Application Note

# Screening for Drug Induced Mitochondrial Dysfunction: Cellular Metabolism and Bioenergetics Analysis

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## Key Features

- Determining drug-induced mitochondrial dysfunction
- Evaluating for mitochondrial dysfunction by measuring oxygen consumption
- Identifying inhibition of specific mitochondrial complexes using novel non-antibody based assays

# Introduction

Mitochondria produce the majority of the ATP utilized by mammalian cells through oxidative phosphorylation. The mitochondrion also plays a central role in a number of crucial biochemical processes required for cellular homeostasis and acts as the gatekeeper for apoptosis with the release of cytochrome *c*.[1] In order for the mitochondrion to act in such a role, proper function is essential. Since the majority of the biochemical processes occurring in mitochondria depend on the existence of a membrane potential ($\Delta\psi_M$), it is expected that any phenomenon, pharmacologic or otherwise, that disrupts ΔψM will cause dysfunction and impact cellular function.[2] Because of this, mutations or environmental factors affecting mitochondrial function are at the root of a number of neurodegenerative diseases.[3-5] Mitochondrial dysfunction has also been linked to heart disease, diabetes, and certain types of cancer.[1,6]

A number of drugs on the market with black-box warnings are known to exhibit adverse effects on mitochondrial function that result in cardio- and hepatotoxic side effects.[7] Therefore, methods to screen for mitochondrial toxicity in a high throughput manner are rapidly gaining value in the pharmaceutical industry. Due to the multifaceted biochemistry of the mitochondrion, it is important that the primary screens encompass the whole system to yield generalized information, *i.e.* determine if the compound has a mitochondrial effect or not. The next phase would be to run functional assays in order to understand which components are being affected and what the mechanism is that causes any effect. In this report, a series of assays (white boxes in **Figure 1**) were employed to determine the mitochondrial toxicity of a panel of five antipsychotic compounds (**Table 1**) of which some were known to affect mitochondrial function.

