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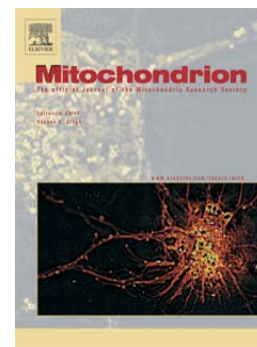
A high-throughput screen for mitochondrial function reveals known and novel mitochondrial toxicants in a library of environmental agents

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**Title:**

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

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Biosensor

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Cell-based assay

**Abbreviations:**

SAR: 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

**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 mitochondrial 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 mitotoxics, and could be used to quickly evaluate human health risks from mitotoxics 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 mitochondrial 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 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. 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)-phenylhydrazine (FCCP) was purchased from Sigma Aldrich.

### 2.2 Biosensor plate assay to determine oxygen consumption effects of envirottoxins

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% CO<sub>2</sub> 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 hours. 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:

$$\% \text{ Basal OCR} = \text{OCR after addition of rotenone} / \text{OCR before addition of rotenone}.$$

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

### 2.4 Cell viability assay by Sulforhodamine B method

The HeLa cells were seeded 50,000 cells/well in 100  $\mu$ L 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<sub>2</sub>. Next day, the cells were treated with the test compounds diluted in the serum-free culture media (100  $\mu$ L) at 1, 3, 10, 30  $\mu$ M 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 hours post-chemical library treatment, and luminescence readings for ATP content were collected after 24 hours of chemical treatment. The fold change from baseline (FCB) was calculated by normalizing post-incubation readings to the T<sub>0</sub> 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  $\pm 2 \times$  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  $\mu$ M 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 hours and 2 hours 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  $1.00 \pm 0.09$  SD after the 2-hour treatment. We use the criterion of  $\pm 2XSD$  (i.e.  $100\% - 2XSD \leq 4.5\%$  of normally distributed data) as significant, thus values above 1.18 or below 0.82 FCV were considered significant hits (i.e.  $\pm 18\% = \pm 2XSD$  from vehicle control). After screening the envirotxin 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**).

**Table 1.** The table to the right indicates compounds that have an effect on oxygen consumption in RGC-5 cells. ‘% change’ refers to mean fold change of oxygen biosensor fluorescence between the compound-treated and vehicle control-treated cells. Each compound was tested at 10  $\mu$ M concentration for 2 hours prior to output reading. Significant compounds were indicated as those that modulated oxygen abundance by greater than mean 2x SD.

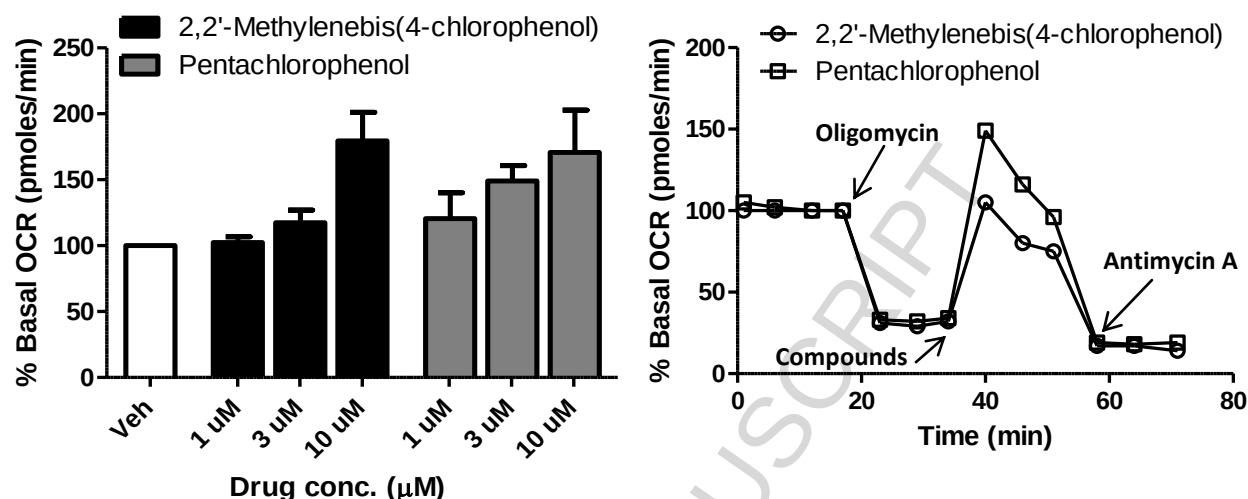
### 3.2 The two most potent stimulators of mitochondrial O<sub>2</sub> consumption are uncouplers.

