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High-throughput respirometric assay identifies predictive toxicophore of mitochondrial injury

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Abstract

Many environmental chemicals and drugs negatively affect human health through deleterious effects on mitochondrial function. Currently there is no chemical library of mitochondrial toxicants, and no reliable methods for predicting mitochondrial toxicity. We hypothesized that discrete toxicophores defined by distinct chemical entities can identify previously unidentified mitochondrial toxicants. We used a respirometric assay to screen 1760 compounds (5 μ M) from the LOPAC and ChemBridge DIVERSet libraries. Thirty-one of the assayed compounds decreased uncoupled respiration, a stress test for mitochondrial dysfunction, prior to a decrease in cell viability and reduced the oxygen consumption rate in isolated mitochondria. The mitochondrial toxicants were grouped by chemical similarity and two clusters containing four compounds each were identified. Cheminformatic analysis of one of the clusters identified previously uncharacterized mitochondrial toxicants from the ChemBridge DIVERSet. This approach will enable the identification of mitochondrial toxicants and advance the prediction of mitochondrial toxicity for both drug discovery and risk assessment.

Keywords

mitochondria; toxicophore; renal proximal tubule cells; LOPAC; ChemBridge; Seahorse Biosciences Extracellular Flux

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Introduction

Mitochondria are essential organelles with significant roles in energy production, regulation of intracellular homeostasis, reduction/oxidation balance, proliferation, and apoptosis^{1,2}. The bioenergetic functions of the mitochondria require control of the size and number of mitochondrial units, the efficiency of both electron transport and oxidative phosphorylation, and the availability of required substrates³. Any insult with deleterious effects on mitochondrial function will result in decreased production of adenosine triphosphate (ATP), increased formation of harmful reactive oxygen species, alterations in ionic homeostasis, and/or cell injury and death.

Mitochondrial damage is the primary target underlying many organ toxicities including hepatotoxicity, cardiotoxicity, and acute kidney injury (AKI)⁴. In addition, sub-lethal mitochondrial toxicity makes cells more vulnerable to other non-mitochondrial stressors, and severe mitochondrial dysfunction can lead to catastrophic damage resulting in apoptosis or necrosis. Many compounds including pesticides, antivirals, antibiotics, analgesics, chemotherapeutics, and cholesterol-lowering drugs have been reported to induce mitochondrial dysfunction⁵⁻⁹. Mitochondrial toxicity is a major cause for concern for preclinical therapeutic candidate failures as well as post market drug withdrawals^{3,10,11}. In the last ten years “off target” mitochondrial impairment has been identified as a contributing factor in the etiology of drug-induced organ toxicity¹¹. The Food and Drug Administration has issued 528 Black Box Warnings for patients through 2010, and many of those were because of mitochondrial toxicity¹². Thus, it is critical to test for mitochondrial toxicity early in drug development to avoid increasing the progression of existing mitochondrial diseases or causing other life threatening pathologies.

As researchers have discovered more mitochondrial-related mechanisms underlying disease, the demand for more flexible and higher throughput methods of assessing mitochondrial function have increased^{13,14}. Measurements of cellular O₂ consumption rates (OCR) reflect the influence of multiple hormonal effects, regulated transporters and pathways, and signaling cascades. Therefore, OCR represents the functional capacity of the mitochondria, and ultimately overall cellular health. Respirometry methods have largely relied on Clark electrode chambers that are not conducive to high-throughput screening (HTS)^{15,16}. The Seahorse Biosciences 96-well extracellular flux analyzer (XF-96) is a multi-well plate-based assay that uses fluorescent detectors and addresses the need for higher throughput cellular respirometric measurements¹⁷. The injection of mitochondrial probes can be used to determine and quantify the sources of OCR. For example, after determining the basal OCR, injection of the proton ionophore, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) uncouples oxygen consumption from the production of ATP resulting in maximal respiration, a stress test to measure maximal activity of the electron transport chain¹⁸. This technology represents a significant advance in throughput for assessment of cellular mitochondrial function.

Various animal-based experimental methods have been developed to evaluate toxicological responses, based on the assumption that adverse effects in laboratory animals are closely related to adverse effects in humans^{19,20}. While this method of testing provides informative

toxicity information, these assays are low throughput and expensive in terms of money, time, and animal lives¹⁹. There is increased interest among the scientific community in the development, translation, validation, and use of non-*in vivo* alternative test methods for making regulatory decisions^{21,22}. The Environmental Protection Agency, the National Toxicology Program, and the National Institutes of Health Chemical Genomics Center are collaborating to identify a battery of cell-based screens to prioritize compounds for further toxicological evaluation¹⁹.

The majority of *in vitro* drug toxicity evaluations are conducted with immortalized cell lines and grown under culture conditions that do not mimic those found *in vivo*^{23,24}. These immortalized cells have lost differentiated cell functions, and derive ATP from glycolysis, not oxidative phosphorylation. Many of these cell lines are cultured in media containing artificially high concentrations of glucose. Under these conditions cellular OCR are low, and cells are more resistant to xenobiotics that impair mitochondrial function.

Primary cultures of rabbit renal proximal tubule cells (RPTC) utilized in this study were grown under improved culture conditions with optimized glucose-free media supplemented with 6 mM sodium lactate and increased oxygen supply^{25,26}. RPTC grown under these conditions remain polarized, maintain their differentiated functions, and exhibit respiration and gluconeogenesis rates comparable to *in vivo* renal proximal tubule cells^{14,25,26}. In addition to the improved culture conditions, RPTC are a clinically relevant platform to study mitochondrial toxicity. Mitochondrial impairment typically affects tissues exposed to high concentrations of a compound (kidney and liver), and the most aerobically dependent tissues (kidney and heart)^{7,27,28}. Furthermore, RPTC have an extensive group of transporters allowing intracellular transport of small molecules.