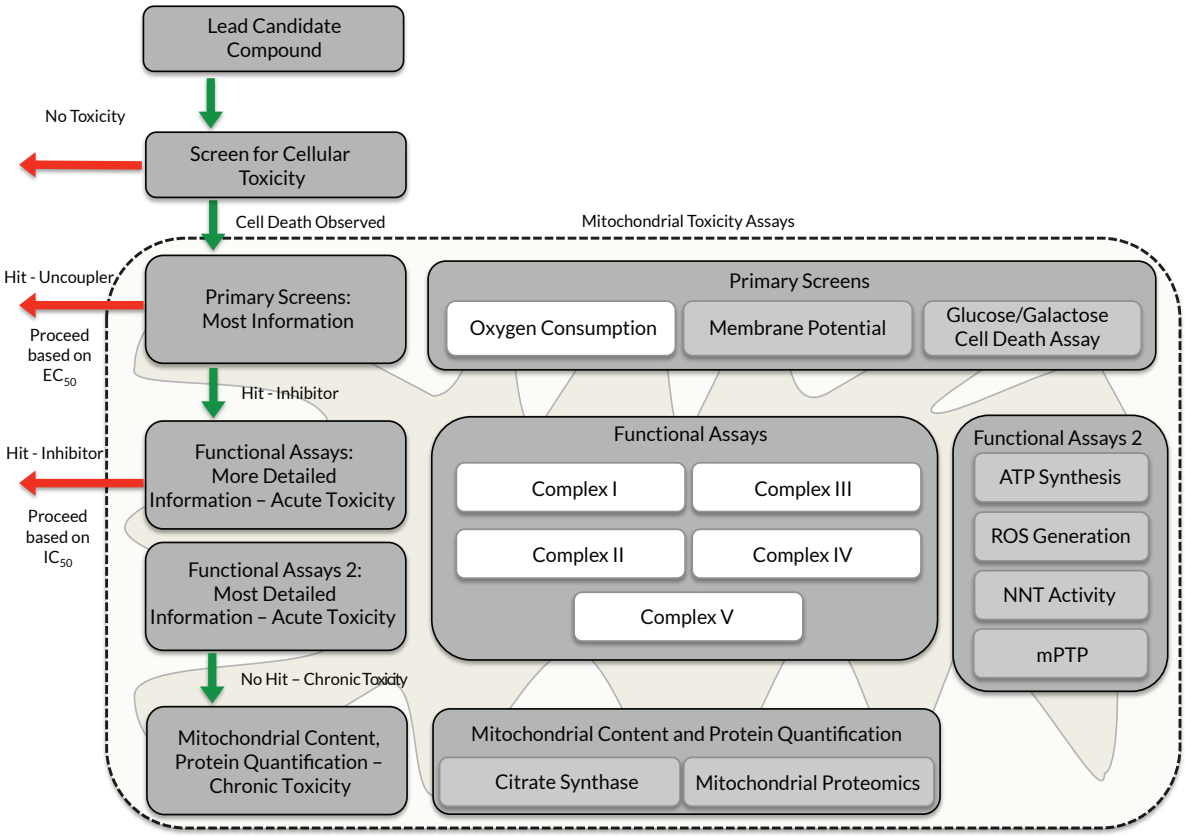



**Figure 1.** Screening assays used to determine mitochondrial toxicity

# Methods and Materials

## *General Information*

All reagents were obtained from Cayman Chemical Company unless otherwise noted. Compounds were dissolved in DMSO and added to 96-well mother plates with a maximal concentration of 100 mM (100X). Duplicate 10-point ½-log dilutions were performed from these stocks using a robotic liquid handling system. The Z'-factor was calculated from 12 positive controls and 12 vehicle wells. Using the liquid handler, 1 µl of liquid from each mother plate was transferred into each assay plate with a final reaction volume of 100 µl. The final concentration of DMSO in each assay was 1%. Mitochondrial electron transport chain (ETC) activity screens were carried out using a Synergy H4 plate reader (BioTek Instruments) at 25°C. All assays utilized and compounds tested can be found in **Table 2**.

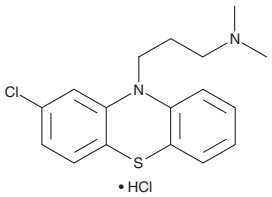
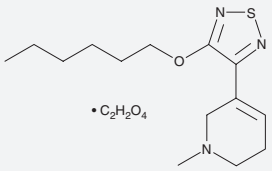
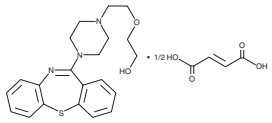
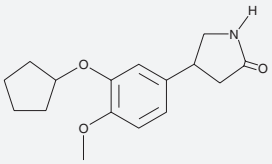
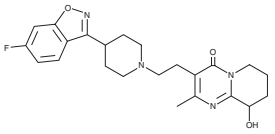
## *Mitochondrial Isolation*

Freshly isolated mouse liver mitochondria were obtained by differential centrifugation within 2 hours prior to the start of each experiment. Briefly, livers were placed into ice-cold mitochondrial isolation medium (MIM) containing sucrose (250 mM), Tris (10 mM, pH 7.4, at 4°C) and EGTA (1 mM) and minced finely. Tissue was washed with ice-cold MIM, and homogenized using a 40 ml Dounce homogenizer (pestle A). Tissue homogenate was centrifuged at 1,000 × g for 3 minutes to pellet cellular debris. The supernatant was centrifuged at 10,000 × g for 10 minutes and the resulting supernatant discarded. The pellet was suspended in MIM and the high speed centrifugation step repeated. This step was repeated an additional 3 times with the final pellet homogenized in ~800 µl of MIM. Protein concentration was determined using the BCA method (Life Technologies). The respiratory control ratio (RCR) was determined for this prep by measuring the rate of state three respiration (phosphorylating) over the rate of state four respiration (quiescent). RCR values for experiments with freshly isolated mitochondria were >5, which is the expected value for liver mitochondria. State three and four respiration rates were measured using a Clark-type oxygen electrode (YSI Lifesciences).

## *Primary Assay Oxygen Consumption*

Plate based oxygen consumption assays were performed in 96-well format using phosphorescent oxygen probe (**Item# 600801**) in a fluorescent plate reader equipped with 340±50 nm excitation and 650±50 nm emission filters (BMG Labtech). Oxygen consumption experiments were carried out in respiration medium containing HEPES (10 mM, pH 7.4, at 37°C), KCl (120 mM), sucrose (25 mM), succinate (10 mM), malate (10 mM), glutamate (5 mM), $MgCl_2$ (5 mM), KH2PO4 (5 mM), ADP (2 mM), EGTA (1 mM), and a final mitochondrial protein content of 0.12 mg/ml. Test compounds were transferred to each assay plate as indicated above. Oxygen consumption rates were determined as lifetime of probe (µs) vs. time (min). Rates were then normalized as percentages of vehicle control. The positive control used for the inhibition of oxygen consumption was antimycin A at a maximal concentration of 10 µM.