To follow up on our primary screen of the environmental pollutants impairing mitochondrial function, we picked the two most potent activators of mitochondrial O<sub>2</sub> 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<sub>2</sub> consumption (**Figure 1A**). In addition, both of the environmental toxins overcame oligomycin inhibition (**Figure 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 $\mu$ M) 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.

| Modulators of mitochondrial oxygen consumption in RGC-5 cells |                 |
|---------------------------------------------------------------|-----------------|
| Compound                                                      | % change, 2 hrs |
| <i>Activators</i>                                             |                 |
| 2,2'-Methylenebis(4-chlorophenol)                             | 124             |
| Pentachlorophenol                                             | 86              |
| 4,6-Dinitro-o-cresol                                          | 60              |
| 2-methylheptyl-4,6-dinitrophenyl Crotonate                    | 34              |
| 2,3,4,6-Tetrachlorophenol                                     | 25              |
| $\alpha$ -Cypermethrin                                        | 25              |
| Naphthalene                                                   | 22              |
| p-Dichloro-benzene                                            | 22              |
| Clopyralid                                                    | 21              |
| Pyrethrum                                                     | 20              |
| 2-Naphthoxyacetic acid                                        | 19              |
| DEHP                                                          | 19              |
| des-N-Isopropyl isofenphos oxygen analog                      | 19              |
| Dichlorprop                                                   | 19              |
| Parathion                                                     | 19              |
| Trichloroacetic acid                                          | 19              |
| CDEC                                                          | 18              |
| Gibberellic acid                                              | 18              |
| <i>Inhibitors</i>                                             |                 |
| Rotenone                                                      | -35             |
| Triton X-100                                                  | -26             |
| 2,4-Dichlorophenoxy acetic acid, isopropyl ester              | -24             |
| 2,4,5-Trichlorophenol                                         | -22             |
| Tedion                                                        | -20             |
| Bifenthrin                                                    | -18             |
| Sanmarton                                                     | -15             |





**Figure 1.** Concentration-dependent uncoupling effect of environmental pollutants. (A) Concentration-dependent increase in oxygen consumption by environmental pollutants in HeLa cells. The cells were treated with the compounds (1, 3, 10 μM) and OCR was measured. The data is presented as average % of basal OCR + standard deviation from three independent observations. (B) Reversal of oligomycin-induced inhibition of mitochondrial oxygen consumption by the environmental pollutants. The cells were first treated with oligomycin (1 μM) followed by the compounds and Antimycin A (1 μM) as indicated. The data is presented as % of Basal OCR from one representative experiment.

## 4. Discussion

### 4.1 High-throughput chemical screen identifies environmental toxicants with a known mitochondrial mechanism of action

Mitochondrial toxicity has been in focus in the recent times and several assays for mitochondrial toxicity/function have been developed by different groups (Andreux et al., 2014; Hynes et al., 2013; Hynes et al., 2012). These, screens are however more complex and tedious compared to the HTS screening method utilized in this study that can be completed in 3hrs. This study utilized a previously reported high-throughput screen (Sahdeo et al., 2014) to assay a library of 176 known environmental toxicants (Morisseau et al., 2009) for potential effects on mitochondrial function. Numerous known mitochondrial inhibitors were validated with this method. Namely, many chlorinated phenols were found to have a strong effect on mitochondrial

function including 2,2'-Methylenebis(4-chlorophenol), Pentachlorophenol, 2,3,4,6-Tetrachlorophenol, p-dichlorobenzene, 2,4-Dichlorophenoxyacetic acid isopropyl ester, and 2,4,5-Trichlorophenol (**Table 1**). These findings support previous reports that chlorophenols are mitochondrial toxins (Attene-Ramos et al., 2013). Further, phenol ring substitutions are known to have an effect on mitochondrial respiration (Ravanel et al., 1985; Stockdale and Selwyn, 1971), whereby they uncouple and inhibit mitochondrial complex I in plants (Ravanel et al., 1985). The high lipophilicity of chlorophenols allows them to partition into lipophilic mitochondrial bilayers; consequently, the toxic uncoupling effect is due to the protonophoric capability of the chemicals, allowing shuttling of electrons across the inner mitochondrial membrane (Dyken and Will, 2008).

Other mitochondrial inhibitors and uncouplers identified in this screen have been previously reported. Our data illustrate that rotenone is the most potent inhibitor of RGC-5 mitochondrial respiration in this screen, and rotenone is a classical mitochondrial complex I inhibitor (Ernster et al., 1963) (**Table 1**). Additionally, 4,6-Dinitro-o-cresol was shown to be one of the most potent uncouplers in this screen (**Table 1**), and previous literature supports this finding (Castilho et al., 1997).

#### *4.2 Novel mitochondrial mode of toxicological action identified for environmental chemicals.*

In addition to this assay verifying known mitochondrial inhibitors, it also revealed that several commonly used agricultural chemicals: 2,4D, Isophenphos, 2-Naphthoxyacetic acid, alter mitochondrial function in neural cells *in vitro*.

