

In vitro detection of pharmaceutical compounds that disturb mitochondrial functions

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Introduction

There is a need for predictive *in vitro* assays in the pharmaceutical industry to avoid unexpected toxicity in pre-clinical species and man. A large number of drugs that have been withdrawn from the market or stopped in development due to hepatotoxicity and cardiotoxicity have been reported to disturb mitochondrial functions (Ref 1). In this study, Huh7 cells were exposed to a number of pharmaceutical drugs and the effect on bioenergetics was monitored in the Seahorse XF24 analyzer. The aims of this study were to investigate:

- the sensitivity and specificity of the assay in detection of drugs that are known to disturb mitochondrial functions
- the possibility to perform assays that are specific for the different respiratory complexes in this system

Material and methods

The human hepatoma cells Huh7 were exposed to 70 pharmaceutical drugs and oxygen consumption rate (OCR) and extra-cellular acidification rate (ECAR) were monitored in real time on the Seahorse XF24 analyzer.

During the assay, the cells were kept in non-buffering DMEM medium and each compound was injected into triplicate wells. The concentration of the drugs were increased to 1, 10, 30, 100 μ M, over time by injections from the ports in the sensor cartridge. For drugs that were cytotoxic in this dose-range (by LDH leakage) the highest non-cytotoxic concentration was used as a maximum dose. The values before and after injection were compared and analyzed with an AUC ANOVA. A compound was assigned "positive in the assay" if OCR or ECAR was significantly different compared to control.

In the complex specific assays the cells were permeabilized with digitonine (7.5 μ g/mL) before being assayed in respiration buffer (Ref 2).

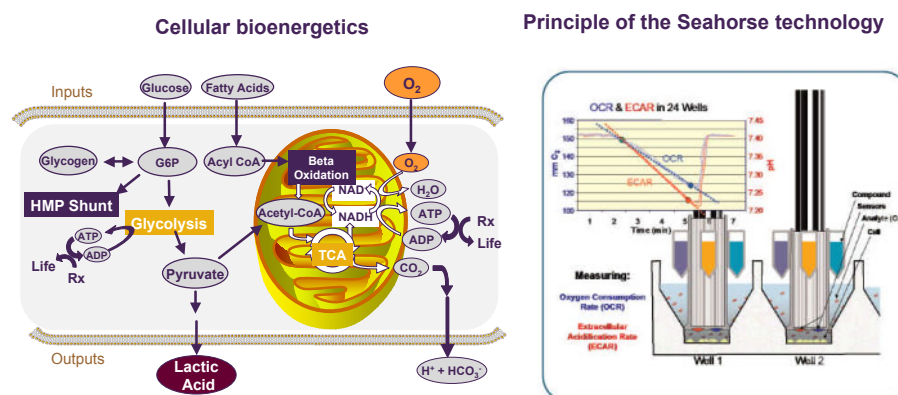


Table 1. Classification of the test drugs with respect to hepatotoxicity in man and known effects on mitochondria respiratory functions

Class	Definition	Number of drugs	Number of drugs with reported effects in mitochondria*	Drugs with reported effects in mitochondria at concentrations <100 μ M (number)
Severe	Drugs that have been withdrawn or not licensed due to hepatotoxicity in clinical studies, Black box warnings	22	7	Amiodarone, Tolcapone, Nefazodone, Flutamide, Ketoconazole, Troglitazone (6)
High	Warnings on idiosyncratic DILI	19	11	Chlorpromazine, Ticlopidine, Tacrine, Diclofenac, Disiram, Leflunomide (6)
Low	Liver enzyme elevations, rare idiosyncratic liver toxicity	11	5	Rosiglitazone, Buspirone (2)
None	No reported DILI	18	3	Pinacidil (1)
Total		70	26	

* Annotations based on literature mining

Results

Results from a representative experiment. OCR and ECAR after injection of test drugs to D: 1 μ M, C: 10 μ M, B: 30 μ M, A: 100 μ M

OCR and ECAR are shown in % of basal rate.