**Table 1.** Antipsychotic compounds screened for mitochondrial toxicity

| Compound | Structure | Target Receptors | Known Mitochondrial Effect? | Reference |
|---|---|---|---|---|
| Chlorpromazine (hydrochloride) |  | Dopamine, Serotonin 5-$HT_1$, and 5-$HT_2$, α-Adrenergic, Muscarinic | Yes - Complex V, I, ANT inhibition | 8-12 |
| Xanomeline oxalate |  | Muscarinic | No Data - However, oxalate has been linked to ROSgeneration/ mitochondrial dysfunction | 13-18 |
| Quetiapine (hemifumarate) |  | Dopamine $D_{1-3}$, Serotonin 5-$HT_{1A}$, 5-$HT_{2A}$ and 5-$HT_7$, $\alpha_{1a}$-, $\alpha_{1b}$-, $\alpha_{2c}$-Andrenergic, Histamine $H_1$ | Yes - Complex I inhibition | 11, 19-21 |
| Rolipram |  | PDE4 | No Data | 22-24 |
| Paliperidone |  | Dopamine $D_2$ and $D_3$, $\alpha_1$- and $\alpha_2$-Adrenergic, Histamine $H_1$, Serotonin 5-$HT_{1D}$ and 5-$HT_{2A}$ | Mixed - affects mitochondrial proteins, yet can restore function in quinolimic acid toxicity | 12, 25-28 |

# *Functional Assays*

## Complex I

Complex I activity was determined using the MitoCheck® Complex I Activity Assay Kit. This assay measures the rotenone sensitive rate of NADH oxidation by complex I in isolated bovine heart mitochondria. To prevent oxidation of ubiquinol by complex III, KCN (1 mM) was present to inhibit the downstream ETC. The positive control used for this assay was rotenone at a starting concentration of 10 µM.

## Complex II

Complex II activity was determined using the MitoCheck® Complex II Activity Assay Kit, which measures the succinate dependent rate of DCPIP reduction in isolated bovine heart mitochondria. To prevent oxidation of ubiquinone by complex III, and reverse electron transfer from complex II to complex I, antimycin A (10 µM), KCN (1 mM), and rotenone (1 µM) were present for all experiments. The positive control used for this assay was 2-thenoyltrifluoroacetone (TTFA) at a starting concentration of 10 mM.

## Complex III

Complex III activity was determined using the MitoCheck® Complex II/III Activity Assay Kit. This assay measures the rate of cytochrome c reduction by the passage of electrons from complex II to complex III *via* ubiquinone in isolated bovine heart mitochondria. To prevent oxidation of cytochrome c by complex IV, KCN (1 mM) was present for all experiments. The positive control used for this assay was antimycin A at a starting concentration of 10 µM.

### Complex IV

Complex IV activity was determined using the MitoCheck® Complex IV Activity Assay Kit, which measures the rate of cytochrome c oxidation by complex IV in isolated bovine heart mitochondria. The positive control used for this assay was KCN at a starting concentration of 10 mM.

### Complex V

Complex V activity was determined using the MitoCheck® Complex V Activity Assay Kit. This kit measures the rate of NADH reduction resulting from a series of coupled reactions linked to the hydrolysis of ATP by complex V in isolated bovine heart mitochondria. The positive control used for this assay was oligomycin at a starting concentration of 10 µM. Rotenone (1 µM) was present for all experiments to prevent NADH oxidation by complex I. Counter screens to test for inhibition of nonspecific ATPases were carried out in the presence of 10 µM oligomycin.

### Data Analysis

Absorbance vs. time was plotted for each assay. The rates were determined by plotting the linear portion of the curve. The rate vs. concentration was plotted and $IC_{50}$ values were calculated using 4-paramater nonlinear regression fit in Prism 6.05 (GraphPad Software, Inc.). The means ± standard deviation are shown for all assays.

## Results and Discussion

A panel of five antipsychotic compounds were screened for mitochondrial toxicity. The effects of each compound on oxygen consumption rates were measured. Freshly isolated mitochondria were essential for this study since frozen mitochondria are unable to undergo oxidative phosphorylation due to inner membrane damage upon freezing. In a freshly isolated mitochondrial system, proton translocation by the ETC results in the generation of $\Delta\psi_M$, which is proportional to the oxygen consumption rate. Because of this, any perturbation of ΔψM would subsequently result in a change in the oxygen consumption rate. Since phosphorylation of ADP by complex V utilizes $\Delta\psi_M$, inhibition of ATP synthesis would result in a decreased utilization of $\Delta\psi_M$, and a transition from state three respiration (phosphorylating) to state four respiration (non-phosphorylating). This change is commonly observed in coupled mitochondria upon the addition of the complex V inhibitor, oligomycin. In order to detect the inhibitory effects of the compounds on both the ETC and oxidative phosphorylation, the oxygen consumption experiments were performed with freshly isolated, coupled mitochondria, instead of frozen, uncoupled mitochondria.

