Screening and validation of mitochondrial inhibitors and uncouplers using a novel toxicity index derived from mitochondrial oxygen consumption rates

Yoonseok Kam, Lisa Winer, George W. Rogers, Melanie Schwalfenberg, Natalia Romero
Cell Analysis Division,
Agilent Technologies, MA., USA

SOT 2022

Agilent Trusted Answers

Introduction

Mitochondrial toxicity is a common issue with therapeutic development, as most eukaryotic cells employ mitochondria to produce the majority of ATP required for metabolic function and regulate key cellular processes.

Hence, sensitive, specific and accurate detection of mitochondrial toxicity is a key consideration during the development of therapeutic compounds to decrease both drug candidate attrition and post-market drug withdrawals.

Direct measurement of mitochondrial oxygen consumption can be used as a specific and sensitive indicator to assess drug-induced mitochondrial toxicity.

Introduced is a mitochondrial toxicity assay workflow using a novel parameter, Mito Tox Index (MTI), derived from real-time oxygen consumption rates (OCRs) measured by the Agilent Seahorse XF Pro Analyzer.

Using Seahorse XF Mito Tox Assay kit in conjunction with the XF Pro Analyzer and dedicated software features enables streamlined, sensitive detection and characterization of mitochondrial toxicants.

Assay Design

Parametric assessment of mitochondrial toxicity using the Mito Tox Index and detection of OxPhos inhibitors

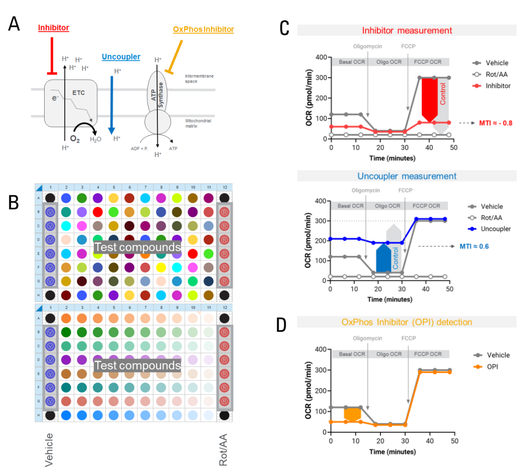


Figure 1. XF Seahorse Mito Tox Assay design and concept of calculating Mito Tox Index (MTI) and detecting potential OxPhos inhibitors (OPIs)

A. Three types of mitochondrial toxicity are assessable by measuring OCR with the XF Seahorse technology.
B. XF Mito Tox assay template designs for screening and dose-response analysis. The assay requires two control groups: Vehicle and Rot/AA.
C. The MTI is quantitative measurement of mitochondrial toxicity. Toxicity due to inhibition is defined as a decrease in FCCP OCR compared of the vehicle group, resulting in a negative MTI value (between 0 and -1). Toxicity due to uncoupling is defined as an increase in Oligo OCR compared to the vehicle group, resulting in a positive MTI value (between 0 and 1).
D. If a test compound treatment results in a decrease in Basal OCR, but does not result in significant decrease in FCCP OCR, then the compound is categorized as a potential OPI. It is suggested to perform downstream assays (dose-response or other orthogonal assays) to further investigate and characterize this type of toxicity.

Results and Discussion

Applying the XF Mito Tox Assay for toxicity screening

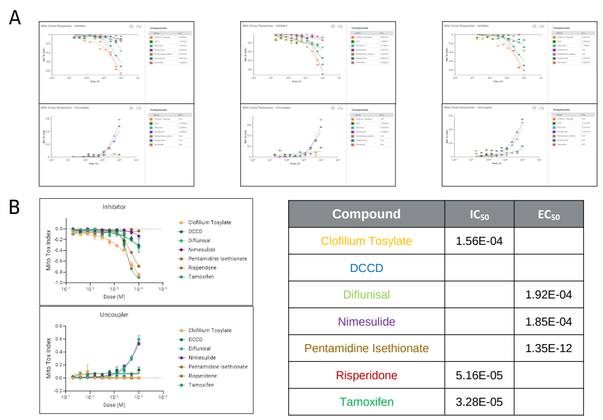
Compound	Inhibitor	Uncoupler	OPI
Azathioprine			
Clofilium Tosylate	-0.85 ± 0.03		
DCCD	-0.31 ± 0.11	0.14 ± 0.05	
Diflunisal	-0.18 ± 0.09	0.56 ± 0.08	
Fenofibrate	-0.09 ± 0.06		
Nimesulide	-0.11 ± 0.10	0.50 ± 0.08	
Pentamidine Isethionate			
Risperidone	-0.73 ± 0.03		

