



Target identification of drug induced mitochondrial toxicity using immunocapture based OXPHOS activity assays

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Abstract

Mitochondrial dysfunction has been shown to be a pharmacotoxicological response to a variety of currently-marketed drugs. In order to reduce attrition due to mitochondrial toxicity, high throughput-applicable screens are needed for early stage drug discovery. We describe, here, a set of immunocapture based assays to identify compounds that directly inhibit four of the oxidative phosphorylation (OXPHOS) complexes: I, II, IV, and V. Intra- and inter-assay variation were determined and specificity tested by using classical mitochondrial inhibitors. Twenty drugs, some with known mitochondrial toxicity and others with no known mitochondrial liability, were studied. Direct inhibition of one or more of the OXPHOS complexes was identified for many of the drugs. Novel information was obtained for several drugs including ones with previously unknown effects on oxidative phosphorylation. A major advantage of the immunocapture approach is that it can be used throughout drug screening from early compound evaluation to clinical trials.

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1. Introduction

It is estimated that only 10% of Investigational New Drugs (IND) are approved by the FDA, with 30% failing to reach the market as a result of toxicity issues. It is also widely estimated that, on average, the launch of a drug in the US requires a decade of R&D, and costs approximately \$800 million–1 billion (Kola and Landis, 2004). To reduce

this attrition, high throughput-applicable screens are needed to identify unacceptable levels of toxicity early in the drug discovery and pre-clinical stages. Many drug failures or withdrawals have been associated with mitochondrial toxicity, such as tolcapone, troglitazone and amiodarone (Chan et al., 2005).

Mitochondria are the energy powerhouses of cells and make approximately 95% of the cell's ATP, utilizing oxidative phosphorylation (OXPHOS). This process is carried out by the oxidative phosphorylation complexes. Free energy released by oxidation of substrates within the mitochondrial matrix is used to reduce the electron carriers, NAD (nicotinamide adenine dinucleotide) and FAD (flavin adenine dinucleotide) to form NADH and FADH₂ which, in turn, donate electrons to Complex I (NADH-ubiquinone oxidoreductase) and Complex II (succinate-ubiquinone oxidoreductase), respectively. Electrons from Complexes I and II are transferred to Complex III (ubiquinol-cytochrome *c* oxidoreductase) and Complex IV (cytochrome *c* oxidase), and the energy that is released during electron transfer is

Abbreviations: ATP, adenosine 5' triphosphate; BSA, bovine serum albumin; CV, coefficient of variation; FDA, Food and Drug Administration; HMG-CoA reductase, hydroxy methyl glutaryl Coenzyme A reductase; HTS, high throughput screening; IND, investigational new drug; NADH, nicotinamide adenine dinucleotide, reduced; NCE, new chemical entity; NSAID, non-steroidal anti inflammatory drug; NRTI, nucleoside reverse transcriptase inhibitor; OXPHOS, oxidative phosphorylation; R&D, research and development; SAR, structure-activity relationship; SD, standard deviation; TBS, tris buffered saline.

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used to build a chemiosmotic gradient across the inner mitochondrial membrane. This gradient is used by Complex V (the ATP synthase, F_1F_0) for ATP synthesis (Wallace and Starkov, 2000). Mitochondria also play a crucial role in apoptosis (programmed cell death), fatty acid oxidation, heme synthesis and steroid synthesis. They contain at least 600 different proteins (Taylor et al., 2003), 13 of which are encoded on mitochondrial DNA (mtDNA) and made using the mitochondrion's own replication machinery.

Considering the complexity of mitochondria, it is not surprising that they are often the target of drug-induced toxicity. Mitochondrial injury can occur through several mechanisms, such as (a) uncoupling of electron transport from ATP synthesis (nonsteroidal anti-inflammatory drugs (NSAIDs), sulfonamides, fatty acids), (b) redox-cycling (quinones, nitroarenes), (c) opening of the mitochondrial permeability transition pore (bile acids, anticancer drugs), (d) depletion of mtDNA (nucleoside reverse transcriptase inhibitors (NRTIs)), (e) inhibition of the Krebs cycle (fluoroacetate), (f) inhibition of β -fatty acid oxidation (tetracyclines, NSAIDs), (g) inhibition of transporters and (h) inhibition of the OXPHOS complexes. Not surprisingly, this multitude of mechanisms has precluded the identification of simple structure–activity relationships (SAR) by which to predict mitochondrial liability of compounds. Therefore, we are seeking to develop a screening paradigm which optimally removes mitotoxic compounds early and provides mechanistic information for improved compound synthesis.

There are several screens of overall mitochondrial toxicity that have been used in early screening of compounds, including measurement of dehydrogenase activities using Alamar Blue™ and determination of ATP production using the luciferin-luciferase luminescent assay (Schoonen et al., 2005). Also recently, an HTS applicable method to monitor mitochondrial oxygen consumption in isolated mitochondria has been introduced (Hynes et al., 2006). This method distinguishes between uncouplers and inhibitors of mitochondrial respiration. However, this assay does not identify mitochondrial enzyme(s) whose inhibition is directly responsible for any observed toxicity.

Here, we describe five high-throughput microplate-based activity assays of the oxidative phosphorylation complexes. In four assays, a 96-well plate coated with a monoclonal antibody raised against one of the OXPHOS complexes is used to immunocapture a functionally active complex from small amounts of biological material. We show that the assays are reproducible and have low intra- and inter-assay coefficients of variation using bovine heart as a source of mitochondria. Furthermore, we show the specificity of the Complex I, II, IV and V activity assays by using their respective classical inhibitors rotenone, TTFA, KCN, and oligomycin. For assay validation, 20 compounds, both with reported and non-reported mitochondrial toxicity profiles, were then evaluated with these assays. A Complex II + III activity assay was also included as part of the screening procedure, although this assay did not rely on immunocap-

tured material. All five activity assays allow for rapid screening of drugs and provide a high-throughput, low cost method of identifying mitochondrial toxicity *in vitro*. We confirmed the oxidative phosphorylation inhibition previously described in the literature. In addition, we were able to obtain new information on several compounds.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (St Louis, MO) and Toronto Research Chemicals (Toronto, Canada) and were of the highest purity available. Nunc Maxisorp clear bottom 96-well plates were purchased from Fisher (Hampton, NH). Protein G plates were from Pierce (Rockford, IL). All monoclonal antibodies were from MitoSciences Inc. (Eugene, OR).