Primary screens, measuring oxygen consumption (**Figure 1**), revealed that four of the five compounds resulted in mitochondrial perturbation (**Figure 2**). Treatment with the known complex III inhibitor antimycin A, as a control, resulted in complete inhibition of mitochondrial oxygen consumption with an $IC_{50}$ value of 12.2 nM (**Figure 2A**). The test compounds resulted in partial inhibition of mitochondrial oxygen consumption, suggesting inhibition of state three respiration (*via* ATP synthase or adenine nucleotide translocase (ANT) inhibition) or partial inhibition of the ETC at either complex I or complex II. Observed $IC_{50}$ values for mitochondrial oxygen consumption were 1.2 µM for chlorpromazine (80% inhibition), 4.5 µM for xanomeline oxalate (90% inhibition), 10.2 µM for quetiapine (40% inhibition) and 54 µM for paliperidone (40% inhibition). To determine the mechanism by which oxygen consumption was inhibited, compounds were then screened against a panel of functional assays (**Figure 1**). This would reveal any inhibitory effects on complexes I-IV of the ETC and complex V. **Figure 3** shows the positive control dose response curves for each of these assays.

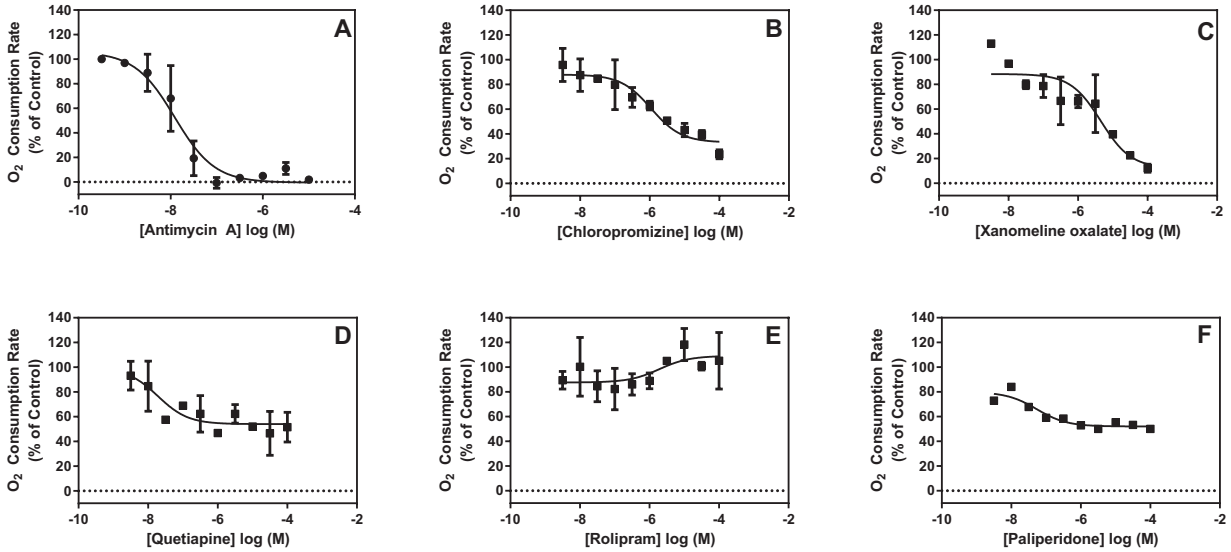



**Figure 2.** Dose response curves for oxygen consumption rates. $IC_{50}$ values were 12.2 nM for antimycin A **A.**, 1.2 µM for chlorpromazine **B.**, 4.5 µM for xanomeline oxalate **C.**, 10.2 µM for quetiapine **D.**, no effect for rolipram at the concentrations tested and **E.**, 54 µM for paliperidone **F**.

