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Short communication

A high-throughput screen for mitochondrial function reveals known and novel mitochondrial toxicants in a library of environmental agents

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ABSTRACT

Mitochondrial toxicity is emerging as a major mechanism underlying serious human health consequences. This work performs a high-throughput screen (HTS) of 176 environmental chemicals for mitochondrial toxicity utilizing a previously reported biosensor platform. This established HTS confirmed known mitochondrial toxins and identified novel mitotochondrial uncouplers such as 2, 2′-Methylenebis(4-chlorophenol) and pentachlorophenol. It also identified a mitochondrial 'structure activity relationship' (SAR) in the sense that multiple environmental chlorophenols are mitochondrial inhibitors and uncouplers. This study demonstrates proof-of-concept that a mitochondrial HTS assay detects known and novel environmental mitotoxicants, and could be used to quickly evaluate human health risks from mitotoxicants in the environment.

1. Introduction

Mitochondria are critical for numerous cellular and biochemical processes such as oxygen sensing, ATP production, cell signaling, differentiation, and apoptosis (McBride et al., 2006). Proper neuronal function especially relies on mitochondria, as synaptic transmission requires a high metabolic demand (Kann and Kovács, 2007). The distribution of mitochondria along dendritic neurons is critical for synaptic plasticity (Li et al., 2004), and mitochondrial damage leads to selective neurodegeneration caused by increased oxidative stress (Wang and Michaelis, 2010).

Due to the deleterious human health consequences of mitochondrial defects, extensive toxicological and pharmacological studies have focused on the susceptibility of these organelles to both environmental and genetic damage (Scatena et al., 2007). In recent times, the growing threat to the mitochondrial function by environmental pollutants/toxins has been highlighted by several reports (Brunst et al., 2015; Caito and Aschner, 2015; Meyer et al., 2013). Numerous environmental chemicals such as MPTP, rotenone, dimethylbenzanthracene (DMBA), and naphthalene are already known to negatively affect mitochondrial function, (Backer and Weinstein, 1980; Ernster et al., 1963; Harmon and Sanborn, 1982; Nicklas et al., 1987), and mitochondrial exposure to certain environmental compounds has been implicated in the etiology of some forms of Parkinson's disease (Sherer et al., 2002). Although numerous chemicals used in industry have potentially negative health consequences, the toxicity of these compounds is often not easily tractable due to extensive monetary or labor investment.

This study utilizes the previously optimized high-throughput assay (Sahdeo et al., 2014) to screen the effects on mitochondrial function of a library of 176 known environmental toxicants (Morisseau et al., 2009) in the RGC-5 rat retinal ganglion cell line. The results confirm the known mitochondrial toxicant effects of certain industrially used chemicals, and they also point to novel environmental pollutants that were previously unknown to impair mitochondrial function. The chlorinated phenols or chlorophenols in the environment were identified as a group of compounds that causes mitotochondrial uncoupling. The efficiency and speed of this assay serves as a powerful tool for the screening of new industrial compound effects on mitochondrial toxicity.

2. Materials and methods

2.1. Cell line and chemical library

The RGC-5 retinal ganglion cell line and HeLa cells were purchased from American Type Culture Collection (Manassas, VA) and grown at

Abbreviations: AR, Structure activity relationship; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ATP, Adenosine triphosphate; CDEC, Carbamodithioic acid, diethyl-, 2-chloro-2-propenyl ester; OCR, Oxygen consumption rate.

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37 °C in a humidified atmosphere containing 5% CO_2. RGC-5 cells were grown in DMEM supplemented with 2 mM glutamine, 50 μg/mL uridine and 10% fetal bovine serum (FBS). HeLa cells were grown in DMEM supplemented with 10% FBS, 100 mM Pyruvate, 2 mM L-glutamine, 50 μg/mL uridine and antibiotics (50 units/mL of penicillin + 50 μg/mL of streptomycin). The library of 176 environmental chemicals utilized for screening is as detailed in Morisseau et al., 2009. The 4-(trifluoromethoxy)-phenylhydrazone (FCCP) was purchased from Sigma Aldrich.

2.2. Biosensor plate assay to determine oxygen consumption effects of envirotoxins

RGC-5 cells were grown in the media specified above and once confluent, they were trypsinized and resuspended in assay buffer (phenol-red free DMEM supplemented with 1 mM Na pyruvate and 10% FBS), and cell concentration and viability were determined using a Vi-Cell counter (Beckman Coulter). Cells were aliquoted (70,000 cells, 90 μL media/well) into 384-well oxygen biosensor plates (BD Biosciences) and allowed to equilibrate for 20–30 min (Sahdeo et al., 2014). The library of 176 environmental toxicants (Morisseau et al., 2009) was initially dissolved in DMSO (10 mM) and was diluted in PBS (100 μM). The compounds (10 μL), DMSO or FCCP was added in their respective wells, and fluorescence was monitored using a Polar Star Omega plate reader (BMG Labtech, Germany) set at 37 °C. For the endpoint assay, fluorescence was monitored at 0, and 2 h post addition, and plates were incubated at 37 °C under 5% CO2 between readings. The final conc. of DMSO was 0.1% and DMSO and FCCP were used as negative and positive controls respectively (Sahdeo et al., 2014). Each compound was evaluated in triplicate ($n = 3$).