2.1. Isolation of bovine heart mitochondria

All steps were carried out at 4°C with ice-cold buffers. Bovine ventricular tissue was cut into small pieces, added to 1.5 volumes of Buffer A (250 mM sucrose, 10 mM Tris–HCl (Tris(hydroxymethyl)aminomethane hydrochloride), 0.2 mM EDTA, pH 7.8) and homogenized, first in a blender and then with a Polytron probe in 15 second intervals until smooth. The pH of the homogenate was maintained at 7.8 by addition of 2 M Tris. The homogenate was centrifuged at 1000g for 15 min at 4°C to pellet the debris, and the supernatant filtered through four layers of cheesecloth and recentrifuged at 1000g for 10 min at 4°C. The pellet was discarded and the supernatant centrifuged at 12,000g for 15 min at 4°C to sediment the mitochondria. The mitochondria were washed twice by resuspending in four volumes of Buffer B (250 mM sucrose, 10 mM Tris–HCl, 0.2 mM EDTA, 0.5 mM PMSF (phenylmethylsulfonyl fluoride), pH 7.8) and centrifuging at 12,000g for 15 min at 4°C. The final pellet was resuspended in a small volume of Buffer B, and the mitochondrial protein concentration determined by the BCA (bicinchoninic acid, Pierce) method. The mitochondria were frozen in 10–20 mg/ml aliquots at –80°C. Mitochondria remain functional for the described assays for at least six months.

2.2. Solubilization of bovine heart mitochondria

Bovine heart mitochondria had to be solubilized prior to assaying Complex I, II, IV and V activities. Mitochondria were diluted to 5.5 mg/ml in 50 mM Tris–HCl, 1% (v/v) Protease Inhibitor cocktail (Sigma), pH 7.5 and solubilized by addition of a 1/10th volume of 10% (w/v) *n*-dodecyl β -D maltopyranoside (Anatrace). The final protein concentration was 5 mg/ml and the final detergent concentration was 1%. The sample was left on ice for 20 min and centrifuged at 25,000g in a microfuge centrifuge at 4°C for 20 min. The supernatant was diluted to the appropriate concentration for each assay (see below) and used for measuring Complex I, II, IV and V activities.

2.3. Measurement of activities of individual OXPHOS complexes

All activity assays were done in 96-well plates. For drug treatments, compound stock solutions were prepared in dimethyl sulfoxide (DMSO), added to multichannel Dilux™ Dilution Reservoirs (ISC BioExpress, Kaysville, UT) containing the appropriate assay solution and then dispensed into each 96-well plate in quadruplicate wells: in the Complex I, II, IV and V activity assays, measurements for a compound at a given concentration were done in triplicate wells coated with the appropriate immunocapture monoclonal antibody and a single well containing a null capture antibody used as a negative control (the null capture antibody was raised against a neuron-specific RNA binding protein as described in Marusich et al., 1994); in the Complex II+III activity assay, measurements for a compound at a given concentration were done in triplicate wells with bovine heart mitochondria and a single well with no mitochondria (negative control). The final DMSO concentration in all these activity assays was 1.5% (v/v). This concentration of DMSO did not have an inhibitory effect in any of the assays. Each assay was read in a SpectraMax Plus³⁸⁴ plate reader immediately after addition of the assay solution (containing the drugs) to the 96-well plates.

2.3.1. Measurement of complex I activity (NADH-ubiquinone oxidoreductase)

Complex I activity was measured in 96-well format. Protein G plates (Pierce) were coated with an anti-Complex I mAb 20D1AB7 (MS101c, MitoSciences Inc., Eugene OR) at 40 µg/ml in PBS, and incubated overnight at 4 °C. To control for non-specific binding of Complex I, a non-specific mouse mAb was coated in separate wells of each plate as a null capture mAb. The plates were washed three times with 50 mM Tris-HCl, pH 7.5, incubated for 2 h at room temperature with 15 µg/well solubilized bovine heart mitochondria (in 50 mM Tris-HCl, pH 7.5), washed three times with 20 mM Tris-HCl, 50 mM KCl, 0.015% (w/v) *n*-dodecyl β-D maltopyranoside pH 7.5, and incubated for 45 min at 4 °C with 56 µg/ml phosphatidylcholine that had been dissolved in 20 mM Tris-HCl, 50 mM KCl, 0.015% (w/v) *n*-dodecyl β-D maltopyranoside pH 7.5.

Complex I activity was measured by adding an assay solution containing 25 mM KH₂PO₄ pH 7.2, 5 mM MgCl₂, 1.2% (w/v) bovine serum albumin (BSA), 0.15 mM Coenzyme Q₁ and 0.26 mM NADH. The oxidation of NADH was monitored by measuring its decrease in absorbance at 340 nm in a SpectraMax Plus³⁸⁴ plate reader (Molecular Devices) in kinetic mode at 30 °C for 90 min. The rate was linear during this period.

2.3.2. Measurement of complex II activity (succinate-ubiquinone oxidoreductase)

The Complex II activity assay developed by J.G. Murray (MitoSciences Inc.) was measured in 96-well format. Nunc maxisorp plates were coated with an anti-Complex II mAb

4H12BG12AG2 (MS201c, MitoSciences Inc.) at 5 µg/ml in 50 mM KH₂PO₄, pH 7.2, and incubated overnight at 4 °C. To control for non-specific binding of Complex II, a non-specific mouse mAb was coated in separate wells of each plate as a null capture mAb. The plates were then aspirated, blocked with 5% non-fat dry milk (dissolved in 50 mM KH₂PO₄, pH 7.2) for 2 h at room temperature, washed with 50 mM KH₂PO₄, pH 7.2, and incubated with 25 µg/well solubilized bovine heart mitochondria (in 20 mM KH₂PO₄, pH 7.2, 0.015% (w/v) *n*-dodecyl β-D maltopyranoside) for 2 h at room temperature, and washed twice with 20 mM KH₂PO₄, pH 7.2, 0.015% (w/v) *n*-dodecyl β-D maltopyranoside.