RPTC have been optimized for use with the XF-96 platform, and tested with several specific probes that are known to alter mitochondrial respiration¹⁴. The study by Beeson et. al. showed that RPTC cultured under standard conditions with 17 mM glucose had basal OCR 100-fold lower than rates observed in RPTC grown under the improved culture conditions. RPTC grown under optimized conditions were also treated with nephrotoxins to determine the ability of the XF instrument to measure mitochondrial dysfunction. The results showed that known mitochondrial toxicants (cisplatin, gentamicin, and HgCl₂) caused concentration dependent depression in FCCP-uncoupled OCR with no significant decrease in basal respiration. These data demonstrate that FCCP-uncoupled OCR is a sensitive measure of mitochondrial damage. Based on this information we tested 1760 diverse small molecules for mitochondrial toxicity to identify mitochondrial toxicophores which can be used to define and identify previously unrecognized and future toxicants.

Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the Medical

University of South Carolina protocol number 2974. All efforts were made to minimize animal suffering.

Unless otherwise noted all of the chemicals were purchased from Sigma-Aldrich

Isolation of proximal tubules—Female New Zealand white rabbits (1.5–2.0 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN, USA). Rabbit renal tubules were isolated using the iron oxide perfusion method as described in Nowak et al ²⁵. The resulting proximal tubules were plated on 100-mm tissue culture-grade plastic Petri dishes constantly swirled on an orbital shaker at 80 rpm. The culture medium was a 50:50 mixture of Dulbecco's modified Eagle's essential medium and Ham's F12 (lacking glucose, phenol red, and sodium pyruvate; Gibco BRL) and supplemented with HEPES (15 mM), glutamine (2.5 mM), pyridoxine HCl (1 μ M), sodium bicarbonate (15 mM), and lactate (6 mM). Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 μ g/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 μ M) were added to fresh culture medium. After three days the cells were trypsinized and re-plated onto XF-96 microplates at a concentration of 18,000 cells/well and maintained in a 37°C incubator for two days prior to experimentation ²⁴.

RPTC respirometric assay—The OCR measurements were performed using a Seahorse Bioscience XF-96 instrument according to the protocol outlined in Beeson et al ¹⁴. Each experimental plate was treated with vehicle controls (DMSO < 0.5%), a positive control (SRT1720, 10 μ M), blank controls, and the appropriate concentration of the compound of interest. The XF96 protocol consists of five measurements of basal OCR (1 measurement/ 1.5 min), injection of FCCP (0.5 μ M), and three measurements of uncoupled OCR (1 measurement/ 1.5 min). The consumption rates were calculated from the continuous average slope of the O₂ partitioning among plastic, atmosphere, and cellular uptake ¹⁷. Quality control evaluations included the basal and uncoupled rates of the vehicle control, positive control, and variances between duplicate treatment wells. Based on preliminary studies the toxicity threshold value was < 0.85 for the mean ratio of (chemical treatment FCCP-OCR/ vehicle control FCCP-OCR). This threshold is 1 S.D. below the historic mean for the vehicle control.

Assessment of RPTC viability—RPTC were plated into 96-well clear bottom black plates (Corning #3603) at a concentration of 30,000 cells/well. RPTC were washed and fixed with 4 % neutral buffer formalin solution. Each well was stained with 100 nM 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Calbiochem). Samples were analyzed by fluorescence microscopy using 350-nm excitation and 486-nm emission wavelengths in the IN Cell Analyzer 2000 (GE Healthcare). Analysis was performed using InCell Analyzer 3.7 software running the dual area object analysis. The parameters for normal nuclear morphology and size were set between 100–200 μ m². Micronuclei were determined to be 50% of control nuclei area. The number of normal, micro- and bi-nucleated cells were counted in four fields for each well. Cell count/viability was calculated by subtracting the number of altered nuclei from the total number of nuclei.

Mitochondrial isolation—Renal cortical mitochondria were isolated from female New Zealand White rabbits (2kg), as previously described²⁹. Kidney cortex was minced and homogenized in ice-cold mitochondrial isolation buffer (0.27 M sucrose, 5 mM Tris·HCl, and 1 mM EGTA, pH 7.4). Nuclei and cellular debris were pelleted by centrifugation at $1,000 \times g$ for 10 min. The supernatant was centrifuged at $10,000 \times g$ for 5 min, resulting in a crude mitochondrial pellet. The pellet was washed once in ice-cold mitochondrial isolation buffer and resuspended in mitochondrial incubation buffer (130 mM KCl, 9 mM Tris-PO₄, 4 mM Tris·HCl, and 1 mM EGTA, pH 7.4). The mitochondria were diluted in mitochondrial assay buffer (137 mM KCl, 2 mM KH₂PO₄, 2.5 mM MgCl₂, 20 mM HEPES, 0.5 mM EGTA, 0.2% fatty-acid free BSA, pH 7.4) containing mitochondrial substrates (6mM pyruvate, 5 mM malate).