2.3. Oxygen consumption measurements using a Seahorse XF-24

For oxygen consumption measurement, 50,000 HeLa cells (ATCC) per well were seeded onto 24 well plates (Seahorse Biosciences, Billerica, MA) in previously specified culture medium and allowed to attach for 16 h. For respiration analysis, media was changed to un-buffered cultured assay DMEM media containing 200 mM glutamax, 100 mM sodium pyruvate, 25 mM glucose, pH 7.4 without phenol red. Cells were pre-equilibrated for 20 min. The oxygen consumption rate (OCR) was recorded using a Seahorse XF-24 instrument and measured oxygen consumption per minute in pmol (pmol/min). The OCR was measured before (basal) and after addition of the test compounds at the specified concentrations. Percentage of the basal OCR was calculated using the formula:

% Basal OCR = OCR after addition of rotenone/OCR before addition of rotenone.

Final concentrations of various compounds in the mechanistic experiments were: Oligomycin: 1 μM, test compounds: 10 μM, and Antimycin A: 1 μM.

2.4. Cell viability assay by sulforhodamine B method

The HeLa cells were seeded 50,000 cells/well in 100 μL of the culture media in a 96-well plate and allowed to attach to the plate for overnight by incubating at 37 °C under 5% CO_2. Next day, the cells were treated with the test compounds diluted in the serum-free culture media (100 μL) at 1, 3, 10, 30 μM final assay conc. and incubated for 24 h. At the end of the 24 h incubation the cell viability was measured by sulforhodamine B method (Skehan et al., 1990). Data were processed as % of the untreated control.

2.5. Data analysis and statistics

Fluorescence readings representing oxygen consumption were recorded at 2 h post-chemical library treatment, and luminescence readings for ATP content were collected after 24 h of chemical treatment. The fold change from baseline (FCB) was calculated by normalizing post-incubation readings to the T_0 reading. This normalization helped to minimize effects due to well-to-well variation and eliminated potential false positives generated by compound fluorescence. FCB responses for drug-treated wells were then normalized to the average FCB for the 16 vehicle-treated wells producing the fold change from vehicle (FCV) value for each well.

Fold change from vehicle (FCV) = FCB of drug-treated wells/Average FCB from vehicle wells

Fold change from baseline (FCB) = Values at T_{2h}/untreated baseline value at T_0

Significant hits were scored as vehicle mean ± 2× standard deviation. The FCV values of significant hits were then converted to percent change, as indicated. Oxygen consumption data are presented by mean % of control + standard deviation.

3. Results

3.1. High-throughput screen of environmental toxicant library reveals modulators of mitochondrial function

This study identifies common environmental chemicals that alter mitochondrial oxygen consumption. The processes of substrate oxidation and oxygen consumption are "coupled" with chemical phosphorylation during mitochondrial ATP synthesis (Cross et al., 1949). A previously characterized high-throughput protocol (Sahdeo et al., 2014) was used to screen the effects of 176 known and structurally diverse environmental chemicals (Morisseau et al., 2009) on mitochondrial oxygen consumption. The RGC-5 retinal ganglion neuron cell line was used for this assay as it showed a high dynamic range, i.e. high signal to noise ratio and these cells are known to be sensitive to mitochondrial stress (Ju et al., 2007; Kamalden et al., 2012; Marella et al., 2010). A 2-hour endpoint after 10 μM compound treatment revealed that a number of chemicals inhibited or stimulated mitochondrial O_2 consumption in RGC-5 neural cells. Oxygen consumption rates per well were compared between 0 h and 2 h of chemical treatment in order to generate fold change from vehicle (FCV) values. The O_2 consumption FCV average of 16 vehicle-treated wells was 1.00 ± 0.09 SD after the 2-hour treatment. We use the criterion of ±2× SD (i.e. 100% – 2XSD ≤ 4.5% of normally distributed data) as significant, thus values above 1.18 or below 0.82 FCV were considered significant hits (i.e. ±18% = ±2× SD from vehicle control). After screening the envirotoxin library and using these criteria, 18 compounds significantly stimulated mitochondrial O_2 consumption, and 7 compounds inhibited mitochondrial function, for an overall 25/176 = 14% of the environmental toxins affected mitochondrial function (Table 1).

3.2. The two most potent stimulators of mitochondrial O_2 consumption are uncouplers

To follow up on our primary screen of the environmental pollutants impairing mitochondrial function, we picked the two most potent activators of mitochondrial O_2 consumption from Table 1, using a Seahorse XF extracellular flux analyzer. 2,2′-Methylenebis(4-chlorophenol) and pentachlorophenol showed a concentration- dependent increase in mitochondrial O_2 consumption (Fig. 1A). In addition, both of the environmental toxins overcame oligomycin inhibition (Fig. 1B), confirming their mitochondrial uncoupling activity.

We also tested the dose dependent effects of 2,2′-Methylenebis(4-chlorophenol) and pentachlorophenol on cell viability at (1, 3, 10 μM) by the sulforhodamine B method, neither treatment produced a decrease of 15% or more in viability, demonstrating that the mitotoxic effect precedes any loss of cell viability.