Many commonly used herbicides were found in this screen to affect mitochondrial function. Since there is an existing connection between inhibition of mitochondrial function and Parkinson's disease (PD), and the epidemiological findings of increased PD in agricultural workers and farmers, there could be a connection between increased exposure to mitotoxigenic herbicides and pesticides and increased PD in agricultural workers (Sherer et al., 2002). Namely, this study detected that 2,4 Dichlorophenoxyacetic acid (2,4D) isopropyl ester, one of the first and still very widely used broadleaf herbicides (Song, 2014), reduced oxygen consumption in RGC-5 cells (**Table 1**). This compound might thus have harmful effects on human health by way of impairing neuronal mitochondrial function. Further, the herbicide CDEC, whose active

ingredient is sulfallate (Bacon, 1979), is reported here to be a potent inhibitor of mitochondrial function and a short-term mitochondrial uncoupler in cells (**Table 1**).

Another pest control agent showing mitochondrial uncoupling in RGC-5 cells was an organophosphate oxygen analog of des-N-isopropyl isofenphos (**Table 1**). Isofenphos is an insecticide with toxicity toward a variety of agricultural pests (Roberts and Hutson, 1999), and previous reports have shown that isofenphos causes changes in glucose metabolism in K562 myeloid blast cells, resulting in leukemogenic effects (Boros et al., 2001). Additionally, many organophosphates have been shown to impair mitochondrial function, although the effects of des-N-isopropyl isofenphos were not previously described (Kaur et al., 2007).

In addition to chemicals that prevent excess plant and insect proliferation, two plant hormones that are used to promote agricultural growth, auxin and gibberellic acid (Davies, 2010), were shown in this screen to affect mitochondrial function. The synthetic auxin 2-Naphthoxyacetic acid was found in this study to increase mitochondrial O<sub>2</sub> consumption in RGC-5 cells after two hours of exposure (**Table 1**). Gibberellic acid, a promoter of plant growth in many agricultural crops such as barley, (Chrispeels and Varner, 1967) was also found to be a potential uncoupler in human neural cells (**Table 1**).

One important thing to mention here is that none of the representative mitochondrial toxicants showed major decrease in cell viability. This can be an effect of the high glucose content in the media. It has been previously reported that the high glucose concentration (25 mM) in the media can poise the cells towards higher glycolysis and can reduce the cytotoxicity of the mitochondrial toxicants (Marroquin et al., 2007). The glucose content, however, is unlikely to interfere with the mitochondrial effects of these compounds.

This work showing the toxicity of compounds in neural mitochondria *in vitro* provide an impetus to further study the consequences of these commonly used environmental chemicals on human health.

#### 4.3 Conclusions

In summary, the utilization of a previously characterized high-throughput assay (Sahdeo et al., 2014) was found to validate known mitochondrial function toxicity of commonly used

compounds, and it serves as a useful tool to demonstrate previously uncharacterized potentially toxic effects of commonly used environmental chemicals. Of the 176 environmental compounds in the library, 25/176 or 15% were observed to have a mitochondrial effect. The high-throughput nature of this screen provides an efficient method to identify compounds that are toxic, and it further provides details into their mechanism of mitochondrial toxicity, i.e. stimulator (and potential uncoupler) or inhibitor. The assay identifies known mitotoxins such as rotenone, and also suggests that 2,4D, isophenphos and 2-Naphthoxyacetic acid as potential mitotoxins that exist in the agricultural environment and should be further evaluated for their impact on human health. Furthermore, the assay reveals that multiple chlorinated phenols in the environment have strong SAR for affecting mitochondrial function. This system is a relatively quick, 3hr, automated screen and could be used in the pharmaceutical prioritization context for drug development (Dyken et al. 2008; Hynes et al. 2013). It could also be used in the context of regulatory (FDA/EPA) screening of pesticides, herbicides or other compounds released into the environment as a rapid screen for mitotoxins that could impact human neurological health.

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### **Conflict of Interest**

The authors declare there are no conflicts of interest.

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## Highlights

- A rapid high-throughput mitochondrial activity screen identifies mitoactives in an environmental toxicant library.
- Environmental pollutants 2,4D; Isophenphos and 2-Natpthoxyacetic acid cause mitochondrial dysfunction.
- Chlorinated phenols in general impair mitochondrial function.
- Environmental compounds 2,2'-Methylenebis(4-chlorophenol) and pentachlorophenol are potent mitochondrial uncouplers.
- 2,2'-Methylenebis(4-chlorophenol) is widely used as veterinary fungicide, anthelmintic, and anti-protozoan while pentachlorophenol an acutely toxic pesticide is banned in many countries in the world except the USA.