Determining Mechanisms of Mitochondrial Toxicity using Agilent Seahorse XF Technology

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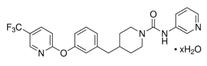
Agilent Trusted Answers

Introduction

- *Mitochondrial dysfunction is increasingly implicated in the etiology of drug induced toxicities and is a major reason for safety-related compound attrition and post-market drug withdrawals. Most assays currently used for mitochondrial toxicity studies provide limited mechanistic information. There is a need for more reproducible assays that provide mechanistic information to support selection of the most promising lead compounds while screening out off-target effects.*
- *Here, a standard workflow is presented to enable a comprehensive characterization of the mechanism of action of mitotoxic compounds. Using the XF Real-Time ATP Rate assay in intact cells (HepG2) we identified several compounds with mitotoxic but not cytotoxic effects after acute (1 hr) or chronic (18 hr) treatment.*
- *Fatty acid amide hydrolase (FAAH) is an integral membrane enzyme that hydrolyzes the endocannabinoid anandamide and related signaling lipids. Interestingly, it was found that the FAAH inhibitor, PF-3845, induced an acute decrease of mitochondrial ATP production that is compensated with an increase in glycolytic activity without significant changes in total ATP production or cell viability.*



PF-3845

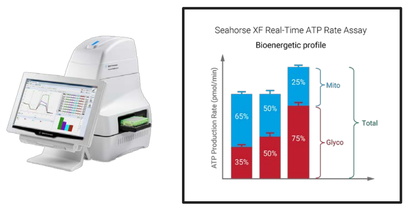
- *Additionally, using the cell permeabilizing agent, XF PMP, the mechanism of action of this mitotoxic compound was identified at the molecular level.*
- *The proposed workflow can be used for deeper evaluation of safety of lead compounds, identify off-target effects or to characterize the mechanism of mitotoxicity observed in pre-clinical studies.*

Experimental

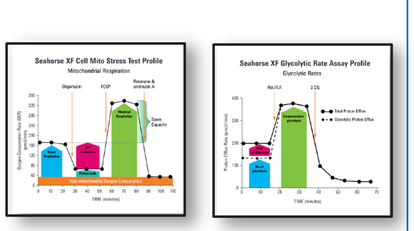
Workflow to study the mechanism of action of mitotoxic compounds using Agilent Seahorse XF Technology.

Prepare serial dilutions of compound(s): the use of the Bravo Automated Liquid Handling Platform reduces hands-on time and can make assay performance more robust.

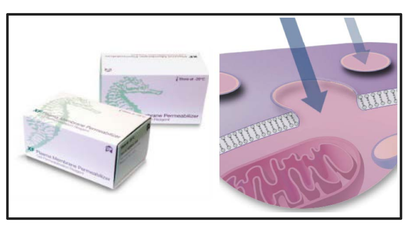
Identify in real-time compounds that affect cell metabolism or induce mitochondrial or cell toxicity using the Seahorse XF Real-Time ATP Rate Assay.



Confirm the main metabolic pathways affected by selected compounds using gold-standard assessment of metabolic function: obtain information of the effect on mitochondrial and glycolytic function under basal or stressed conditions.



Explore mechanism of action of selected compounds using XF Plasma Membrane Permeabilizer (PMP): identify and characterize key mitochondrial components affected by mitotoxic compound.



Results and Discussion

Real-time analysis of changes in mitochondrial, glycolytic and total ATP production rate after acute treatment with an experimental compound.

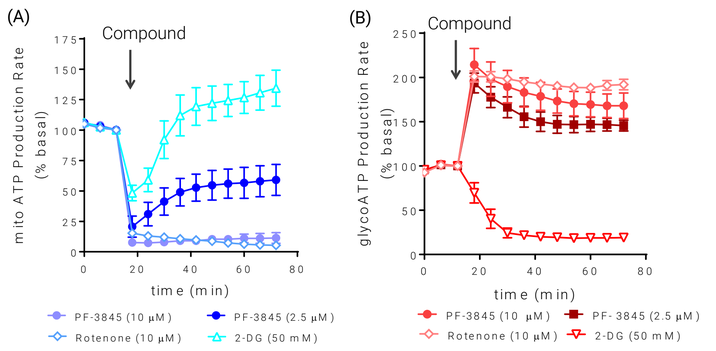
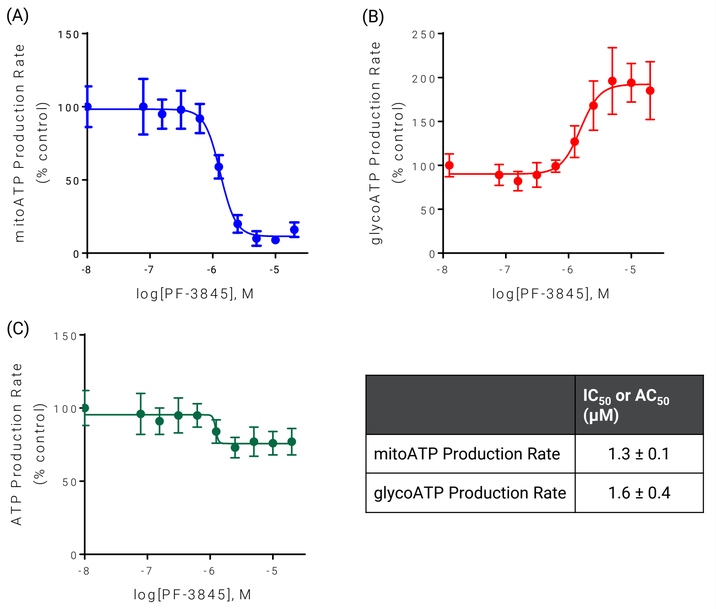