Red- Flutamide
Blue- Tolcapone
Pink- Acetaminophen
Turquoise- Propranolol
Orange- DMSO control

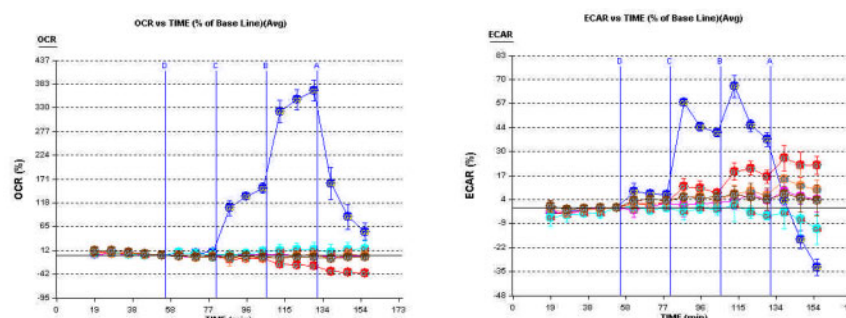


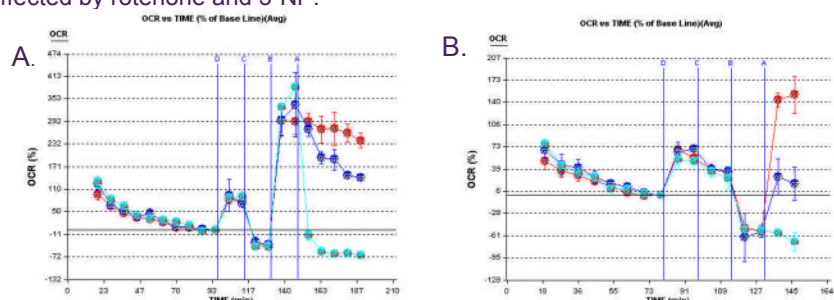
Table 2. Sensitivity and specificity of the assay

Class	All drugs with reported effects on mitochondria respiratory functions			Drugs with a reported effects on mitochondria respiratory functions at concentrations <100 μ M			Drugs with no reported effects on mitochondria respiratory functions		
	No. of drugs	Found positive	Sensitivity	No. of drugs	Found positive	Sensitivity	No. of drugs	Found negative	Specificity
Severe	7	5	0.71	6	5*	0.83	15	12	0.80
High	11	6	0.55	6	4	0.67	8	6	0.75
Low	5	2	0.40	2	2	1.0	6	3	0.50
None	3	0	0	1	0	0	15	12	0.80
Total	26	13	0.50	15	11	0.73	44	33	0.75

*amiodarone tested negative up to maximum 72 μ M (low solubility)

Experiments in permeabilized Huh7 cells

Electrons were transferred to complex I by malate and glutamate (10 mM in buffer) or to complex II by succinate (injection 10 mM). Rotenone inhibited OCR by electrons entering via complex I but not via complex II (Fig A). In contrast, 3-NP inhibited OCR from electrons entering via complex II but not complex I (Fig B). Complex II and IV were unaffected by rotenone and 3-NP.



Injection D: 1.5 mM ADP
Injection C: 0.5 μ M rotenone
Injection B: 10 mM succinate
Injection A: 3-NP (0, 1, 10 mM)

Injection D: 1.5 mM ADP
Injection C: 3-NP (0, 1, 10 mM)
Injection B: 0.5 μ M rotenone
Injection A: 10 mM succinate

Conclusions

- The assay showed a high sensitivity and specificity for drugs that affect mitochondrial functions amongst drugs that frequently causes hepatotoxicity in man, and may be useful in detection of compounds with this liability
- The Seahorse analyser together with permeabilized cells may be utilized in mechanistic studies to identify the respiratory complex that is affected by a particular compound

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References: 1) Dykens A. and Will Y. Drug Discovery Today (12) 17/18, 2007
2) MipNet Protocols, Oroboros, <http://www.orooboros.at/index.php?id=orooboros-protocols>