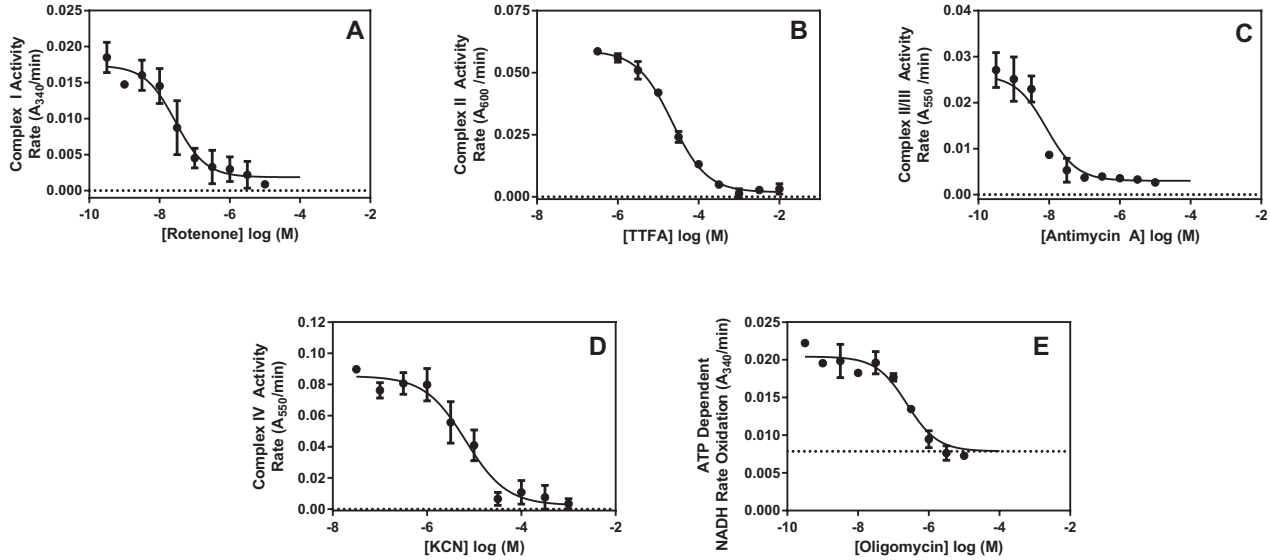



**Figure 3.** Positive control dose response curves for MitoCheck® ETC activity assays for complexes I-IV and complex V. Respective $IC_{50}$ values for each complex were 28 nM (rotenone - complex I, **A.**, 22 µM (TTFA – complex II, **B.**, 8.3 nM (antimycin A - complex III, **C.** 6.6 µM (KCN – complex IV, **D.**, and 241 nM (oligomycin – complex V, E. Z'-values for all data included.

Screens for complex I inhibition revealed that four out of five compounds tested inhibited complex I with respective $IC_{50}$ values of 105 µM for chlorpromazine, 54.9 µM for xanomeline oxalate, and 35 µM for paliperidone (**Figure 4B**). While inhibition was observed with quetiapine, an accurate $IC_{50}$ could not be calculated from the concentrations tested due to limited solubility in aqueous buffers. Rolipram did not inhibit complex I activity at the concentrations tested. These data support the oxygen consumption rate experiments, indicating that a partial, but not full inhibition of the ETC is present. Should full inhibition at complex I and II, complex III, or complex IV be present, oxygen consumption would cease as electrons would be unable to reach the terminal site of complex IV, where $O_2$ is reduced. Additionally, should the ETC be fully inhibited, the resulting dose response curve would be similar to that with the complex III inhibitor antimycin A, showing full inhibition of oxygen consumption. However, full inhibition of the ETC was not observed, and this was further verified by a lack of inhibition by any of the tested compounds when measuring complex II, III, or IV activity (data not shown).