Figure 1 : HepG2 cells (2.0 x10^4/well) were treated with serial dilutions of small compounds from a commercial library and changes in mitochondrial (A), glycolytic (B) and total (C) ATP production rates were followed over time using the Agilent Seahorse XF Real-Time ATP Rate assay. Rotenone and 2-DG were included as positive controls of inhibition of mitochondrial and glycolytic function, respectively. The figures show data corresponding to 2 of 9 concentrations of the compound PF-3845 tested. Data represents the average ± SEM of 3 independent experiments, with one technical replicate per experiment.

✓ PF-3845 acute treatment promotes a decrease in mitochondrial ATP production in HepG2 cells, compensated by an increase in glycolytic ATP production without significant effect in total ATP production or cell viability (data not shown).

Dose-response curves of ATP production rates after acute treatment of HepG2 cells with different concentrations of the FAAH inhibitor PF-3845.



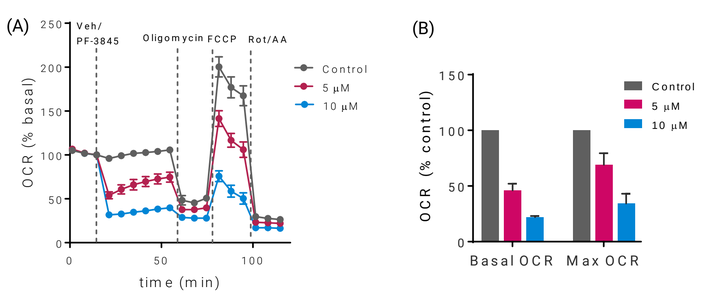
	IC_{50} or AC_{50} (µM)
mitoATP Production Rate	1.3 ± 0.1
glycoATP Production Rate	1.6 ± 0.4

Figure 2 : HepG2 cells (2.0 x10^4/well) were treated with serial dilutions of the FAAH inhibitor PF-3845 and mitochondrial (A), glycolytic (B) and total (C) ATP production rates were calculated 12 min after compound injection using he Agilent Seahorse XF Real-Time ATP Rate assay. Graphs show data corresponding to average ± SEM of 3 independent experiments. IC50/AC50 doses were calculated using GraphPad Prism 7.02 Software.

✓ Dose- response curves of mitoATP Production Rate after treatment with PF-3845 have an IC_{50} of 1.3 µM, very similar to the AC_{50} for glycolytic ATP production suggesting that increase in glycolytic activity is a consequence of mitochondrial dysfunction.

Confirming inhibition of mitochondrial function and increase of glycolytic activity using XF Cell Mito Stress Test and XF Glycolytic Rate Assay

XF Cell Mito Stress Test



XF Glycolytic Rate Assay

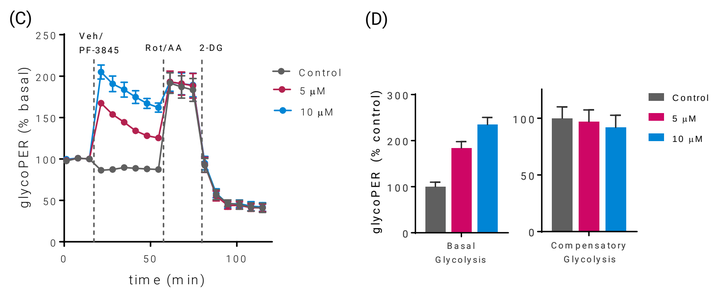


Figure 3 : Agilent Seahorse XF Cell Mito Stress Test profile (A) or Agilent Seahorse XF Glycolytic Rate Assay (C) profile of HepG2 cells (2.0 x10^4/well) after acute injection of the FFAH inhibitor PF-3845.(B) and (D) represent the calculated parameters of the corresponding assays using Seahorse XF Report Generators. Data represent average ± SEM of 3 independent experiments.

✓ Acute treatment of HepG2 cells with PF- 3845 induces a dose-dependent inhibition of basal and maximal respiration, confirming that mitochondrial activity is affected after drug treatment. Glycolytic activity after treatment is increased, however there is no effect in compensatory glycolysis (maximal glycolytic activity after addition of a mitochondrial inhibitor).

Results and Discussion

Evaluation of mitochondrial electron transport activities in permeabilized cells: using XF PMP to locate the target of mitochondrial dysfunction.