Figure 2. Functional screening of mitochondrial toxicity using the MTI and detection of OPI

HepG2 cells were exposed to a test set of 8 compounds (100 µM each) for 1 hour. The mitochondrial toxicity of the compounds was categorized as an inhibitor, uncoupler, or OPI. Identified were 2 inhibitors, 2 uncouplers, and 1 instance of OPI detection.

A. 3 independent assay results reported as MTI bar charts, MTI heat maps, and OPI detection maps (n=8).
B. The 3 data sets were combined (left graph, mean ± SEM) and summarized (right table).

Dose-response analysis of mitochondrial inhibitors and uncouplers using MTI



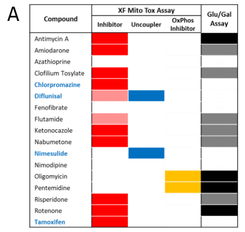
Compound	IC_{50}	EC_{50}
Clofilium Tosylate	1.56E-04	
DCCD		
Diflunisal		1.92E-04
Nimesulide		1.85E-04
Pentamidine Isethionate		1.35E-12
Risperidone	5.16E-05	
Tamoxifen	3.28E-05	

Figure 3. Dose-response evaluation of mitochondrial toxicity

HepG2 cells were exposed to compounds at increasing concentrations for 1 hour prior to the OCR measurement. MTI values were calculated and plotted vs. compound dose and IC_{50} and EC_{50} values were determined.were evaluated for the dose-response to a set of test compounds.

A. 3 independent assays results reported as MTI-based dose-response curves.
B. The 3 data sets were combined (left graphs, mean ± SEM) and summarized (right table). Presented are relevant IC_{50} and EC_{50} values.

Enhanced identification and quantitation of mitochondrial toxicity



B

Toxicity Identification	MTI		OxPhos Inhibitor	Glu/Gal Test
	Inhibitor	Uncoupler		
Yes	<= -0.3	>= 0.5	Yes	>= 3
Maybe	<= -0.2	>= 0.2	No	>= 2
No	> -0.2	< 0.2		< 2

Figure 4 Summary and Comparison of XF Mito Tox Assay vs. Glu/Gal-Total ATP assay.

HepG2 cells were exposed to compounds listed and the mitochondrial toxicity was evaluated by using XF Mito Tox or Glu/Gal Total ATP assays. Compared to Glu/Gal assay, the XF method identified more instances of significant mitochondrial toxicity as well as having the distinct advantage of providing preliminary information as to the mode of toxicity; inhibitor, uncoupler, or OPI.

A. Results of 17 compound Mito tox screen using XF Mito Tox Assay vs. Glu/Gal-Total ATP assay.
B. Toxicity "hit" criteria for XF MTI/OPI and Glu/Gal assays. The threshold were assigned arbitrarily.

Mitochondrial toxicity creening of hepatotoxic compound library

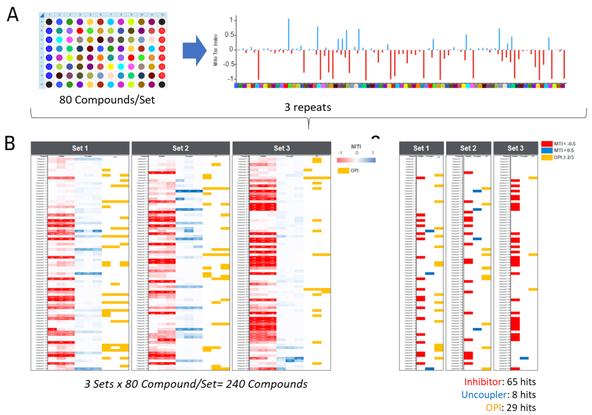


Figure 5. Screening of a hepatotoxic compound library using XF Mito Tox Assay

To demonstrate utility, the XF Mito Tox assay was applied to a library of 240 hepatotoxic compounds (SCREEN-WELL® Hepatotoxicity library, Enzo Life Sciences). The library was divided into 3 subsets of 80 compounds, and each subset of 80 compounds was screened using triplicate assays performed on different days. The Agilent Bravo liquid handling system was used for the media change and the compound administration.