Complex II activity was measured by adding an assay solution containing 25 mM KH₂PO₄, pH 7.2, 20 mM sodium succinate, 65 µM Coenzyme Q₂, 50 µM dichlorophenolindophenol and 0.115% (w/v) *n*-dodecyl β-D maltopyranoside. The decrease in absorbance of dichlorophenolindophenol was measured at 600 nm in a SpectraMax Plus³⁸⁴ plate reader in kinetic mode at room temperature for 30 min. The rate was linear during this period.

2.3.3. Measurement of complex II+III activity (succinate-cytochrome *c* oxidoreductase)

Complex II+III activity was measured in 96-well format based on a method described by Kramer et al. (2005) with modifications. Bovine heart mitochondria (3 µg/well) was added to an assay solution containing 25 mM sodium succinate, 37 mM KH₂PO₄, 12 µM rotenone, 0.18 mM KCN, and 200 µM oxidized cytochrome *c* (Sigma C3131), pH 7.5. The reduction of cytochrome *c* was monitored by measuring its increase in absorbance at 550 nm in a SpectraMax Plus³⁸⁴ plate reader in kinetic mode for 5 min at room temperature. The rate was linear during this period.

2.3.4. Measurement of complex IV activity (cytochrome *c* oxidase)

The Complex IV activity assay was measured in 96-well format as described by Murray et al. (2007). Briefly, Nunc maxisorp plates were coated with an anti-Complex IV mAb 31E91B82G9 (MS401c, MitoSciences Inc.) at 5 µg/ml in 50 mM KH₂PO₄, pH 7.2, and incubated overnight at 4 °C. To control for non-specific binding of Complex IV, a non-specific mouse mAb was coated in separate wells of each plate as a null capture mAb. The plates were then aspirated, blocked with 5% non-fat dry milk (dissolved in 50 mM KH₂PO₄, pH 7.2) for 2 h at room temperature, washed with 50 mM KH₂PO₄, pH 7.2, incubated with 0.1 µg/well solubilized bovine heart mitochondria (in 20 mM KH₂PO₄, pH 7.2, 0.015% (w/v) *n*-dodecyl β-D maltopyranoside) for 3 h at room temperature, and washed twice with 20 mM KH₂PO₄, pH 7.2, 0.015% (w/v) *n*-dodecyl β-D maltopyranoside.

Complex IV activity was measured by adding an assay solution containing 60 µM reduced cytochrome *c*, 20 mM KH₂PO₄, pH 7.2, and 0.015% (w/v) *n*-dodecyl β-D maltopyranoside. The oxidation of reduced cytochrome *c* was monitored by measuring its decrease in absorbance at 550 nm

in a SpectraMax Plus³⁸⁴ plate reader in kinetic mode at room temperature for 20 min. The rate was linear during this period.

2.3.5. Measurement of complex V activity (F_1F_0 -ATPase)

Complex V activity was measured in 96-well format as described by Aggeler et al. (2002) with slight modifications. Nunc maxisorp plates were coated with goat anti-mouse IgG-Fc (Fc γ for all subclasses, Jackson ImmunoResearch, Westgrove, PA) at 5 μ g/ml in TBS (50 mM Tris–HCl, 0.5 M NaCl, pH 7.5), incubated overnight at 4°C, washed three times with TBS, coated with an anti-Complex V capture mAb 12F4AD8AF8 (MS501 c, MitoSciences Inc.) at 5 μ g/ml in TBS containing 2.5% (w/v) BSA and incubated overnight at 4°C. To control for non-specific binding of Complex V, a non-specific mouse mAb was coated in separate wells of each plate as a null capture mAb. The plates were washed three times in TBS and incubated for 2 h at room temperature with 20 μ g/well solubilized bovine heart mitochondria (in TBS containing 2.5% BSA), and then washed three times with TBS.

Complex V activity was measured by addition of an ADP-coupled assay solution containing 25 mM HEPES, 25 mM KCl, 2 mM MgCl₂, 2 mM phosphoenolpyruvate, 2 mM ATP, 0.5 mM NADH, 30 units/ml Pyruvate Kinase and 30 units/ml L-Lactic Dehydrogenase, pH 7.5. The decrease in absorbance of NADH was measured at 340 nm in a SpectraMax Plus³⁸⁴ plate reader in kinetic mode at 30°C for 30 min. The rate was linear during this period.

2.4. Data analysis

Absorbance values obtained during all activity assays on the SpectraMax Plus³⁸⁴ plate reader were exported from SoftmaxPro to either Excel or SigmaPlot. 100% activity (i.e., no inhibition) for each complex was determined as the mean of the triplicate measurement in absence of compound – negative control value in absence of compound. IC₅₀ values were generated using a four-parameter logistic equation. For establishment of significance, 1-way ANOVA was performed followed by Tukey's *post hoc* test. The following statistical significance values were reported: * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

Table 1
The intra- and inter-assay coefficients of variation of the five OXPHOS activity assays

	n	Complex I Activity		Complex II Activity		Complex II + III Activity		Complex IV Activity		Complex V Activity	
		Mean \pm SD (mOD/min)	CV (%)	Mean \pm SD (mOD/min)	CV (%)	Mean \pm SD (mOD/min)	CV (%)	Mean \pm SD (mOD/min)	CV (%)	Mean \pm SD (mOD/min)	CV (%)
Day 1	3	3.46 \pm 0.28	8.1	20.31 \pm 2.40	11.8	105.37 \pm 1.95	1.9	4.57 \pm 0.51	11.2	13.32 \pm 0.67	5.0
Day 2	3	3.94 \pm 0.41	10.4	19.97 \pm 2.36	11.8	101.74 \pm 2.39	2.4	4.91 \pm 0.36	7.3	13.50 \pm 1.16	8.6
Day 3	3	3.94 \pm 0.30	7.6	21.52 \pm 2.73	12.7	94.02 \pm 4.62	4.91	4.71 \pm 0.49	10.4	12.83 \pm 1.08	8.4
Day 4	3	3.43 \pm 0.28	8.2	18.46 \pm 1.98	10.7	107.34 \pm 4.78	4.45	4.35 \pm 0.42	9.7	12.26 \pm 1.17	9.5
Inter assay Day 1–4		3.69 \pm 0.32	8.7	20.06 \pm 2.38	11.9	102.12 \pm 3.66	3.6	4.63 \pm 0.45	9.7	12.97 \pm 1.04	8.0

Data are described as Mean \pm SD and coefficient of variation (CV). Further details are described in Section 2.

