White Paper

Multiplex Detection of Oxidative Phosphorylation (OXPHOS) as a Mechanistic Indicator for Mitochondrial Health and Toxicity

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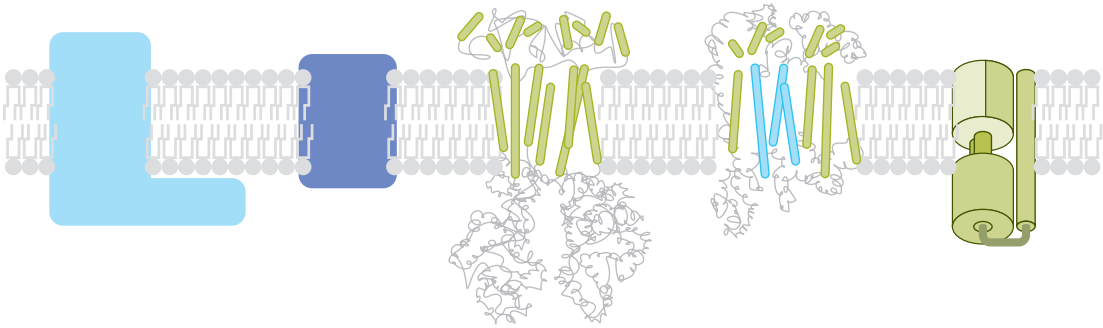
About the Author

Wei Zheng, M.Sc., Ph.D., obtained his master's degree in Physiology and Biophysics and Ph.D. in Neuroscience. He has more than 10 years expertise designing and developing novel immunoassays. He currently leads the multiplex immunoassay team focusing on discovery and validation of novel diagnostic, prognostic and predictive safety biomarkers at EMD Millipore.



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Introduction

Mitochondria are unique and complex organelles that perform essential functions in many aspects of cell biology. The dominant function of mitochondria is the production of more than 90% of the cell's energy in the form of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS). The OXPHOS system, comprised of five multimeric enzyme complexes (complex I-V or CI-V), drives the synthesis of ATP in most cells. Two mobile carriers, ubiquinone and cytochrome c, are used to transfer electrons from NADH, produced by the oxidation of carbohydrates and lipids, to molecular oxygen (Figure 1). This creates a proton electrochemical gradient that is harnessed by Complex V to synthesize ATP.

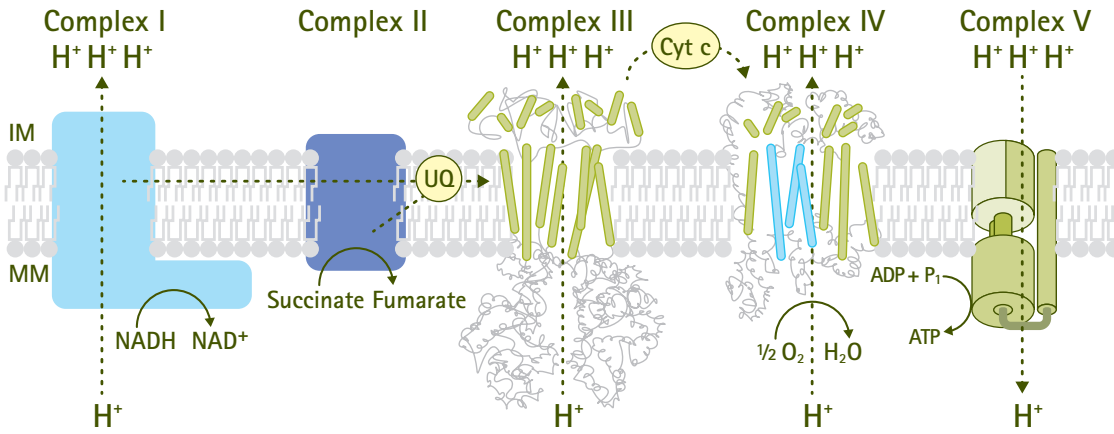


Figure 1. OXPHOS pathway in the mitochondrial membrane. Five multimeric enzyme complexes together catalyze the transfer of electrons from NADH to oxygen, mediated in part by mobile carriers ubiquinone (UQ) and cytochrome c (Cyt c). The complexes use the energy obtained from electron transport to pump protons (H^+) across the membrane, creating an electrochemical gradient ultimately used to generate stored energy (ATP).