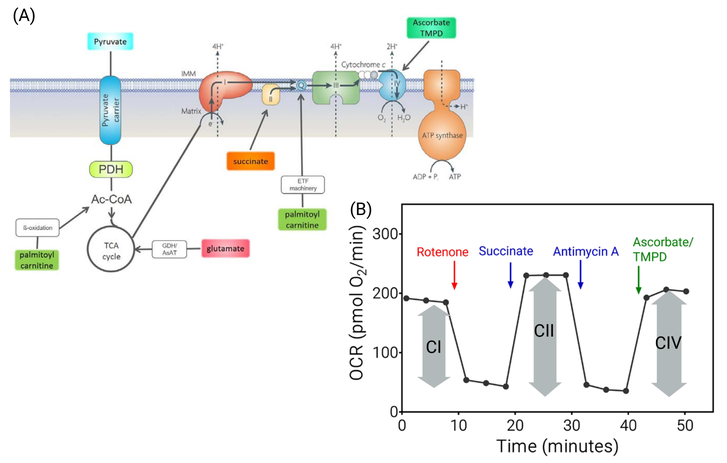


Figure 4: The XF Plasma Membrane Permeabilizer (XF PMP) reagent forms pores in the cellular plasma membrane without damaging mitochondrial membrane; allowing for control over mitochondrial substrate provision without the need for mitochondrial isolation or use of detergents. (A) Different oxidizable substrates can feed into different parts of the respiratory chain. (B) Measurement of OCR after sequential addition of the specific substrates and inhibitors offered to permeabilized cells allows measurement of the activity of the different components of the respiratory chain.

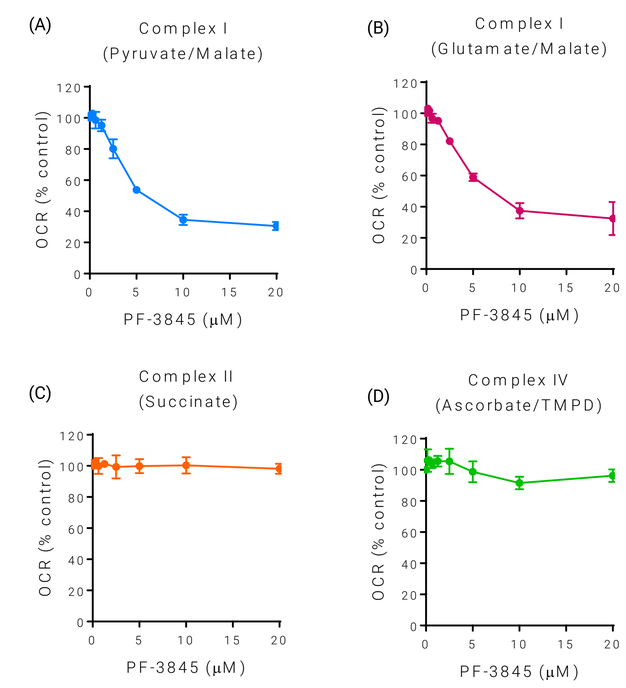


Figure 5: HepG2 cells (2.0 x10^4/well) were permeabilized using XF PMP (3 nM) treated with different concentrations of PF-3845 and immediately assayed for Complex I activity using Pyruvate/Malate or Glutamate/Malate as substrates, A and B, respectively; Complex II activity using Succinate/rotenone (C) or Complex IV activity (Ascorbate/TMPD) (D) in the presence of ADP using a Seahorse XF Analyzer according to (1,2). Graphs show the average ± SD of 3 independent experiments.

✓ Acute treatment of HepG2 cells with PF-3845 inhibits complex I activity independently of the substrate used to provide electrons without affecting complex II or complex IV activity.

Conclusions

- Seahorse XF workflows allow direct detection of metabolic switches induced by mito-toxic compounds.
- The XF Real-time ATP Rate assay can measure the rate of ATP production from the two key energy pathways (glycolysis and mitochondrial respiration) simultaneously, delivering a dual pathway kinetic picture of the drug response.
- The combined use of the XF Cell Mito Stress Test and the XF Glycolytic Rate Assay allows a thorough and multi-parametric understanding of the effect of toxic compounds on both major pathways of cell energy metabolism.
- Complementary mitochondrial function studies using XF Plasma Membrane Permeabilizer (PMP) for detailed mitochondrial function analysis allows a complete understanding of the molecular basis and mechanism of mito-toxicity.

References

1. Using PMP to Measure Mitochondrial ETC Complex Activity in Limited Biomaterials. (Technical Overview). https://www.agilent.com/cs/library/technicaloverviews/public/XF-PMP-Limited-Tech-Brief-WEB.pdf

2. Assessing Mitochondrial Respiratory Complexes Using Cells Permeabilized XF Plasma Membrane Permeabilizer (PMP). (Technical Overview). https://www.agilent.com/cs/library/technicaloverviews/public/5991-7157EN.pdf