Mitochondrial respirometric assay—The OCR measurements were performed using a Seahorse Bioscience XF-96 instrument according to the protocol outlined in Rogers et al¹³. The XF96 protocol consists of two measurements of State 2 OCR (2 min mix /2 min measure), injection of ADP (4 μ M) preceding two measurements of State 3 OCR (0.5 min mix /2 min measure), and injection of FCCP (1 μ M) preceding two measurements of uncoupled OCR (0.5 min mix /2 min measure). The consumption rates were calculated from the continuous average slope of the O₂ partitioning among plastic, atmosphere, and cellular uptake¹⁷. Mitochondrial OCR was normalized to total protein in each well.

Toxicophore modeling of potential mitochondrially toxic compounds—

Modeling, simulations and visualizations were performed using MOE Version 2010.10 (Chemical Computing Group Inc.). Computational procedures were performed using a Dell XPS T3400 with an Intel Core2 Duo processor E8500@3.16 GHz with 3GB RAM using a Windows XP OS. The Sigma LOPAC and the ChemBridge DiverSet were clustered using the Tanimoto score with MACCS structural keys fingerprinting (*Maccs II*; Molecular Design Ltd.). Using tables of the compounds represented as Simplified Molecular Input Line Entry Specification (SMILES) strings were imported into MOE as a mdb database. Molecules were rigidly aligned manually, and then subjected to MOE flexible body refinement (configuration limit 100, alpha 1, gradient test 0.01, RMSD tolerance 0.5, maximum steps 500). Consensus toxicophores were calculated using a distance parameter (tolerance) maintained at the default value of 1.2 angstroms and a threshold of 100%. Toxicophore feature projections including Pi rings (PiN), H-bond acceptors (Don2), and H-bond acceptors (Acc2) were not included in the analysis.

Statistical Analysis—Data are presented as mean $\pm$ s.e.m. and were tested for normality. The cellular and mitochondrial respiration data failed a normality test, therefore a Kruskal-Wallis one-way ANOVA on ranks was conducted. Multiple means were compared utilizing Dunn's post hoc test and were considered statistically different when $P < 0.05$. RPTCs and mitochondria isolated from a single animal represented an individual experiment ($N=1$) and were repeated until an N four was obtained.

Results

High-throughput screen in RPTC identifies potential mitochondrial toxicants

To assess the dynamic range of mitochondrial function, a HTS was conducted on RPTC exposed to both pharmacologically defined and random small molecules. Differentiated RPTC were plated onto specialized 96-well-plates, treated with 5 μ M of the test compounds for 24 h, and evaluated on the XF-96 for basal and FCCP-uncoupled OCR (FCCP-OCR). The ligand database was chosen to maximize diversity and the relatively high dose and long incubation period were chosen for the initial screen in order to maximize the number of compounds with potential mitochondrial toxicity. A total of 1760 compounds were tested: 1280 chemicals from the Sigma Library of Pharmacologically Active Compounds (LOPAC) and a random 480 compound subset from the ChemBridge DIVERSet of 50,000 small molecules.

Vehicle and blank control wells were present on each plate. Quality control evaluations were based on the control basal and uncoupled respiration rates, the responsiveness of the cells to positive controls, and evaluation of the variances between two duplicate wells for each compound. Maximum accepted variances ranged from 12–15%, which is within the accuracy of the instrument. Beeson et. al., established that RPTC exposed to mitochondrial toxicants exhibited decreases in FCCP-OCR equal to or greater than 20% even in the absence of altered basal respiratory capacity¹⁴. The results of the screen identified compounds that alter mitochondrial respiration by either increasing or decreasing FCCP-OCR relative to the vehicle control (Fig. 1a). For the purpose of this study, a compound was classified as a potential toxicant based on a mean ratio of: (chemical-treated FCCP-OCR)/(vehicle control FCCP-OCR) < 0.85. This value is $\pm$ 1 standard deviation (SD) from the mean ratio for the vehicle control. From our analysis we identified 33 compounds that caused a significant decrease in FCCP-uncoupled OCR compared to the vehicle control when treated with 5 μ M for 24 hr (Fig. 1b Supp. Table 1).

Compounds confirmed as mitochondrial toxicants in RPTC

To determine whether observed decreases in FCCP-OCR were the result of mitochondrial toxicity or cell death, RPTC were exposed to 5 μ M of the 33 potential mitochondrial toxicants for 1, 6, or 24 hr and examined for changes in FCCP-OCR and cell count. This combination of time points allowed for the identification of both fast and slow acting mitochondrial toxicants, and those that cause cell death concurrent with mitochondrial toxicity.

To evaluate cell count RPTC nuclei were stained with DAPI (4',6-diamidino-2-phenylindole), visualized by fluorescence microscopy, and counted using the GE Health Sciences INCell 1000 automated imager. Compounds were classified as decreasing mitochondrial respiratory capacity based a FCCP-OCR $\leq$ 85% of the vehicle control and as decreasing cell viability based a cell count $\leq$ 85% of the vehicle control. At each time point, the compounds were separated into four groups: 1) no effect on mitochondrial function or cell viability, 2) change in mitochondrial function with no effect on cell viability, 3) no

effect on mitochondrial function and a decrease in cell viability, and 4) change in mitochondrial function and a decrease in cell viability.