The OXPHOS enzymes contain subunits of dual genetic origin, nuclear and mitochondrial, with the exception of the Complex II subunits (which are all encoded by the nuclear genome). The nuclear DNA (nDNA)-encoded subunits are synthesized on cytosolic ribosomes and imported into the mitochondria, where they assemble together with mitochondrial DNA (mtDNA)-encoded subunits and prosthetic groups to build up the OXPHOS complexes[1]. The overall process of OXPHOS is tightly controlled by transcriptional regulation, substrate feedback inhibition and post-translational modifications including phosphorylation and acetylation. Inefficient electron transfer through complexes I-IV cause mitochondrial dysfunctions in part due to the loss of energy metabolism, but also due to the induction of toxic reactive oxygen species (ROS).

Mitochondrial dysfunction is associated with multiple human diseases. These include relatively common disorders, such as cancer[2], diabetes[3], and neurodegenerative disease[4]. Beyond these common disorders, which are all age-related, mitochondrial dysfunction has also been associated with the aging process itself [5,6]. Also, studies have increasingly revealed that the toxic effects of certain drugs are due to those drugs causing mitochondrial dysfunction. Many different classes of drugs can compromise mitochondrial function. The US Food and Drug Administration (FDA) has given Black Box warnings (requiring packaging inserts) to drugs that have been shown to have the most potent effects on mitochondria, and has even removed some of these drugs from the market.

Not surprisingly, there is a growing need to analyze mitochondrial health, impairment and toxicity in the study of human diseases and during the drug development process. Since OXPHOS carries out energy production, the dominant function of mitochondria, assessment of OXPHOS expression and function logically becomes a key part of mitochondrial analysis. In this paper, we describe a method for multiplexed analysis of OXPHOS from cell lysate and tissue extract using the MILLIPLEX® MAP Human Oxidative Phosphorylation (OXPHOS) and the MILLIPLEX® MAP Rat/Mouse Oxidative Phosphorylation (OXPHOS) Magnetic Bead Panels, showing sensitive and reproducible detection of drug-induced mitochondrial impairment in human and rodent cells.

Materials and Methods

Assays. MILLIPLEX® MAP Human OXPHOS Magnetic Bead Panel (Cat. No. HOXPSMAG-16K) is available as a premixed panel and is to be used for the simultaneous detection of Complex I, II, III, IV, V, and NNT in human cell lysate or tissue extract. For the full names of the analytes, see Table 1.

MILLIPLEX® map Rat/Mouse OXPHOS Magnetic Bead Panel (Cat. No. RMOXPSMAG-17K) is available as a premixed panel and is to be used for the simultaneous detection of Complex I, III, and V in rat/mouse cell lysate or tissue extract. For the full names of the analytes, see Table 1.

Mitochondrial Complex IV (Human) Activity Assay Kit (Cat. No. AAMT004) was used to determine the activity of the cytochrome c oxidase enzyme (Complex IV) in human samples. Complex IV is immunocaptured within the wells and activity is determined colorimetrically by following the oxidation of reduced cytochrome c as an absorbance decrease at 550 nm.

Sample Preparation. Cell lysates from human cancer cell lines (HepG2, HL-60, A431, HeLa, HEK293), rat H9C2 myoblast, and MCA-RH7777 hepatoma cells were prepared according to the cell lysate preparation procedures described in the assay protocols for the multiplex kits. Tissue extracts from various human, rat and mouse organ tissues were prepared according to the tissue extract sample preparation procedures described in the assay protocols for the multiplex kits.

Treatment and Analysis. HepG2 hepatocellular carcinoma cells were cultured to 95% confluency then treated with either 20 µM of chloramphenicol or 20 µM of ddC (2'-3'-dideoxycytidine) for 6 days, during which the cells were split in conditioned media if necessary. In addition, HepG2 cells were cultured and treated with two members of the thiazolidinedione class of anti-diabetes drugs, either rosiglitazone (50 µM for 2 days) or troglitazone (20 µM for 2 days). DMSO mock-treated cells were used as controls. At the end of the treatment, the cell lysates were harvested. Equal amounts of cell lysate (10 µg) were analyzed in triplicate with the MILLIPLEX® MAP Human OXPHOS Magnetic Bead Panel following the recommended assay protocol. Drug-induced mitochondrial impairment was evaluated by normalizing the quantity of each analyte to mock-treated cells as a percentage.