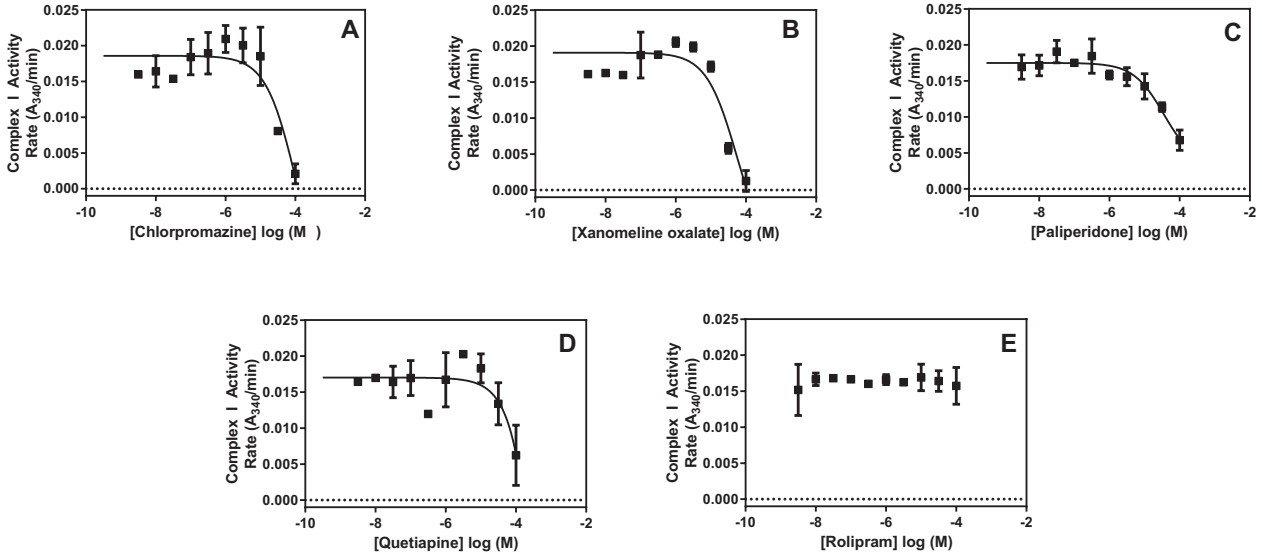



**Figure 4.** Dose response curves for complex I activity. Respective $IC_{50}$ values were 105 µM for chlorpromazine **A.**, 54.9 µM for xanomeline oxalate **B.**, 35 µM for paliperidone **C.** $IC_{50}$ values for quetiapine could not to be calculated from the data provided **D.** rolipram did not inhibit complex I at the concentrations tested **E.**

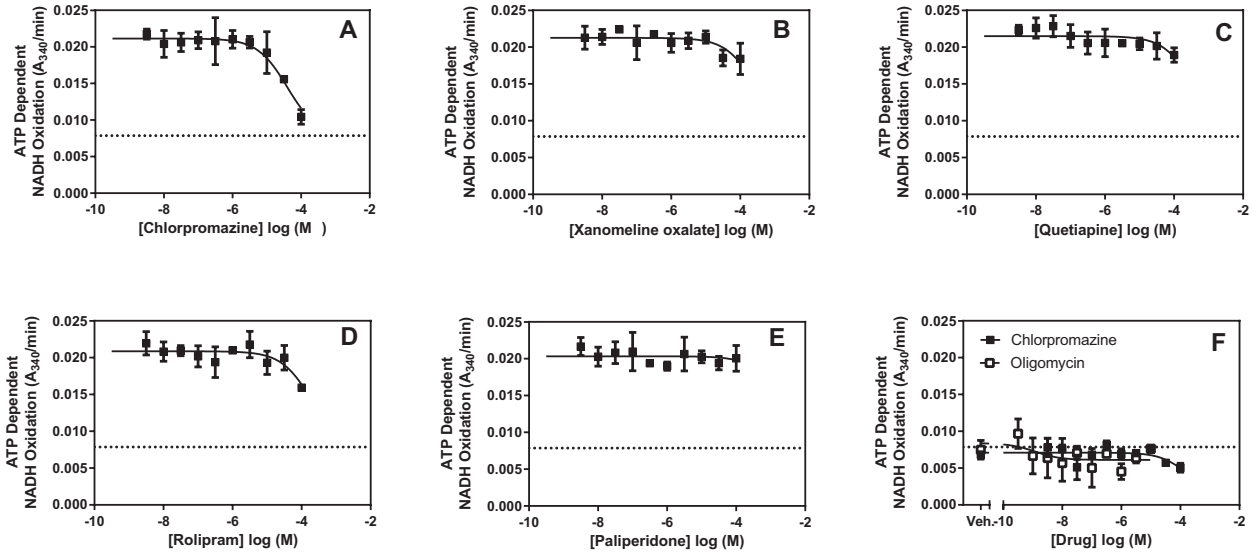



**Figure 5**. Dose response curves for ATP-dependent NADH oxidation (complex V activity). $IC_{50}$ values for complex V inhibition were 39 µM for chlorpromazine **A.**, 289 µM for xanomeline oxalate **B.**, 379 µM for quetiapine **C.** 188 µM for rolipram **D.** and no inhibitory effect from paliperidone at the concentrations tested **E.** Panel **F** shows a counter screen with saturating concentrations of oligomycin showing the chlorpromazine is not functioning as an inhibitor of non-specific ATPases or the coupling enzymes.