3. Results

3.1. Validation of the immunocapture based OXPHOS assays: intra- and inter-assay variation

As with all newly developed assays, the first step was to establish the intra- and inter-assay variation to ensure that quantitative, meaningful and reproducible results could be obtained on a daily basis. Thus, the Complex I, II, IV and V immunocapture activity assays and, for completeness, a Complex II + III activity assay were performed in 96-well format as described in detail in Section 2. Mitochondria from bovine heart were used as the biological material due to the relative ease of purification of large amounts of the organelle from this readily available tissue. However, as discussed later, these assays can be done with different tissues and different species and do not require prior isolation of mitochondria. Each measurement was done in triplicate in wells coated with the appropriate immunocapture antibody for the Complex I, II, IV and V assays. In the case of the Complex II + III assay, intact mitochondria were used. Table 1 summarizes the intra-assay and inter-assay coefficients of variation for each of the assays. The intra- and inter-assay variations were less than 15% (Table 1), an acceptable value for continuation of our studies.

3.2. Specificity of the immunocapture based OXPHOS assays: effect of classical inhibitors

The Complex I, II, IV and V activity assays as well as the Complex II + III assay were evaluated with classical inhibitors of OXPHOS complexes in order to test their specificity and to provide a baseline toxicity against which other compounds could potentially be compared. Rotenone, a classical inhibitor of Complex I, blocks electron transfer from Fe–S centers to the ubiquinone binding site within this complex (Horgan et al., 1968). Fig. 1a shows that rotenone was a potent inhibitor of the activity of Complex I immunocaptured from bovine heart mitochondria. Thus, Complex I activity was 85% sensitive to rotenone; the IC₅₀ was 17.3 nM. Rotenone did not inhibit Complex II, II + III, IV or V (data not shown).

2-Thenoyltrifluoroacetone (TTFA) inhibits Complex II by binding to this complex's ubiquinone-binding sites

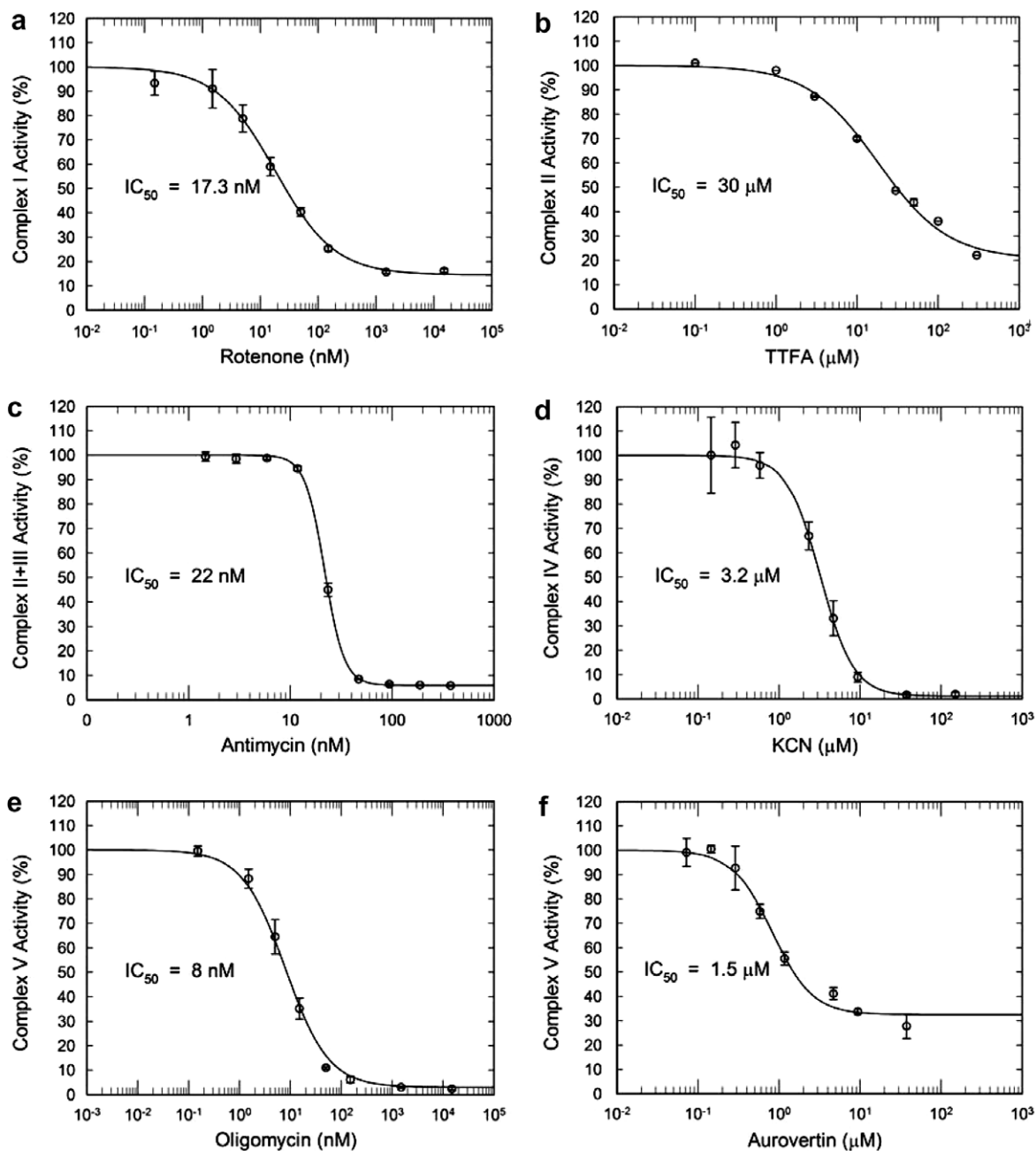


Fig. 1. Effect of rotenone on Complex I activity (a), TTFA on Complex II activity (b), Antimycin on Complex II + III activity (c), KCN on Complex IV activity (d), Oligomycin on Complex V activity (e), and Aurovertin on Complex V activity (f). 100% represents the activity of uninhibited enzyme. Each data point is the mean of triplicate measurements. The error bars represent calculated standard deviations. The line is a best fit using a four-parameter logistic equation.