Rat MCA-RH7777 hepatoma cells were cultured to 95% confluency then treated with either 500 µM of clofibrate or 20 µM of ddC for 6 days to induce mitochondrial toxicity. DMSO mock-treated cells were used as controls. During the treatments, the cells were split in conditioned media if necessary. At the end of the treatments, the cell lysates were prepared according to the procedures described in the protocol for the multiplex kit. 20 µg of the cell lysates were analyzed with the MILLIPLEX® MAP Rat/Mouse OXPHOS Magnetic Bead Panel according to the assay protocol. Drug-induced mitochondrial toxicity was evaluated by normalizing the signal from each analyte to the mock-treated cells as a percentage.

OXPHOS Complex	Full Name	Total No. of Subunits	No. of mtDNA-Encoded Subunits
Complex I	NADH–ubiquinone oxidoreductase	45	7
Complex II	Succinate ubiquinone oxidoreductase	4	0
Complex III	Ubiquinone cytochrome c oxidoreductase	11	1
Complex IV	Cytochrome c oxidase	13	3
Complex V	ATP synthase	17	2
NNT	Nicotinamide nucleotide transhydrogenase	N/A	N/A

Table 1: Full names of analytes detected using MILLIPLEX® MAP panels and numbers of subunits included in each complex.

Results

Correlation between OXPHOS complex quantity and activity. Each of the OXPHOS enzyme complexes (Complex I-V) is comprised of multiple subunits (see Table 1). It is well documented that the multimeric complex structures must stay intact in order for the OXPHOS complexes to maintain their enzymatic activities[7,8,9,10,11]. The reagents and sample preparation procedures for the MILLIPLEX® MAP OXPHOS Magnetic Bead Panels (both Human and Rat/Mouse) were optimized and validated to keep the OXPHOS complexes intact, thus maintaining the enzymatic activities of the OXPHOS complexes. The antibody pairs used in the assays were carefully selected to bind to the different subunits of each OXPHOS complex, and therefore to achieve the detection of the intact OXPHOS complexes rather than any single subunits.

To assess the correlation between OXPHOS complex activity and the protein quantity, HepG2 cells were treated with ddC to induce mitochondrial toxicity. During the treatment, the cells were split 1:2 into conditioned media whenever the cell population doubled (at 95% confluency). At each doubling, one flask of cells was harvested and the cell lysate prepared according to the procedures described in the MILLIPLEX® MAP Human OXPHOS Magnetic Bead Panel assay protocol. The same amount of cell lysates were analyzed with the Human OXPHOS Magnetic Bead Panel and the Mitochondrial Complex IV (Human) Activity Assay Kit according to the respective assay protocols. The quantity and activity of Complex IV from treated cells were normalized to DMSO mock-treated cells as a percentage (Figure 2).

At each cell doubling, Complex IV activity decreased due to ddC-induced mitochondrial toxicity. Significantly, Complex IV activity decreases correlated very well with its complex quantity changes obtained from the MILLIPLEX® MAP Human OXPHOS Magnetic Bead Panel (Figure 2). Similar results were obtained for other complexes as well (data not shown). These results together suggested that the intact OXPHOS complex quantities as measured using the MILLIPLEX® MAP OXPHOS Magnetic Bead Panel could be used as surrogate readout for OXPHOS complex enzymatic activity and therefore as a functional indicator for the overall mitochondria respiratory activity.