Because full ETC inhibition was not observed in any of the assays performed, it was hypothesized that the observed inhibition of oxygen consumption (**Figure 2**) was due to a partial inhibition of complex V. However, results from complex V activity screens revealed that, with the exception of chlorpromazine ($IC_{50}$ = 39 µM), none of the compounds tested significantly inhibited complex V activity (**Figure 5**). These data suggest an alternative mechanism by which oxygen consumption is inhibited, such as, the inhibition of the ANT10, which is responsible for the import of ADP (and export of ATP) into the mitochondrial matrix. Because inhibitors of ANT prevent ADP from reaching complex V, inhibitors of ANT or complex V would have identical effects on state three respiration, making them indiscernible without a direct screen for one or the other. Another possible mechanism is that the tested compounds also influence the import of substrates required for oxygen

consumption, although the data from the functional assays suggest that this is unlikely. While the exact mechanism by which chlorpromazine and xanomeline oxalate affect mitochondrial function is somewhat ambiguous, the data show clear evidence of mitochondrial inhibition. In the future, further experiments will be conducted to further elucidate the exact mechanism in which these compounds perturb mitochondrial function. In summary, the data presented show the ability of the available assays (**Figure 1**) to accurately and reproducibly detect perturbations inmitochondrial function providing a means by which to screen compounds for mitochondrial toxicity.

**Table 2.** Cayman Assay Kits and Compounds Utilized

| Primary Screens | Item No. |
|---|---|
| Oxygen Consumption Rate Assay Kit (MitoXpress®- Xtra HS Method) | 600800 |

| Functional Assays | Item No. |
|---|---|
| MitoCheck® Complex I Activity Assay Kit | 700930 |
| MitoCheck® Complex II Activity Assay Kit | 700940 |
| MitoCheck® Complex II/III Activity Assay Kit | 700950 |
| MitoCheck® Complex IV Activity Assay Kit | 700990 |
| MitoCheck® Complex V Activity Assay Kit | 701000 |

| Compounds Tested | Item No. |
|---|---|
| Chlorpromazine (hydrochloride) | 16129 |
| Xanomeline oxalate | 10790 |
| Quetiapine (hemifumarate) | 14152 |
| Rolipram | 10011132 |
| Paliperidone | 15556 |