(Sun et al., 2005). As shown in Fig. 1b, inhibition of immunocaptured Complex II by this compound occurred with an IC_{50} of 30 μ M. The IC_{50} for TTFA inhibition of Complex II in mitochondria (the Complex II + III assay) was 23 μ M (result not shown). TTFA did not inhibit any of the other complexes (data not shown).

Antimycin, an antibiotic that blocks electron transfer from heme b_H to a quinone within Complex III (Schagger et al., 1995), inhibited Complex II + III activity with an IC_{50} of 22 nM (Fig. 1c). Antimycin had no effect on the other complexes (data not shown).

KCN, a noncompetitive inhibitor of Complex IV (Isom and Way, 1984), binds to the heme a_3 site within this complex and blocks electron transfer to oxygen. Fig. 1d demonstrates that Complex IV immunocaptured from bovine heart mitochondria was inhibited by KCN with an IC_{50} of 3.2 μ M. KCN had no effect on the other complexes (data not shown).

Fig. 1e and f show the effect of two Complex V inhibitors, oligomycin and aurovertin. Oligomycin inhibits Complex V when the catalytic F_1 sector of this complex is bound to the proton-conducting F_0 sector (Walker et al., 1995).

90% of Complex V activity immunocaptured from bovine heart mitochondria was inhibited by oligomycin; the IC_{50} was 8 nM. In contrast to oligomycin, aurovertin is an inhibitor of the F_1 sector of Complex V, binding to two of the three catalytic sites (van Raaij et al., 1996). Fig. 1f shows that Complex V activity was inhibited ~70% by aurovertin, with an IC_{50} of 1.5 μ M.

To summarize, the activity of each complex was inhibited by its classical inhibitors at concentrations that ranged from low nanomolar to low micromolar.

3.3. Screening of therapeutic drugs using the immunocapture based OXPHOS assays

The main aim of the assays described in this paper is to systematically and rapidly screen new chemical entities (NCEs) for any direct inhibition of the OXPHOS complexes. The complexes were tested using 20 different drugs, many with previously reported mitochondrial toxicity. Each drug was tested at 50 μ M concentration in triplicates as described in detail in Section 2. The selected drugs were chosen to be representative of many different drug classes and also different observed *in vivo* toxicities. Table 2 summarizes the therapeutic application, the C_{max} and the observed *in vivo* toxicities for each of the compounds tested.

Complex I activity was strongly inhibited (>70%) by nefazodone (Fig. 2a). Approximately 25% inhibition was observed with simvastatin and paroxetine. Slight (<25%), but statistically significant inhibition was observed with amiodarone (Fig. 2a). Slight inhibition was also observed with chlorpromazine, metformin, rosiglitazone and tamoxifen but the differences in comparison with the control were statistically not significant (Fig. 2a). No inhibition, at the concentration tested, was observed for all other compounds.

Compounds were also tested for inhibition of Complex II as described in Section 2. None of the compounds showed any inhibition of Complex II activity (data not shown).

Since no antibody for immunocapturing Complex III activity is available to date, a modification of the traditional coupled spectrophotometric assay for Complex II + III was performed, instead (details are described in Section 2). Simvastatin and tamoxifen inhibited Complex II + III strongly (>50%, >80% inhibition, respectively; Fig. 2b). Weaker inhibition (<50%) was observed with amiodarone, chlorpromazine and paroxetine (Fig. 2b). A small, but significant inhibition (15%) was also observed with nefazodone. All other compounds tested showed no significant inhibition in this assay (Fig. 2b).

Complex IV activity was inhibited the strongest (80%) by tamoxifen (Fig. 2c). 25–50% inhibition was found with simvastatin and nefazodone. A small (~25%), but statistically significant, inhibition was also observed with amiodarone and gefitinib. Statistically non-significant inhibition was also observed with dantrolene and paroxetine.

Complex V activity was strongly inhibited by several of the tested compounds (Fig. 2d). More than 75% inhibition was observed with simvastatin, paroxetine and tamoxifen. 70% inhibition was observed with chlorpromazine and approximately 50% inhibition with amiodarone. Less than 50% inhibition was observed with diclofenac, nefazodone and gefitinib (Fig. 2d). All other compounds had no statistically significant effect on Complex V activity.

For those drugs showing more than 50% inhibition of one or more complexes at 50 μ M, IC_{50} values were determined. A representative example (tamoxifen) of how data were generated is shown in Fig. 3. Table 3 summarizes the IC_{50} values for nefazodone, paroxetine, simvastatin, tamoxifen and chlorpromazine. The major target of inhibition of nefazodone was Complex I with an IC_{50} value of 4 μ M. In

Table 2
Summary of therapeutic application, C_{max} , and observed *in vivo* toxicities of each of the drugs tested

Compound	Mode of Action	Dose per day	C_{max} (μ M)	Observed toxicities
Amiodarone	Antianginal	400–1600 mg/day	0.85	Hepatotoxicity
Benoxaprofen	NSAID	600 mg/day	2.9	Hepatotoxicity
Bezafibrate	Anti-diabetic	400–600 mg/day	16.6	Hepatotoxicity
Chlorpromazine	Antipsychotic	25–75 mg/day	0.9	Cholestasis
Dantrolene	Muscle relaxant	25–400 mg/day	3.9	Hepatotoxicity
Diclofenac	NSAID	150–200 mg/day	7.9	Hepatotoxicity
Fluconazole	Antifungal	50–400 mg/day	8.8	Hepatotoxicity
Gefitinib	Anti-cancer	250 mg/day	No report	No report
Linezolid	Antibacterial	200 mg twice/day	14.46	Optic Neuropathy
Metformin	Anti-diabetic	500 mg twice/day	12.4	Hepatotoxicity
Nefazodone	Anti depressant	100 mg twice/day	0.92	Hepatotoxicity
Paroxetine	Anti depressant	10–60 mg/day	0.06	Hepatotoxicity
Pravastatin	Anti-lipidemic	5–40 mg/day	0.02	Muscle toxicity
Primaquine	Antimalarial	15 mg/day for 14 days	1.07	Neutropenia and Hepatitis
Radicicol	Anti-cancer	No report	No report	No report
Rosiglitazone	Anti-diabetic	4–8 mg/day	1.04	Hepatotoxicity
Simvastatin	Anti-lipidemic	5–40 mg/day	0.02	Muscle toxicity
Tamoxifen	Anti-cancer	20–40 mg/day	0.16	Retinopathy
Trazodone	Anti depressant	400 mg/day in divided doses	5.05	Hepatotoxicity
Trovaflaxacin	Antibacterial	100–300 mg/day	5	Hepatotoxicity