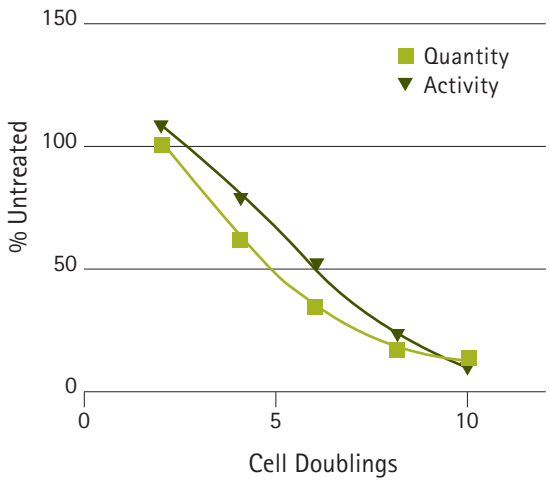


Figure 2. Correlation between OXPHOS Complex IV quantity and activity. HepG2 cells were treated with 4 µM ddC to induce mitochondrial toxicity. Cell lysates were assayed in parallel for quantitation of Complex IV and for detection of Complex IV enzymatic activity. Both quantity and activity were graphed as percentage of the values obtained from untreated cells. Percent quantity and activity of Complex IV showed closely parallel decreases with respect to the number of cell doublings.

Differential expression of OXPHOS complexes in multiple cell lines and tissues. Since OXPHOS provides most of the cellular energy driving almost every cellular process, it is obvious to postulate that different types of cells will possess different respiratory capacities and manifest unique OXPHOS expression profiles.

To assess the differences in OXPHOS for different cell lines and tissues, lysates from multiple human cancer cell lines (HepG2 cells, HL-60 acute promyelocytic leukemia cells, A431 epidermoid carcinoma cells, HeLa cervical cancer cells, HEK293 embryonic kidney cells) and human heart tissue were prepared and analyzed with the MILLIPLEX® MAP Human OXPHOS Magnetic Bead Panel according to the protocol (Figure 3). Rat and mouse heart, brain and liver tissue extracts, rat H9C2 myoblast, and MCA-RH7777 hepatoma cell lysates were prepared and analyzed with the MILLIPLEX® MAP Rat/Mouse OXPHOS Magnetic Bead Panel (Figure 4). Based on the results, it was clear that different cells or tissues demonstrated distinct expression profiles for the OXPHOS complexes. For instance, OXPHOS complex levels were much higher in heart tissues when compared to other tissues, reflecting the higher metabolic rate and energy consumption demanded by the function of the heart.

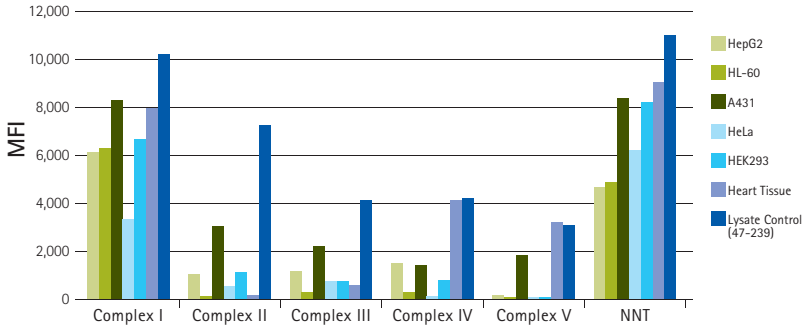


Figure 3. Multiplexed analysis of multiple human cancer cell lines and heart tissue extract with the Human OXPHOS Magnetic Bead Panel. Lysates from different human cancer cell lines (HepG2, HL-60, A431, HeLa, HEK293) and heart tissue were prepared according to the procedures described in the protocol. 6000 ng of cell lysates, 300 ng of heart tissue extract and the Lysate Control (Cat. No. 47-239) were analyzed with the Human OXPHOS Magnetic Bead Panel according to the assay protocol. The Median Fluorescence Intensity (MFI) was measured with a Luminex® system.