After a 1 h exposure to 5 μ M 8 compounds caused a decrease in FCCP-OCR without a decrease in cell count, identifying them as mitochondrial toxicants (Fig. 3). Interestingly, 4 compounds caused an increase in FCCP-OCR without affecting cell count. These four compounds were either cytotoxic or mitochondrially toxic after the longer incubation times suggesting mitochondrial toxicity through an uncoupling of ATP production from oxidative phosphorylation. Therefore 12 of the compounds were identified as fast acting mitochondrial toxicants. After a 6 h exposure 14 compounds caused a decrease in FCCP-OCR without a decrease in cell count (Fig. 4). Ten of these compounds were not toxic after 1 h exposure. Therefore these compounds were identified as moderate mitochondrial toxicants. After a 24 h exposure 15 of the tested compounds caused a decrease FCCP-OCR without a decrease in cell count (Fig. 5). Nine of these compounds were not toxic after a 1 or a 6 h exposure. Therefore these compounds were identified as fast acting mitochondrial toxicants. Only one compound (GW7647) was classified as mitochondrially toxic at all of the time points examined. Thus, of the 33 compounds tested, 31 (94%) of the compounds were classified as fast, moderate or slow acting mitochondrial toxicants after 1, 6 or 24 h exposures (Table 1). Two of the compounds were classified as being cytotoxic with no confirmed mitochondrial toxicity: NSC 95397 and sanguinarine chloride.

Compounds clustered based on chemical similarity

The 31 mitochondrial toxicants were first analyzed for molecular similarity using the Molecular Operating Environment (MOE) software package (Chemical Computing Group, Inc) and compounds clustered based on Tanimoto coefficients (TC). The TC is an association rank of one-dimensional chemical fingerprints that identifies the presence of molecular elements, and facilitates rapid comparisons³⁰. Utilizing the TC associations, a chemogram was generated using Chemmine single linkage hierarchical clustering (Fig. 6a). A heat map was generated for 24 h % control FCCP-OCR indicating highly toxic (red) and moderately toxic (white) compounds as determined by RPTC OCR. A heat map was also generated for time to toxicity indicating fast (1 h; red), medium (6 h; orange), and slow (24 h; white) acting mitochondrial toxicants. The original TC analysis identified two major clusters of chemical similarity including the two TBB and Tryphostin derivatives. These compounds are greater than 30% similar and were among the least toxic. Due to the small size and high degree of chemical similarity, these clusters were not further analyzed.

The remaining 27 compounds were analyzed for similar structural features independent of two-dimensional atom connectivity. The goal of this analysis was to align chemical features in three dimensional chemical space. Analysis of the data indicated that a TC of 0.54 (54% similarity) was optimal for clustering chemically similar compounds within our dataset (Fig. 6b). There were three clusters with only two compounds of moderate chemical similarity: 10058-F4/AS-604850, rotenone/sanguinarine, CB9012591/CB9012096. These clusters were not analyzed further considering that they consisted of only two compounds with highly similar chemotypes. We were able to rationalize 17 of the 31 mitochondrial toxicants into six clusters containing two or more compounds. Two of the identified clusters were further

developed into robust toxicophore models (Fig. 7 and Fig. 8). Cluster A is a xanthine-type cluster comprised of ChemBridge 9017474, 1,3-dimethyl-8-phenylxanthine, 3-isobutyl-1-methylxanthine (IBMX), and calcimycin. Cluster B is a sulfonylbenzene-type cluster comprised of ST-148, ML-7, ChemBridge 9017720 and cefsulodin. Although dequalinium has similar chemical features, it was not incorporated into Cluster B because the extended ten carbon alkene in dequalinium made the three dimensional alignment ineffective. Because of the low number of chemicals in the remaining clusters, no further analysis was performed.

Mitochondrial toxicant clustering to develop mitochondrial specific toxicophores

Toxicophores are developed from the analysis of chemical alignment of toxicants like pharmacophores are developed from the alignment of drug-like chemicals³¹. The observed toxicities of many drugs and pesticides are associated with specific chemical structures within the compounds of interest^{32–34}. The presence of “toxic” structures in novel or untested compounds can be used to predict potential toxicity based on the assumption that compounds containing the same features, or toxicophore, are likely to cause similar toxic effects³⁵. As such, we propose that toxicophore modeling based on *in vitro* mitochondrial toxicity data can be used to predict deleterious effects of novel compounds on mitochondrial function.

The compounds within Clusters A and B were aligned based on the presence of a chemical feature within 100% of the components. It is important to note that the final toxicophore models contained additional features not present in the base chemical scaffolds used to rationalize the alignments. The alignments were refined first in two and then three dimensions, and resulted in the formation of two toxicophores containing features of conserved chemical similarity. The four compounds in Cluster A were aligned in chemical space: ChemBridge 9017474, 1,3-dimethyl-8-phenylxanthine, calcimycin and 3-isobutyl-1-methylxanthine (IBMX) (Fig. 7a). The alignment was refined, and resulted in a toxicophore with a xanthine-type core containing eight features of conserved chemical similarity (Fig. 7b). Calcimycin was included in Cluster A due to the structural similarity of the benzoxazole within the xanthine. Features in this model are as follows: F1 is a bioisostere of $-\text{CO}_2$ or an H-bond acceptor, F2 is a H-bond donor or acceptor, F3 and F8 are aromatic, F4 and F6 are H-bond acceptors, F5 is hydrophobic, and F7 is a hydrophobic or H-bond donor (Fig. 7c). Distances (in Å) are F1-F2: 3.36, F2-F3: 1.27, F3-F4: 1.26, F4-F5: 3.13, F5-F6: 2.40, F6-F7: 2.94, F7-F1: 3.10, F1-F8: 3.19, and F8-F3: 2.33.