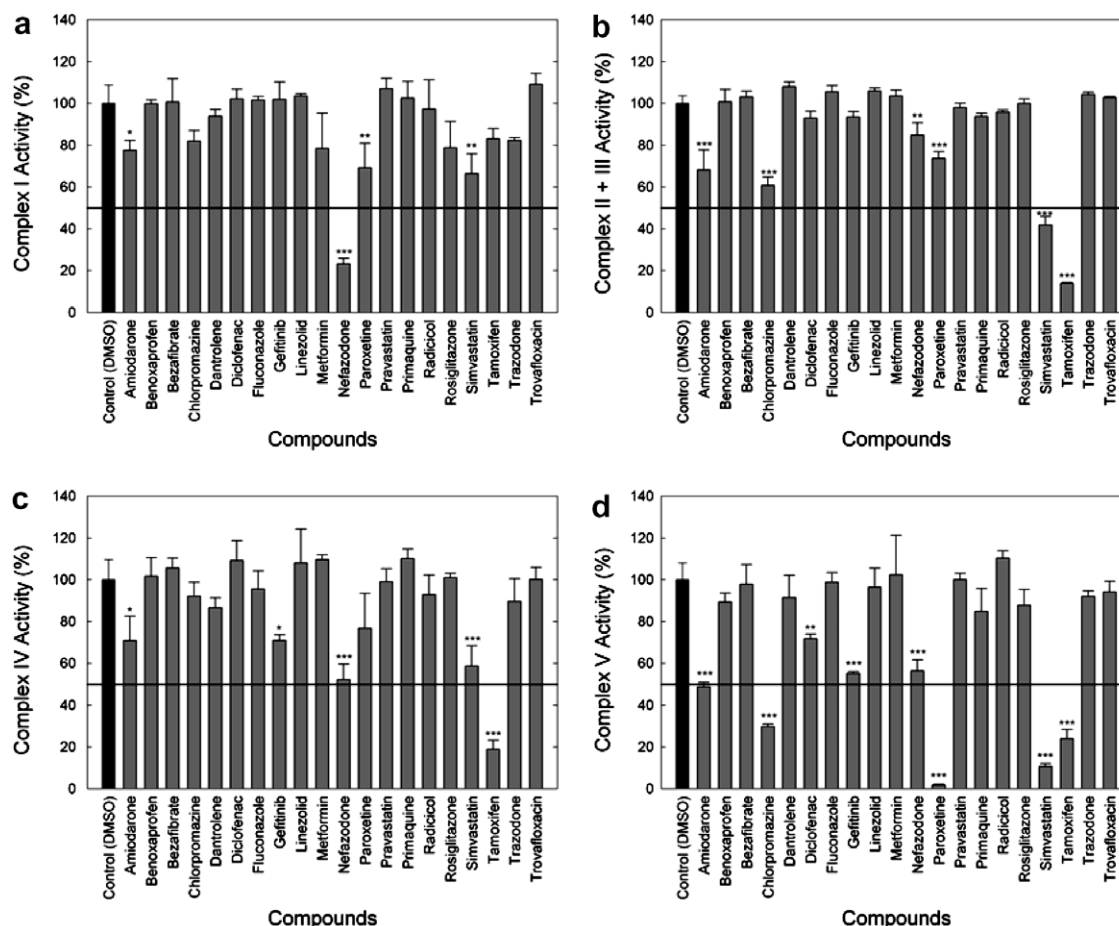


Fig. 2. Effect of 20 drugs (50 μM) on Complex I activity (a), Complex II + III activity (b), Complex IV activity (c) and Complex V activity (d). 100% represents the activity of uninhibited enzyme. Data are expressed as mean ± SD ($n = 3$). Statistically significant values compared with the control are reported as follows: *: for $p < 0.05$, **: for $p < 0.01$ and ***: for $p < 0.001$.

Table 3

IC₅₀ values of the compounds that inhibited >50% at the initial concentration (50 μM) tested

	Complex I	Complex II	Complex II + III	Complex IV	Complex V
	IC ₅₀ (μM)	IC ₅₀ (μM)	IC ₅₀ (μM)	IC ₅₀ (μM)	IC ₅₀ (μM)
Nefazodone	4	0	>50	>50	>50
Paroxetine	>50	0	>50	>50	1.6
Simvastatin	>50	0	30	>50	29
Tamoxifen	>50	0	15	26.6	8.1
Chlorpromazine	>50	0	>50	0	26

0 indicates that no inhibition was observed for that particular complex at the single dose concentration (50 μM) tested.

addition, nefazodone also inhibited Complexes II + III, IV and V but at less than 50% and hence IC₅₀ values were not generated. Paroxetine inhibited Complex V with an IC₅₀ value of 1.6 μM. Simvastatin had multiple targets with almost equal inhibition of Complex II + III and Complex V at ~30 μM. Simvastatin also inhibited Complex I and IV, but to less than 50% at the concentration tested. Tamoxifen inhibited multiple complexes, with Complex V being the most affected OXPHOS complex, with an IC₅₀ value of 8.1 μM (Table 3). Chlorpromazine inhibited Complex V the most, with an IC₅₀ value of 26 μM (Table 3).