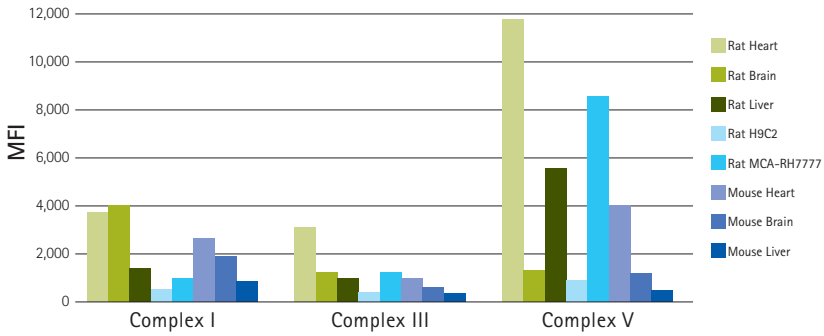


Figure 4. Multiplexed analysis of multiple rat and mouse cell lysates and tissue extracts with the Rat/Mouse OXPHOS Magnetic Bead Panel. Rat and mouse heart, brain and liver tissue extracts, rat H9C2 myoblast, MCA-RH7777 hepatoma cell lysates were prepared according to the procedures described in the protocol. 1,300 ng of heart tissue extracts and 10,000 ng of other lysates/tissue extracts were analyzed with the Rat/Mouse OXPHOS Magnetic Bead Panel according to the assay protocol. The Median Fluorescence Intensity (MFI) was measured with a Luminex® system.

Drug-induced mitochondrial toxicity and impairment. Recently, studies have suggested that drug-induced mitochondrial toxicity could be the underlying mechanism for many of the toxicity effects observed in some of the drugs withdrawn from the market due to drug safety concerns. For example, troglitazone (toxic to liver), cerivastatin (damages skeletal muscle) and tolcapone (toxic to liver) all are thought to manifest their toxic effects to the respective organs at least partially through impairing mitochondria[12, 13, 14, 15].

Drug-induced mitochondrial toxicity can occur by a variety of mechanisms. First, certain nucleotide reverse transcriptase inhibitors (NRTIs) can inhibit the polymerase that replicates mitochondrial DNA, blocking mitochondrial biogenesis and damaging tissues (such as muscle, liver and adipose tissues) in which mitochondrial function is critical. Other drugs, such as many broad spectrum antibiotics that disrupt bacterial protein synthesis, can disrupt mitochondrial proteins synthesis, and consequently mitochondrial biogenesis. This can lead to clinical manifestations such as ototoxicity and nephrotoxicity[16].

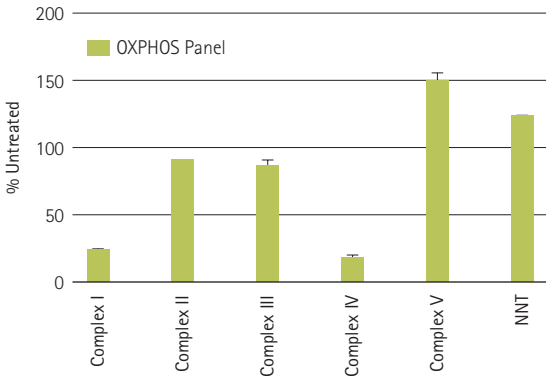
Mitochondrial status has been classically monitored via oxygen consumption using low-throughput polarographic electrodes. A higher throughput method has been developed that uses a phosphorescent probe in 96-well format to report mitochondrial respiration[17]. This technology has primarily been used with isolated mitochondria, therefore, adding time-consuming organelle isolation steps and potentially over-predicting drug effects because compounds have unrestricted access to mitochondria and are not subject to metabolism (either activation or degradation). With the MILLIPLEX® MAP OXPHOS Magnetic Bead Panels (for Human and Rat/Mouse), it is possible to

assess mitochondrial impairment induced by drugs or in disease using cell lysates and tissue extracts. The advantages of this approach include higher throughput, reproducibility and ease of use, without the need to isolate the mitochondria. Moreover, by enabling the precise quantitation of pathway proteins, these panels can provide valuable insights into the mechanisms of mitochondrial impairments.