The four molecules in Cluster B were analyzed and only three overlapped in chemical space: ST-148, ML-7, and ChemBridge 9017720 (Fig. 8a). Cefsulodin did not match the alignment. The alignment was refined, and resulted in a toxicophore containing seven features of conserved chemical similarity (Fig. 8b). Features in this model are as follows: F1-F7. F1: is a cation and H-bond donor, F2: is a hydrophobic, F3 and F4: are H-bond acceptors, F5 and F6: are aromatic or hydrophobic, F7: is hydrophobic (Fig. 8c). Distances (in Å) are F1-F2: 2.45, F1-F3: 2.89, F2-F4: 3.34, F3-F4: 2.64, F3-F5: 3.92, F4-F6: 4.15, F5-F6: 2.98, F6-F7: 4.59, and F7-F5: 3.93.

Compounds examined for toxicity in isolated renal mitochondria

The OCR in intact cells reflects a complex interplay of biological parameters including the rates of energy demand and production as well as the nature, availability, and transport of substrates, the effects of signaling cascades that impinge on mitochondrial function, and the overall mass/volume of mitochondria per cell. As a result, a secondary assay using isolated mitochondria was used¹³.

To confirm that the compounds in Cluster A and Cluster B cause mitochondrial toxicity, we isolated renal mitochondria from the kidney cortex of Female New Zealand White rabbits as previously described³⁶. Isolated mitochondria were exposed to 5 μ M of the compounds of interest for 5, 10 or 20 min and evaluated for State 3 OCR. State 3 OCR is defined as ADP-stimulated respiratory rate in the presence of O₂ and the substrates pyruvate and malate. Mitochondria exposed to 5 μ M calcimycin exhibited a reduction in State 3 OCR relative to DMSO control at all time points examined (Fig. 7d). After the 20 min exposures to ChemBridge 9017474, 1,3-dimethyl-8-phenylxanthine, and 3-isobutyl-1-methylxanthine State 3 OCR was reduced. Mitochondria exposed to both ML-7 and ST-148 exhibited a reduction in State 3 OCR relative to DMSO controls after the 20 min exposure (Fig. 8d). These data confirm that both Toxicophore A and Toxicophore B represent compounds that are mitochondrially toxic.

The 7-point toxicophore model of Toxicophore B was used to virtually search the ChemBridge DIVERSet for similar compounds. This library contains 50,080 unique drug-like compounds that represent a wide range of chemical diversity. These compounds were used to create a 1,420,467 entry conformer library and an *in silico* search identified 28 compounds containing all seven chemical features with a root mean square deviation (RMSD) < 1 Å for all features.

RPTC were exposed to 5 μ M of the 28 identified ChemBridge compounds for 24 hr and examined for changes in FCCP-OCR. This assay identified six compounds that cause at least a 15% decrease in FCCP-OCR compared to the vehicle control. One of the identified compounds was CB9017720, which was utilized to design the toxicophore. Five of the identified compounds were novel compounds: ChemBridge 904454, 5301163, 5951505, 5357507, and 6399416 (Fig. 9a, Supp. Table 2). These five compounds were examined for their effect on State 3 OCR 5, 10 and 20 min after a 5 μ M exposure (Fig. 9b). Isolated mitochondria dosed with 5 μ M of ChemBridge compounds 5301163, 5951505, and 5357507 showed significant decreases in State 3 OCR relative to DMSO after 20 min of exposure. Thus, toxicophore B was successfully used to identify novel mitochondrial toxicants.

Discussion

As the generators of cellular energy, mitochondria are essential and highly vulnerable organelles. Disruption of mitochondrial function can be catastrophic to cells, organs, and ultimately whole organisms. Currently there is limited information correlating mitochondrial toxicity with chemical structure. In addition, many studies of mitochondrial toxicity utilize cell lines that are primarily glycolytic and therefore less sensitive to perturbations in mitochondrial respiration/function. In this study we utilized a recently developed phenotypic

respirometric assay to screen for mitochondrial toxicants in primary cultures of RPTC. This assay was utilized to gather toxicity data from two chemically diverse libraries, the Sigma LOPAC and a 480 compound subset of the ChemBridge DIVERSet (1760 compounds total). Although these libraries are not characterized as enriched for toxicants, this screen identified 33 compounds that cause a significant decrease in FCCP-uncoupled OCR compared to the vehicle control when given at 5 μ M for 24 hours (Fig. 1). These compounds were identified as potential mitochondrial toxicants.

The initial screen was conducted at 24 h to ensure that all potential mitochondrial toxicants were identified. Those potential mitochondrial toxicants observed at 24 h were further tested after 1 and 6 h incubations to identify compounds that act quickly or slowly to produce mitochondrial toxicity before cell death occurs. Exposure of RPTC to 5 μ M of the 33 potential mitochondrial toxicants for 1 h revealed that 12 caused a decrease in mitochondrial respiration without a significant loss of cell viability. These 12 compounds were identified as fast-acting mitochondrial toxicants (Fig. 3). Four compounds caused an increase in FCCP-OCR without affecting cell count, suggesting mitochondrial toxicity through an uncoupling of ATP production from oxidative phosphorylation. Exposure of RPTC to the 33 potential mitochondrial toxicants for 6 h and 24 h identified an additional ten and an additional nine compounds, respectively, that produce mitochondrial toxicity (Fig. 4 and 5).