4. Discussion

There is mounting evidence of the mitotoxicity of certain anti-diabetic drugs, anti-lipidemic drugs, anti-cancer drugs, antibacterials, and antivirals. To identify and manage mitochondrial mediated drug toxicity, low cost, high throughput-adaptable early screens are needed. Step one in any paradigm is an HTS screen that identifies “generic” mitotoxicity. Our recent studies (Hynes et al., 2006) show that a novel HTS applicable oxygen consumption assay provides such a screen and has the advantage that it identifies those compounds that are uncouplers of OXPHOS. Here, we

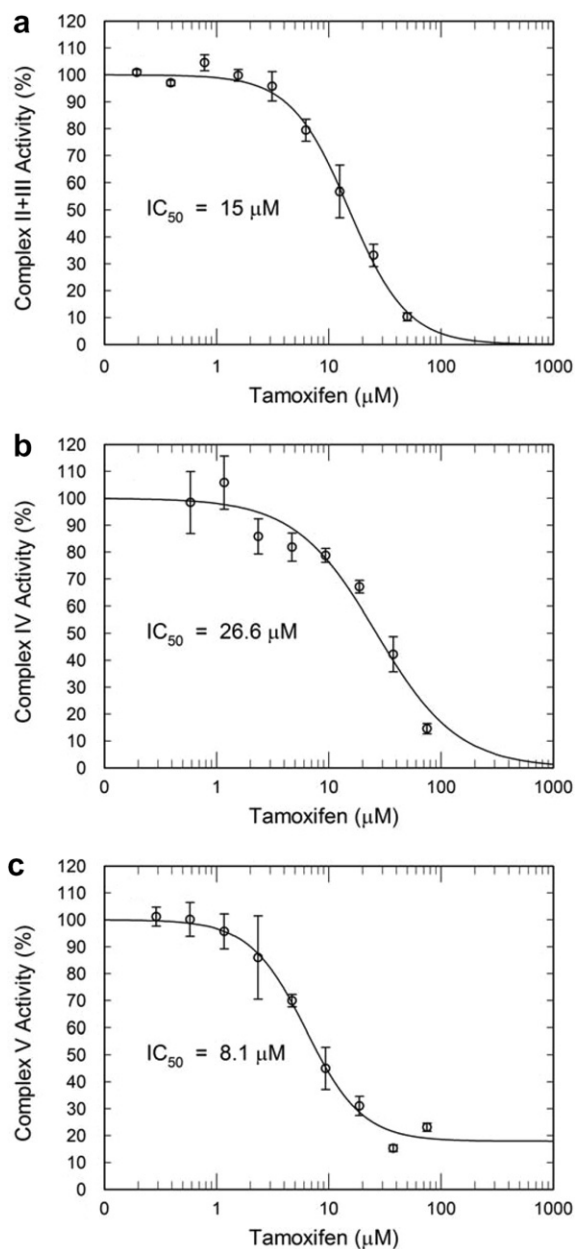


Fig. 3. Effect of tamoxifen on Complex II + III activity (a), Complex IV activity (b) and Complex V activity (c). 100% represents the activity of uninhibited enzyme. Data are expressed as mean \pm SD ($n = 3$). The line is a best fit using a four-parameter logistic equation.

show that the combination of five high-throughput assays, one for each of the OXPHOS complexes, adds important additional information by identifying the enzyme target of compounds. This mechanistic information facilitates SAR (structure–activity relationship) studies when safer compounds need to be synthesized. Moreover, the analysis can provide the mitochondrial toxicity biomarker for subsequent cell and animal studies.

All the assays described here were performed using isolated bovine heart mitochondria which can be prepared on a large scale, aliquoted and stored at -80°C for at least 6 months without loss of individual enzyme function. This ensures consistency in HTS over periods of time and elimi-

nates the need for daily mitochondrial preparations. All five assays had excellent reproducibility and showed low intra- and inter-assay variation (Table 1). Each assay was inhibited by its appropriate classical mitochondrial inhibitor (Fig. 1) at concentrations that are in agreement with published data (Wallace and Starkov, 2000).

Assays for the OXPHOS complexes have been described before. Such assays use intact mitochondria or inner membrane particles. The most commonly used method in toxicology studies monitors oxygen consumption polarographically in rat liver mitochondria in the presence of different substrates (glutamate, malate) and with various inhibitors (antimycin, cyanide) to functionally isolate the different reactions. The immunocapture based assays described here have major advantages over previously used methods. Complex V is not easily measured in concert with the electron transfer complexes in intact mitochondria. When physically isolated in an immunocapture assay as described here, Complex V is freed of other ATP/ADP using enzymes. NADH/NAD utilizing enzymes are amply present in mitochondria and confound accurate Complex I activity measurements. Complex I isolated by an immunocapture assay, as described here, is freed of other NADH/NAD utilizing enzymes. Additionally, the immunocapture assays do not require prior isolation of mitochondria; instead, the enzymes can be immunocaptured directly from cell culture material or tissue extract. Hence, the assays can be conducted with different types of tissues from different species where the antibodies are able to immunocapture the relevant enzymes. Therefore, in any drug discovery project, the same assays can be used for early detection of toxicity as well as in animal studies. Moreover, in a lateral flow device format, these assays can be used in clinical trials.

Here we evaluated the immunocapture assays with 20 drugs with an initial screen at $50\ \mu\text{M}$ concentration which is, in all cases, at least five times the C_{max} concentration (Table 2). For any compound showing inhibition greater than 50% at $50\ \mu\text{M}$, an IC_{50} was determined. Previous studies had identified mitochondrial toxicity of several of the drugs chosen. Others had no previously reported mitochondrial liability. Most of those examined are in current therapeutic use.

In most cases, the systematic analysis of all five OXPHOS complexes conducted here identified and extended the information already available about the mitochondrial toxicity of the compounds. Amiodarone has been reported to have multiple effects on mitochondria including inhibition of the OXPHOS complexes, uncoupling of OXPHOS and inhibition of fatty acid oxidation (Fromenty et al., 1990a,b; Prasad Rao et al., 1986). We found that amiodarone inhibited Complex I, II + III, IV and V activity (Fig. 2a–d).

Chlorpromazine has been shown to inhibit multiple OXPHOS complexes (Chazotte and Vanderkooi, 1981). Bullough et al. (1985) reported 50% inhibition of F_1 -ATPase activity from bovine mitochondria with $50\ \mu\text{M}$ chlorpromazine. The drug inhibited Complex II + III and, in particular, Complex V in our studies (Fig. 2). The drug, diclofenac, inhibited Complex V (Fig. 2d), in agreement with other reports (Moreno-Sanchez et al., 1999).