## References


1. Brookes, P.S., Yoon, Y., Robotham, J.L., *et al.* Calcium, ATP, and ROS: A mitochondrial love-hate triangle. *Am. J. Physiol. Cell Physiol.* **287(4)**, C817-833 (2004).
2. Sone, N., Yoshida, M., Hirata, H., *et al.* Adenosine triphosphate synthesis by electrochemical proton gradient in vesicles reconstituted from purified adenosine triphosphatase and phospholipids of thermophilic bacterium. *J. Biol. Chem.* **252(9)**, 2956-2960 (1977).
3. Wallace, D.C. Mitochondrial diseases in man and mouse. *Science* **283(5407)**, 1482-1488 (1999).
4. Van Erven, P.M., Fischer, J.C., Gabreels, F.J., *et al.* Defect of NADH dehydrogenase in Leigh syndrome. *Acta Neurol. Scand.* **74(2)**, 167 (1986).
5. Kubik, L.L., and Philbert, M.A. The role of astrocyte mitochondria in differential regional susceptibility to environmental neurotoxicants: Tools for understanding neurodegeneration. *Toxicol. Sci.* **144(1)**, 7-16 (2015).
6. Sabharwal, S.S., and Schumacker, P.T. Mitochondrial ROS in cancer: Initiators, amplifiers or an Achilles' heel? Nat. Rev. *Cancer* **14(11)**, 709-721 (2014).
7. Dykens, J.A., and Will, Y. The significance of mitochondrial toxicity testing in drug development. *Drug Discov. Today* **12(17-18)**, 777-785 (2007).
8. Abood, L.G. Effect of chlorpromazine on phosphorylation of brain mitochondria. *Proc. Soc. Exp. Biol. Med.* **88(4)**, 688-690 (1955).
9. Dawkins, M.J., Judah, J.D., and Rees, K.R. The effect of chlorpromazine on the respiratory chain. Cytochrome oxidase. *Biochem. J.* **72(2)**, 204-209 (1959).
10. Dawkins, M.J., Judah, J.D., and Rees, K.R. Action of chlorpromazine. 3. Mitochondrial adenosine triphosphatase and the adenosine triphosphate- adenosine diphosphate exchange. *Biochem. J.* **76**, 200-205 (1960).
11. Modica-Napolitano, J.S., Lagace, C.J., Brennan, W.A., et al. Differential effects of typical and atypical neurolep¬tics on mitochondrial function *in vitro*. *Arch. Pharm. Res.* **26(11)**, 951-959 (2003).
12. Lepping, P., Sambhi, R.S., Whittington, R., *et al.* Clinical relevance of findings in trials of antipsychotics: Systematic review. *Br. J. Psychiatry* **198(5)**, 341-345 (2011).
13. Andersen, M.B., Fink-Jensen, A., Peacock, L., *et al.* The muscarinic $M_1/M_4$ receptor agonist xanomeline exhibits antipsychotic- like activity in *Cebus apella* monkeys.*Neuropsychopharmacology* **28(6)**, 1168-1175 (2003).
14. Heinrich, J.N., Butera, J.A., Carrick, T., *et al.* Pharmacological comparison of muscarinic ligands: Historical versus more recent muscarinic $M_1$-preferring receptor agonists. *Eur. J. Pharmacol.* **605(1-3)**, 53-56 (2009).
15. Jiang, S., Li, Y., Zhang, C., *et al.* $M_1$ muscarinic acetylcholine receptor in Alzheimer's disease. *Neurosci. Bull.* **30(2)**, 295-307 (2014).
16. Romero, A., Cacabelos, R., Oset-Gasque, M.J., *et al.* Novel tacrine-related drugs as potential candidates for the treatment of Alzheimer's disease. *Bioorg. Med. Chem. Lett.* **23(7)**, 1916-1922 (2013).
17. Stanhope, K.J., Mirza, N.R., Bickerdike, M.J., *et al.* The muscarinic receptor agonist xanomeline has an antipsychotic- like profile in the rat. *J. Pharmacol. Exp. Ther.* **299(2)**, 782-792 (2001).
18. McMartin, K.E., and Wallace, K.B. Calcium oxalate monohydrate, a metabolite of ethylene glycol, is toxic for rat renal mito¬chondrial function. *Toxicol. Sci.* **84(1)**, 195-200 (2005).
19. Richelson, E., and Souder, T. Binding of antipsychotic drugs to human brain receptors focus on newer generation compounds. *Life Sci.* **68(1)**, 29-39 (2000).
20. Seeman, P., and Tallerico, T. Antipsychotic drugs which elicit little or no parkinsonism bind more loosely than dopamine to brain $D_2$ receptors, yet occupy high levels of these receptors. *Mol. Psychiatry* **3(2)**, 123-134 (1998).
21. Kroeze, W. K., Hufeisen, S. J., Popadak, B. A., *et al.* $H_1$-histamine receptor affinity predicts short-term weight gain for typical and atypical antipsychotic drugs. *Neuropsycho¬pharmacology 28(3)*, 519-526 (2003).
22. Sommer, N., Loschmann, P. A., Northoff, G. H., *et al.* The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis. *Nat. Med.* **1(3)**, 244-248 (1995).
23. Souness, J. E., Griffin, M., Maslen, C., *et al.* Evidence that cyclic AMP phosphodiesterase inhibitors suppress $TNF_\alpha$ generation from human monocytes by interacting with a 'low-affinity' phosphodiesterase 4 conformer. *Br. J. Pharmacol.* **118(3)**, 649-658 (1996).
24. Tenor, H., Hatzelmann, A., Church, M. K., *et al.* Effects of theophylline and rolipram on leukotriene $C_4$ (LTC4) synthesis and chemotaxis of human eosinophils from normal and atopic subjects. *Br. J. Pharmacol.* **118(7)**, 1727- 1735 (1996).
25. Corena-McLeod Mdel, P., Oliveros, A., Charlesworth, C., *et al.* Paliperidone as a mood stabilizer: A pre-frontal cortex synaptoneurosomal proteomics comparison with lithium and valproic acid after chronic treatment reveals similarities in protein expression. *Brain Res.* **1233**, 8-19 (2008).
26. Kane, J., Canas, F., Kramer, M., *et al.* Treatment of schizophrenia with paliperidone extended-release tablets: A 6-week placebo-controlled trial. *Schizophr. Res.* **90(1-3)**, 147-161 (2007).
27. Mishra, J., and Kumar, A. Improvement of mitochondrial function by paliperidone attenuates quinolinic acid-induced behavioural and neurochemical alterations in rats: Implications in Huntington's disease. *Neurotox. Res.* **26(4)**, 363-381 (2014).
28. Seeman, P., Corbett, R., and Van Tol, H. H. Atypical neuroleptics have low affinity for dopamine $D_2$ receptors or are selective for $D_4$ receptors. *Neuropsychopharmacology* **16(2)**, 93-110; discussion 111-135 (1997).