Twenty-two of the identified compounds were rapidly acting, causing mitochondrial toxicity after 1 or 6 h of exposure. Nine of the identified compounds only demonstrated mitochondrial toxicity after a prolonged exposure of 24 hours. A mitochondrial toxicant can be defined as any compound that causes a deleterious effect on mitochondrial function without a change in viability. For the nine identified slow acting mitochondrial toxicants, mitochondrial dysfunction may be a secondary effect of an alteration in protein translation or cellular signaling. Although mitochondrial toxicity may be a secondary effect, the nine compounds specifically altered cellular respiration contributing to toxicity. Therefore, for the purpose of this study these compounds were also classified as mitochondrial toxicants. Two compounds (NSC 95397 and sanguinarine chloride) induced cell death without causing overt mitochondrial dysfunction under any of the examined experimental conditions. These data indicate the RPTC assay is able to discriminate mitochondrial toxicants from cytotoxicants. Thus, of the 33 compounds tested, 31 (94%) of the compounds were classified as mitochondrial toxicants after 1, 6 or 24 h exposures.

A recent study conducted by Sakamuru et al identified 14 compounds from the LOPAC that disrupted mitochondrial membrane potential³⁷. Five of the compounds identified by Sakamuru were also identified in the current study, confirming that respirometry in RPTC as a viable method for identifying mitochondrial toxicity. Our study also identified an additional 26 mitochondrial toxicants, which highlights that our respirometric assay is more sensitive measure of mitochondrial toxicity when coupled with RPTC.

We examined the 31 mitochondrial toxicants for similarities in chemical structure. After clustering the compounds based on 54% chemical similarity, we identified two clusters (A and B) containing at least four compounds of interest. These clusters were subjected to 3D alignments and these alignments were used to define two toxicophores of mitochondrial

dysfunction (Fig. 7 & 8). In addition, the toxicants from Clusters A and B were evaluated further using isolated mitochondria and were shown to decrease State 3 respiration, confirming that the correlated toxicophore is associated with mitochondrial toxicity (Fig. 7 & 8).

Cluster A is comprised of three compounds from the LOPAC and one compound from the ChemBridge DIVERSet: calcimycin, IBMX, 1,3 dimethyl-8-phenylxanthine, and ChemBridge 9017474. Calcimycin is a Ca^{2+} ionophore and is widely used to study Ca^{2+} -dependent cell injury. Qian et al., showed that exposure of cultured rat hepatocytes to 10 μM calcimycin resulted in increased mitochondrial free Ca^{2+} , mitochondrial depolarization, and the onset of the mitochondrial permeability transition (MPT) ³⁸. IBMX is a competitive phosphodiesterase inhibitor and an adenosine antagonist ^{39,40}. Nissim et al., demonstrated that IBMX exposure inhibited the mitochondrial respiratory chain and reduced OCR during liver perfusion of rats ⁴¹. 1,3 dimethyl-8-phenyltheophylline is a selective A_1 adenosine receptor antagonist; however, there are no known effects of this compound on mitochondrial function ⁴². ChemBridge-9017474 is a novel small molecule with no currently available information regarding mitochondrial or cellular toxicity. Based on the previous studies with calcimycin, it is likely that the compounds in cluster A disrupt mitochondrial membrane potential resulting in dysfunctional mitochondrial respiration.

Cluster B is comprised of two molecules from the LOPAC and one compound from the ChemBridge DIVERSet: ST-148, ML-7 and ChemBridge 9017720. ST-148 is a D_2 dopamine receptor antagonist. Although there are no known effects of this compound on mitochondrial function, there is research indicating that antagonizing the dopamine receptors can result in mitochondrial toxicity⁴³. ML-7 is a selective myosin light chain kinase inhibitor. ML-7 also has no documented mitochondrial toxicity; however, myosins are known to affect organelle transport and are essential for mitochondrial function and localization⁴⁴. ChemBridge 9017720 is a small molecule with no currently available information regarding mitochondrial or cellular toxicity. It is possible that some of the compounds we tested are selectively imported and accumulate within RPTC through organic anion or cation transporters/exchangers, which can affect primary and secondary toxic effects. Future studies will examine the mechanism of toxicity for the two identified toxicophores.

The toxicophore resulting from Cluster B was used to screen the 50,000 small molecule ChemBridge DIVERSet. The *in silico* screen identified 28 compounds that matched seven chemical features that defined the toxicophore. When examined for their effects in RPTC, five compounds caused at least a 15% decrease FCCP-OCR 24 h after a 5 μM exposure (Supp. Table 2). The toxic compounds were examined in isolated mitochondria and four of them caused a decrease in State 3 respiration, confirming these compounds as mitochondrial toxicants (Fig. 9).

The analysis of the toxicity in a random selection of 480 compounds from the ChemBridge DIVERSet allowed for the calculation of predicted random rate of toxicity. Analysis of RTPC using the respirometric assay identified four mitochondrial toxicants providing a random toxic rate of 0.83%. Cluster B was used to identify 28 similar compounds from the

ChemBridge DIVERSet and five displayed mitochondrial toxicity. Thus the utilization of Cluster B resulted in a success toxicity rate of 19% which is highly significant and provides an enrichment factor of 22.3x.

A limitation of a spatial model like the toxicophores presented is that they do not provide the ability to predict potency; only the potential efficacy of the compounds to cause mitochondrial toxicity at the examined concentrations. It is possible that other identified compounds could be toxic if examined under alternative experimental conditions, including higher concentrations. It is also possible that lack of cellular permeability or biotransformation prevented toxicity of some the compounds examined.