We found inhibition of Complex II+III, Complex IV and Complex V with tamoxifen, an anti-cancer drug (Figs. 2, 3a–c). These results are in concordance with Tuquet et al. (2000) who noted strong inhibition of Complex III and slightly weaker inhibition of Complex IV in rat liver mitochondria. Data by Marroquin et al. (2005) indicate that tamoxifen strongly inhibits Complex V. An interesting hypothesis is that the toxicity of tamoxifen could potentially be its therapeutic mode of action. Thus, low concentrations or short term use of tamoxifen could cause dysfunction of Complex III, leading to free radical production which in turn would trigger apoptosis, a beneficial event in cancer therapy. Conversely, long term use of tamoxifen could result in elevated levels of reactive oxygen species, extensive irreversible covalent modification of proteins and, finally, necrosis.

Radicicol is a potent inhibitor of the ATPase activity of the chaperone, Hsp90 (Roe et al., 1999). Hsp90 belongs to a family of ATPases called GHF (Gyrase, Hsp90, MutL) ATPases which have a novel ATP-binding fold that is not found in other ATPases (Dutta and Inouye, 2000). The dissimilarity of the tertiary structure of the ATP binding regions in the GHF ATPases and F_1 ATPase (of Complex V) may explain why radicicol fails to inhibit Complex V.

A number of the drugs proved to have interesting effects on the OXPHOS complexes not previously reported. Nefazodone, an antidepressant, binds to serotonin type 2 receptors with nanomolar affinity, blocking serotonin reuptake. It is metabolized extensively in the liver, suggesting that certain genetic variants of the cytochrome P450 enzymes could play a role in nefazodone idiosyncratic toxicity. Nefazodone inhibited Complex I strongly but also affected Complex II+III, Complex IV and Complex V activity (Fig. 2a–d).

Paroxetine (Paxil), an antidepressant that is a selective serotonin-reuptake inhibitor, was found to cause moderate inhibition of Complex I and Complex II+III activity at 50 μ M (Fig. 2a–d), but was a strong inhibitor of Complex V, causing 50% inhibition at 1.6 μ M (Table 3). This effect has not been reported previously.

Simvastatin and pravastatin are HMG-CoA reductase inhibitors used in lowering cholesterol. Simvastatin inhibited Complex I, II+III, IV and V activity in our studies. In contrast, pravastatin had no observable effect on any of the OXPHOS complexes. The main structural difference between the two statins is a lactone ring present in simvastatin which is replaced by a β -hydroxy acid in pravastatin. One parameter that this difference in structure affects is the log P value. The log P or the partition coefficient of a compound between *n*-octanol and water is a measure of its hydrophobicity. The calculated log P value of simvastatin, 4.94, indicates a significantly higher hydrophobic character than pravastatin (log P, 2.61). It is tempting to speculate that there is a correlation between the log P value and the inhibitory effect of the drug: the higher the log P value, the higher the propensity of hydrophobic interactions between the drug and the OXPHOS complex

and, hence, the greater the likelihood of the complex being inhibited. Notably, other drugs we examined with relatively high log P values are amiodarone (log P, 7.57), tamoxifen (log P, 6.58), chlorpromazine (log P, 5.41) and nefazodone (log P, 4.01), all of which inhibit multiple OXPHOS complexes.

A survey of the compounds studied here shows that some, notably tamoxifen and simvastatin, have significant inhibitory effects on multiple complexes. The effect of compounds on multiple complexes is unlikely to be due to direct inhibition at the catalytic site as the different enzymes have very different substrates and products. The inhibition of multiple complexes by hydrophobic drugs could reflect some block of conformational rearrangements essential for functioning of the enzymes. As assayed here, the complexes are in detergent dispersion and not in membranes and so the broad inhibition is not due to a soap effect. One interesting possibility for further study is whether the drugs alter cardiolipin–protein interactions as this lipid is tightly bound and required for full activity of Complexes I, III, IV and V but not II.

As we proceed to develop an overall strategy for evaluating mitochondrial toxicity, it will be necessary to add assays for investigating other possible routes of drug induced mitochondrial dysfunction. This includes direct effects on substrate and product transfer across the inner mitochondrial membrane and on Ca^{2+} handling by the organelle. It is also necessary to screen for drugs, such as many NRTIs and antibiotics, which alter the biogenesis of the OXPHOS complexes by altering mtDNA replication or mitochondrial protein synthesis. In addition, we do not rule out the possibility that compounds, including the ones discussed here, can be concentrated in the mitochondrion and exert a toxic effect through build up of free radicals or reactive metabolites within the organelle.

Finally, it is important to emphasize that most of the compounds studied here have proved to be important drugs in the therapy of human diseases, with low numbers of reports of serious adverse effects. This is true of several compounds shown here to have considerable mitotoxicity. One obvious explanation is that the concentrations that give the observed mitotoxic effects are considerably higher than the C_{max} . Another issue that requires consideration in any scheme of early identification of mitotoxicity is idiosyncratic toxicity. Humans show considerable variation in mtDNA with polymorphisms known to predispose to Alzheimer's disease, Parkinson's disease and Type II diabetes (Wallace, 1994). Moreover, there are mtDNA mutations leading to diseases of varying severity such as Leber's hereditary optic neuropathy (LHON), mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), myoclonus epilepsy and ragged red fibers syndrome (MERRF) and Leigh disease, all of which show altered levels and activities of the OXPHOS complexes (Luft, 1994). It is possible that polymorphic variants in the sequences of mitochondrial genes for the OXPHOS complexes cause altered sensitivity to drugs. There are

already reports to this effect such as cases in which the rare serious adverse effect of statins unmasks underlying undiagnosed, mild mitochondrial myopathy due to mtDNA mutations (Diaczok and Shali, 2003). An advantage of the immunocapture tests described here is that the OXPPOS complexes can be isolated from cells with different mtDNA sequences to identify any correlation between sequence and drug toxicity.

In summary, our results show that novel immunocapture based assays of OXPPOS introduced here provide a systematic way of evaluating the inhibitory properties of compounds for the OXPPOS complexes. A non-immunocapture approach was used to measure Complex III activity but this will be replaced by an immunocapture based assay once a suitable high affinity antibody is available. The important advantages of the immunocapture assays are accurate measurement of Complex I and Complex V activities, the need for only small amounts of assay material, and the flexibility to use cell and tissue extracts from various species.

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