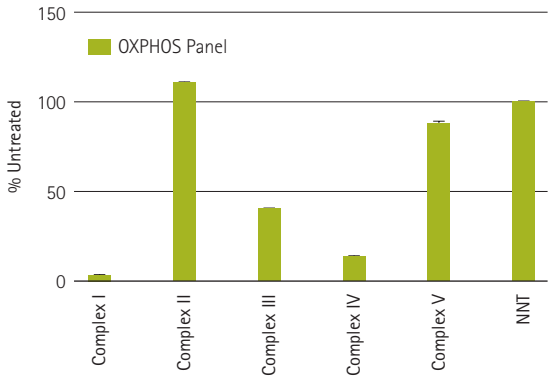
To assess the utility of MILLIPLEX® MAP OXPHOS Magnetic Bead Panels in the detection of drug-induced mitochondrial toxicity, HepG2 cells were treated with either 20 µM of the aminoglycoside antibiotic, chloramphenicol, for 6 days, 20 µM of the NRTI drug ddC for 6 days, or two members of the thiazolidinedione class of anti-diabetes drugs (50 µM rosiglitazone or 20 µM troglitazone) for 2 days. DMSO mock-treated cells were used as controls. At the end of treatment, the cell lysates were harvested and analyzed with the MILLIPLEX® MAP Human OXPHOS Magnetic Bead Panel. For a rodent model system, Rat MCA-RH7777 hepatoma cells were treated with 500 µM of clofibrate or 20 µM of ddC for 6 days to induce mitochondrial toxicity. DMSO mock-treated cells were used as the controls. At the end of the treatment, the cell lysates were harvested and analyzed with the MILLIPLEX® MAP Rat/Mouse OXPHOS Magnetic Bead Panel.

It is well known that certain aminoglycoside antibiotics induce long-term mitochondrial dysfunction by impairing mitochondrial protein synthesis. In this study, chloramphenicol clearly induced mitochondrial impairment with significant reduction of Complexes I and V in HepG2 cells (Figure 5a). In the case of the NRTI drug ddC, the inhibition of mtDNA replication resulted in mitochondrial toxicity with the most significant reduction of Complexes I, III, and IV in HepG2 cells (Figure 5b). These data were also supported by well-documented oxygen consumption reduction within these treatments[18, 19]. It has long been suggested that the hepatotoxicity induced by troglitazone (withdrawn from the market) and increased risk of heart attacks associated with rosiglitazone are associated with glitazone-induced mitochondrial impairment. Interestingly, in this study, both drugs induced notable changes in mitochondrial OXPHOS, with more severe reduction of OXPHOS observed in troglitazone-treated HepG2 cells (Figure 5c and 5d). In rat cells, similar drug-induced mitochondrial toxicities were observed with significant reduction of OXPHOS in drug-treated cells when compared to the mock-treatment (Figure 6). Collectively, these data support the potential application of the MILLIPLEX® MAP OXPHOS Magnetic Bead Panel (both Human and Rat/Mouse) in the study of drug-induced mitochondrial impairment/toxicity. With the mechanistic insights offered by this multiplex assay and its easy-to-use format, it can be incorporated into the drug development process and become a valuable tool in drug safety evaluation.