The predictive toxicophore designed from Cluster B can now serve as a template to search larger chemical libraries to predict mitochondrial toxicity. A preliminary *in silico* screen of the Zinc Clean Leads database of 1.4 million vendor compounds identified 459 (0.03%) compounds that matched seven chemical features of the toxicophore with a RMSD < 1 Å. The models presented in this manuscript were not intended to identify all mitochondrial toxicants. This research does present two discrete models capable of predicting toxicants with variable chemistry within two general structural classes. These data, combined with past and future toxicant models, will provide the ability to identify possible mitochondrial toxicity in novel compounds.

Conclusions

The ability to predict mitochondrial toxicity from chemical structure will be invaluable for both drug development and risk assessment. We conducted a high-throughput respirometric assay in RPTC to create a database of mitochondrial toxicants that we clustered based on chemical similarity. The identified clusters were aligned and used to define atomic resolution toxicophores. We confirmed that the identified toxicophores were toxic to isolated mitochondria. Finally one toxicophore based on the structures of ML-7, ST-148, and ChemBridge 9017720 was used to conduct an *in silico* screen and resulted in the identification of five novel mitochondrial toxicants (Fig. 2). Our research has demonstrated that robust *in vitro* assays can be used as a platform for future *in silico* and cheminformatic studies to identify mitochondrial toxicants. Defining these toxicophores will provide insight into cellular pathways involved in mitochondrial dysfunction. Incorporating these models into established toxicant libraries will provide greater insight for pharmaceutical companies, agricultural companies, and government agencies when designing novel chemicals with a risk of human and environmental exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Respirometric assay conducted in RPTC to create mitochondrial toxicant database.
- Chemically similar mitochondrial toxicants aligned as mitochondrial toxicophores.
- Mitochondrial toxicophore identifies five novel mitochondrial toxicants.

Figure 1. High-throughput respirometric assay identifies mitochondrial toxicants in RPTC
(A) RPTC treated with 5 μ M of experimental compounds for 24 hr. A total of 1760 compounds were tested; 1280 chemicals from the Sigma Library of Pharmacologically Active Compounds and a random 480 compound subset from the ChemBridge DIVERSet of 50,080 small molecules. Data are average FCCP-OCR relative to the vehicle control. (B) 33 compounds from the respirometric assay identified as potential mitochondrial toxicants. Compounds were classified as potential mitochondrial toxicants based on a FCCP-OCR 85% of the vehicle control. Data represented as mean $\pm$ s.e.m. of four biological replicates. The blue line indicates the mean for vehicle control wells.

Figure 2.
Schematic of a respirometric screen to identify mitochondrial toxicants and design a predictive mitochondrial toxicophore.

Figure 3. Cell count and FCCP-OCR in RPTC after 1 h

Compounds were classified as decreasing mitochondrial respiratory capacity based on FCCP-OCR $\leq$ 85% of the vehicle control. Cell count was determined by measuring DAPI stained mono-nuclei. Compounds were classified as decreasing cell viability based a cell count $\leq$ 85% of the vehicle control. Red data points represent compounds that cause a decrease in FCCP-OCR without a decrease in cell count. Green data points represent compounds that cause mitochondrial uncoupling. (A) Average percent vehicle control FCCP-OCR and cell count of RPTC dosed with 5 μ M of potential mitochondrial toxicants for 1 h. (B) Compounds classified as mitochondrial toxicants in RPTC after 1 h exposure to 5 μ M. Data represented as a mean of four biological replicates.

Figure 4. Cell count and FCCP-OCR in RPTC after 6 h

Compounds were classified as decreasing mitochondrial respiratory capacity based on FCCP-OCR $\leq 85\%$ of the vehicle control. Cell count was determined by measuring DAPI stained mono-nuclei. Compounds were classified as decreasing cell viability based on cell count $\leq 85\%$ of the vehicle control. Red data points represent compounds that cause a decrease in FCCP-OCR without a decrease in cell count. Green data points represent compounds that cause mitochondrial uncoupling. (A) Average percent vehicle control FCCP-OCR and cell count of RPTC dosed with 5 μM of potential mitochondrial toxicants for 6 h. (B) Compounds classified as mitochondrial toxicants in RPTC after 6 h exposure to 5 μM . Data represented as a mean of four biological replicates.

Figure 5. Cell count and FCCP-OCR in RPTC after 24 h

Compounds were classified as decreasing mitochondrial respiratory capacity based on FCCP-OCR $\geq 85\%$ of the vehicle control. Cell count was determined by measuring DAPI stained mono-nuclei. Compounds were classified as decreasing cell viability based a cell count $\geq 85\%$ of the vehicle control. Red data points represent compounds that cause a decrease in FCCP-OCR without a decrease in cell count. Green data points represent compounds that cause mitochondrial uncoupling. (A) Average percent vehicle control FCCP-OCR and cell count of RPTC dosed with 5 μM of potential mitochondrial toxicants

for 24 h. (B) Compounds classified as mitochondrial toxicants in RPTC after 24 h exposure to 5 μ M. Data represented as a mean of four biological replicates.

Figure 6. Chemical clustering related to mitochondrial toxicity

MAACS keyed chemical fingerprints were used to cluster compounds based on molecular similarity as measured by Tanimoto Coefficient. (A) The chemogram was generated using Chemmine single linkage hierarchical clustering (<http://chemmine.ucr.edu/>). Three major clusters were identified and numbered within the chemogram. The 24 h % Control FCCP-OCR heat map indicates highly toxic (red) and moderately toxic (white) compounds as determined by RPTC OCR. The time heat map indicates fast (1 h; red), medium (6 h; orange), and slow (24 h; white) acting mitochondrial toxicants. (B) Histogram of identified mitochondrial toxicants clustered by chemical similarity. Chemical similarity determined by a TC cutoff of 0.5 (50 % similarity). The histogram was generated by TC binning, using 166 MACCS bit keys. Data represented as mean $\pm$ s.e.m. of four biological replicates. The blue line indicates the mean for vehicle control wells.

