (Fig. 8). As in the case with ciglitazone, the change in OCR upon addition of troglitazone was accompanied by an increase in ECAR indicating that troglitazone caused mitochondrial impairment in both cell types. Overnight incubation with 33 μM troglitazone reduced the maximum respiratory capacity of HepG2 cells by 35 % (Fig. 6) and that of cardiomyocytes by 15 % (Fig. 8). These results are in agreement with published data indicating that troglitazone causes mitochondrial impairment. Oxygen consumption measurements with isolated rat liver mitochondria showed that troglitazone increased basal respiration (Nadanaciva et al. 2007), caused irreversible opening of the MPTP in mouse liver mitochondria (Masubuchi et al. 2006) and decreased the mitochondrial membrane potential as well as ATP levels in HepG2 cells (Tirmenstein et al. 2002).

Our results showed that rosiglitazone had no effect on the respiration of HepG2 cells but caused a 20 % stimulation of respiration in cardiomyocytes at 100 μM (Fig. 7e). Overnight incubation with 33 μM rosiglitazone had no effect on the ATP-linked respiration of either HepG2 cells (Fig. 6) or cardiomyocytes (Fig. 8). Rosiglitazone caused no impairment in the maximum respiratory capacity of HepG2 cells and only an 8 % decrease in that of cardiomyocytes (Fig. 8). Thus, our results do not support a role for mitochondrial involvement in rosiglitazone-associated cardiac failure.

A factor that needs to be taken into consideration when rank ordering drugs with respect to mitochondrial impairment is the pharmacological efficacious concentration. Hence, knowing the Cmax value of a drug (the therapeutically active average plasma maximum concentration of the drug after a single dose administration in humans) is important. Although all factors that contribute to the toxicity of a drug cannot be mimicked in an in vitro assay, the smaller the ratio, IC_{50} value in an in vitro assay: Cmax value of a drug, the greater the probability that the drug will cause adverse effects (O'Brien et al. 2006). Our results on the TZDs showed that this ratio is >15.6 (>100/6.4) for troglitazone and >100 for rosiglitazone and pioglitazone. Comparison of our results on tolcapone and entacapone showed that the ratio is 0.18 (3.7/20.8) for tolcapone and 8.25 (33/4) for entacapone. This is in accord with reports indicating that entacapone is a safer drug than tolcapone (Haasio et al. 2002).

One observation from our results on the TZDs is that when screening for compounds that cause a particular organ toxicity, there does not appear to be a strict requirement for using a cell type derived only from that organ. Thus, although mitochondrial impairment has been cited as a mechanism for troglitazone-induced hepatotoxicity, our results showed that the mitochondrial impairment caused by this TZD was not restricted to cell types which are derived from the liver, ie troglitazone induced mitochondrial impairment even in cardiomyocytes. This suggests that when screening for drug-induced mitochondrial impairment, one does not necessarily have to use a liver-derived cell type for compounds that cause hepatotoxicity or a cardiac-derived cell type for compounds that cause cardiotoxicity or a renal-derived cell type for compounds that cause nephrotoxicity, particularly when general characteristics of mitochondrial function such as electron transport, ATP synthesis, maintenance of the mitochondrial membrane potential and transient opening of the MPTP are related to the toxicity. These features are universal to mitochondria of all cell types. A similar response to drug-induced mitochondrial impairment in different cell types may be indicative of a systemic effect of the drug on cellular bioenergetics and may manifest itself as toxicity only in the most susceptible organs. While there may be differences in the sensitivity to a compound between two cell types, our results suggest that one could use cell lines/primary cells derived/isolated from a variety of organs to screen for drug-induced mitochondrial impairment which causes changes in respiration. Exceptions to this may include cases where a compound requires a specific mode of transport to cross the cell membrane as in the case of certain aminoglycoside antibiotics which require the megalin receptor (Schmitz et al. 2002).

Conclusion

In summary, our results show that the XF96 Extracellular Flux analyzer can be used as a robust, sensitive platform to perform high throughput screening of compounds for identifying mitochondrial impairment in whole cells. The platform enables real-time monitoring of OCR and ECAR measurements of cells upon acute administration of compounds as well as measurements of ATP-linked respiration and maximum respiratory capacity after longer incubation with compounds. We identified changes in respiration and acidification upon addition of therapeutic agents reported to have a mitochondrial effect. Furthermore, we show that respiration and acidification changes upon addition of the thiazoldinediones were cell-type specific, with the rank order of mitochondrial impairment in whole cells being in accord with the known adverse effects of these drugs.

Conflict of Interest S. Nadanaciva, P. Rana, Y. Will, G.C. Beeson and C.C. Beeson have no conflict of interest. D. Chen and D.A. Ferrick are employees of Seahorse Bioscience.

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