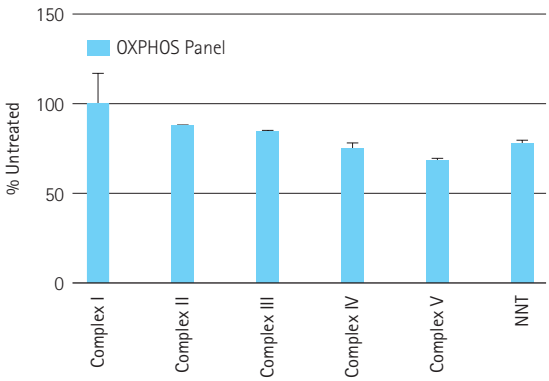
5a. Chloramphenicol 6 day 20 µM



5b. ddc 6 day 20 µM



5c. Rosiglitazone 2 day 50 µM



5d. Troglitazone 2 day 20 µM

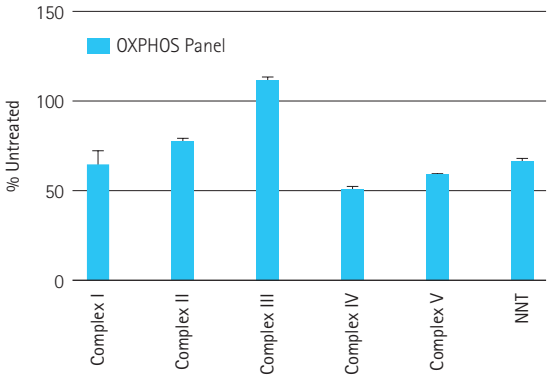


Figure 5. Human OXPHOS changes upon drug-induced mitochondrial toxicity. Human HepG2 cells were treated with either a) an antibiotic, chloramphenicol; b) an NRTI antiviral drug, ddC; c) rosiglitazone; or d) troglitazone to induce mitochondrial toxicity. DMSO mock-treated cells were used as the control. At the end of the treatments, cell lysates were analyzed with the Human OXPHOS Magnetic Bead Panel according to the assay protocol. Drug-induced mitochondrial toxicity was evaluated by normalizing the signal from each analyte to the mock-treated cells as a percentage.

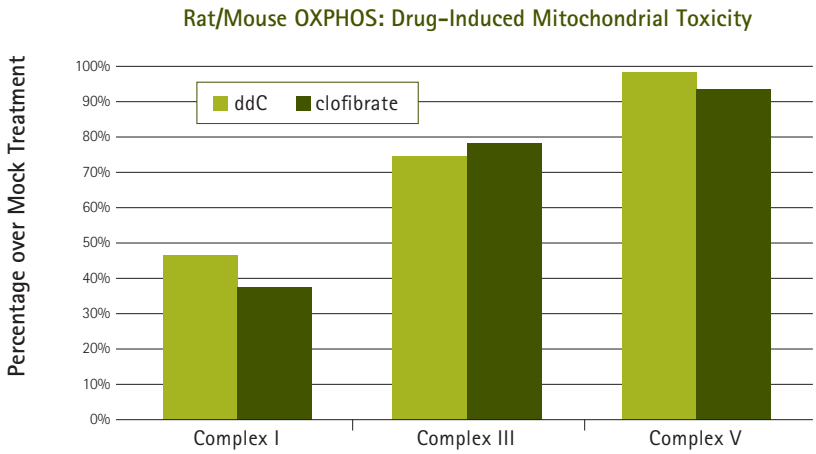


Figure 6. Rat OXPHOS changes upon drug-induced mitochondrial toxicity. Rat MCA-RH7777 cells were treated with either 500 µM of clofibrate or 20 µM of ddC for 6 days to induce mitochondrial toxicity. DMSO mock-treated cells were used as the control. At the end of the treatments, the cell lysates were analyzed with the Rat/Mouse OXPHOS Magnetic Bead Panel according to the assay protocol. Drug-induced mitochondrial toxicity was evaluated by normalizing the signal from each analyte to the mock-treated cells as a percentage. The results indicate that clofibrate and ddC clearly affected the mitochondrial respiratory chain as demonstrated by the significant reduction of Complex I, while Complex V remained relatively unchanged, since Complex V subunit genes are mainly encoded by nuclear DNA.

Conclusions

Significant progress has been made in understanding the links between mitochondrial dysfunction and diverse conditions such as diabetes, Parkinson's disease, Alzheimer's disease, cancer and the aging process. Mitochondrial analyses have drawn renewed interest from both the research and drug development communities. Since the dominant function of mitochondria is the production of cellular energy through OXPHOS, not surprisingly, the assessment of OXPHOS health and impairment has become the focal point of mitochondrial analysis. However, most traditional techniques for such analyses suffer from low throughput, time-consuming mitochondrial isolation steps, and the non-physiological conditions associated with the detachment of mitochondria from the cellular environment.

In contrast to traditional techniques, the MILLIPLEX® MAP OXPHOS Magnetic Bead Panels for human and for rat/mouse conveniently and quantitatively detect intact OXPHOS complexes from cell lysates or tissue extracts in 96-well format. In this study, the presented data support the value of these panels in the detection of intact OXPHOS complexes as functional and mechanistic indicators for mitochondrial health and impairment. Therefore, these panels demonstrate the potential broad applications in the study of mitochondrial dysfunction associated diseases in order to better understand disease mechanisms and to discover potential treatments. These panels can potentially be used in the drug development process to predict the mitochondrial impairment linked drug-induced toxicity, correspondingly reducing late-stage drug attrition and improving drug safety.

Acknowledgement

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