Figure 7. Toxicophore A identifies common features that cause mitochondrial toxicity in RPTC (A) Chemical structures of the four compounds from Cluster A: ChemBridge 9017474, 1,3-Dimethyl-8-phenylxanthine, calcimycin, and 3-Isobutyl-1-methylxanthine for 24 h. (B) Eight feature toxicophore A based on the alignment of cluster A. Each feature was identified in all of the four compounds defining the toxicophore. (C) Distances and feature names of the toxicophore. Distances listed in Å. Features or in this model are as follows: F1 is a bioisostere of -CO_2 an H-bond acceptor, F2 is a H-bond donor or acceptor, F3 and F8 are aromatic, F4 and F6 are H-bond acceptors, F5 is hydrophobic, and F7 is a hydrophobic or H-bond donor. (D) State 3 OCR in isolated mitochondria from rabbit renal cortex dosed with 5 μM of compounds comprising Cluster A for 5, 10, or 20 min. State 3 OCR is defined as ADP-stimulated respiration in the presence of O_2 and the substrates pyruvate and malate. Isolated mitochondria dosed with 5 μM calcimycin showed a significant decrease in State 3 OCR relative to DMSO at all of the time points examined. Isolated mitochondria dosed with 5 μM ChemBridge 9017474, 1,3-dimethyl-8-phenylxanthine, and 3-isobutyl-1-methylxanthine showed a significant decrease in State 3 OCR relative to DMSO after 20 min exposures. Data represented as mean $\text{pmol O}_2/\text{min}/\text{mg protein} \pm \text{s.e.m.}$ of four biological replicates (* $P < 0.05$).

Figure 8. Toxicophore B identifies common features that cause mitochondrial toxicity in RPTC (A) Chemical structures of the three compounds from cluster B that aligned in chemical space: ST-148, ML-7, and ChemBridge 9017720. (B) Seven feature toxicophore based on the alignment of cluster B. Each feature was identified in all three of compounds defining the toxicophore. (C) Distances and feature names of the toxicophore. Distances listed in Å. Features in this model are as follows: F1-F7. F1: is a cation and H-bond donor, F2: is a hydrophobic, F3 and F4: are H-bond acceptors, F5 and F6: are aromatic or hydrophobic, F7: is hydrophobic. (D) State 3 OCR in isolated mitochondria from rabbit renal cortex dosed with 5 μ M of compounds comprising Cluster B for 5, 10, or 20 min. Isolated mitochondria dosed with 5 μ M ML-7 and ST-148 showed significant decreases in State 3 OCR relative to DMSO after a 20 min exposure. Data represented as mean pmol O₂/min/mg protein $\pm$ s.e.m. of four biological replicates (* $P < 0.05$).

Figure 9. Toxicophore B identifies four novel mitochondrial toxicants

The 7-point toxicophore model of Toxicophore B was used to conduct an *in silico* search the ChemBridge DIVERSet of 50,080 unique drug-like compounds. Twenty-seven compounds were identified containing all seven chemical features with a root mean square deviation (RMSD) < 1 Å. (A) Five novel compounds were identified that cause a decrease in FCCP-OCR in RPTC after a 24 h exposure to a 5 µM concentration: ChemBridge 904454, 5301163, 5951505, 5357507, and 6399416 (Supp. Table 2). (B) State 3 OCR in isolated mitochondria from rabbit renal cortex dosed with 5 µM of compounds identified by Toxicophore B for 5, 10, or 20 min. Isolated mitochondria dosed with 5 µM of ChemBridge compounds 5301163, 5951505, and 5357507 showed significant decreases in State 3 OCR relative to DMSO after 20 min of exposure. Isolated mitochondria dosed with 5 µM ChemBridge 904454 and 6399416 showed no significant decrease in State 3 OCR relative to DMSO under any of the experimental conditions. Data represented as mean pmol O₂/min/mg protein ± s.e.m. of four biological replicates (* *P* < 0.05).

Table 1

Fast, moderate, and slow acting mitochondrial toxicants identified in RPTC

Compound	Classification
AC-93253 iodide	Fast
AS 604850	Fast
Cantharidic Acid	Fast
CGP-74514A hydrochloride	Fast
Dequalinium chloride hydrate	Fast
Diphenyleneiodonium chloride	Fast
GW5074	Fast
GW7647	Fast
Rotenone	Fast
Rottlerin	Fast
Tyrphostin A9	Fast
Tyrphostin AG 879	Fast
10058-F4	Moderate
7-Cyclopentyl-5-(4-phenoxy)phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine	Moderate
BRL 15572	Moderate
Calcimycin	Moderate
Emodin	Moderate
Retinoic acid p-hydroxyanilide	Moderate
ST-148	Moderate
TBB	Moderate
TBBz	Moderate
XCT790	Moderate
1,3-Dimethyl-8-phenylxanthine	Slow
3-Isobutyl-1-methylxanthine	Slow
6-Hydroxy-DL-DOPA	Slow
CB9012096	Slow
CB9012591	Slow
CB9017474	Slow
CB9017720	Slow
Cefsulodin sodium salt hydrate	Slow
ML-7	Slow