A. Representative plate map for 80 compounds (plus control groups, Vehicle and R/A), and resulting MTI bar chart.
B. Summary of MTI values and OPI detecttion for 240 compounds.
C. Average of triplicate MTI values in B for the entire data set. MTI reporting thresholds = 0.5 and -0.5 for uncoupling and inhibition, respectively. OPI reporting threshold = 2/3 (i.e. reported as OPI if 2/3 of sample wells are flagged for OPI in panel B.

Conclusions

- A customized XF assay to assess mitochondrial toxicity with the ability to identify and specifically distinguish among different modes of mitochondrial toxicity with high sensitivity. Has been developed.
- This assay introduces and provides a standardized quantitative measurement of the magnitude of the toxicity, the Mitochondrial Toxicity Index (MTI), useful for both screening and dose response types of assays.
- This assay design and the respective output parameters enable rapid, straightforward implementation and intuitive, confident data interpretation when examining mitochondrial toxicity of therapeutic compounds.

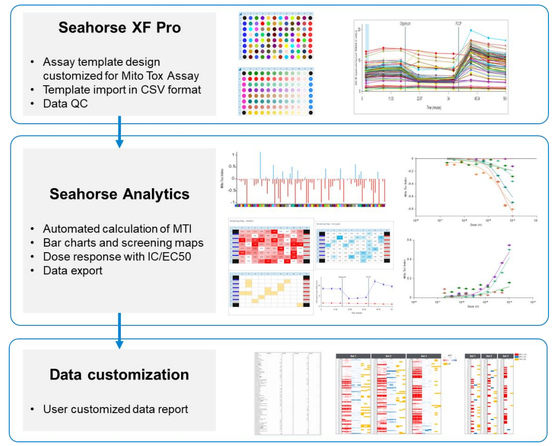


Figure 5. XF Seahorse Mito Tox Assay Workflow

Using the XF Mito Tox assay in conjunction with the XF Pro Analyzer and dedicated software features for assay design and analysis provides a sensitive, streamlined and intuitive method for the detection and assessment of mitochondrial toxicity.

References

Rogers, G.W., Winer, L., Schwalfenberg, M., Romero, N., and Kam, Y. (2022) Principle and Design of Parametric Assessment of Mitochondrial Toxicity Using Agilent Seahorse XF Mito Tox Assay Solution, White Paper

Meyer, J. N., Hartman, J. H., and Mello, D. F. (2018) Mitochondrial Toxicity. *Toxicol. Sci.*, 162(1), 15–23

Will, Y. & Dykens, J. (2014) Mitochondrial toxicity assessment in industry – a decade of technology development and insight. *Expert Opinion on Drug Metabolism & Toxicology*, 10:8, 1061-1067

Tilmant, K., Gerets, H., De Ron, P., Hanon, E., Bento-Pereira, C., Atienzar, F. A. (2018) *In vitro* screening of cell bioenergetics to assess mitochondrial dysfunction in drug development. *Toxicology in Vitro* 52 (2018) 374–383

Dykens, J. A., Marroquin, L. D., Will, Y. (2007) Strategies to reduce late-stage drug attrition due to mitochondrial toxicity. *Expert Opinion on Drug Metabolism & Toxicology*, 7:2, 161-175

Marroquin, L. D., Hynes, J. Dykens, J. A., Jamieson, J. D., Will, Y. (2007) Circumventing the Crabtree Effect: Replacing Media Glucose with Galactose Increases Susceptibility of HepG2 Cells to Mitochondrial Toxicants. *Toxicological Sciences*, 539–547

For Research Use Only. Not for use in diagnostic procedures.
RA44629.5115162037