



Identifying Mitochondrial Toxicities in Primary and Immortalized Hepatocytes

Research Area

Toxicology

Application

Mitochondrial Function:
Identifying drug-induced mitochondrial dysfunction

Cell Type

Hepatocellular carcinoma (HepG2) and cryopreserved, plateable human primary hepatocytes

Work Of

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Tool Box:

- antimycin
- dantrolene
- oligomycin

Keywords:

- *In vitro* toxicity
- primary hepatocytes
- HepG2
- mitochondria
- oxidative phosphorylation
- glycolysis

The Seahorse XF24 Analyzer non-invasively quantifies cellular mitochondrial respiration and glycolytic flux simultaneously. Drugs that inhibit mitochondrial respiration lower oxygen consumption rates (OCR), whereas those that uncouple electron flow from oxidative phosphorylation typically increase OCR. Monitoring extracellular acidification rates (ECAR) quantifies the acceleration of glycolytic flux to compensate for loss of mitochondrial ATP production. Using the XF24, compounds that affect mitochondrial function can be detected in minutes and their EC_{50} values determined, rendering it well-suited for identifying liabilities *in vitro* at early stages of drug discovery

Background

Mitochondria generate most cellular ATP via oxidative phosphorylation and are central to intermediary metabolism, free radical generation, and regulating apoptosis. Most cells tolerate a loss of mitochondrial function until a threshold is reached when lack of ATP endangers the cell's basic functions. Glycolytic flux accelerates to compensate, but this is finite, and at some point, which is different for different cells, they will die. Their crucial role in maintaining cell viability and in many other metabolic pathways renders mitochondrial functions key targets for compound toxicity.

Assessing toxicity in isolated mitochondria is a common method but suffers from, 1) not being able to identify or predict chronic toxicity; 2) less physiological relevance since they are devoid of cellular constituents; and 3) not being able to identify the toxic effects of compounds requiring bioactivation.

The Seahorse XF24 Analyzer addresses the drawbacks of using isolated mitochondria. The XF24 non-invasively quantifies cellular mitochondrial respiration and glycolytic flux simultaneously. The former is monitored using a fluorescent probe that responds to pO_2 , and the latter is calculated indirectly by determining the extracellular acidification rate (ECAR), a byproduct of lactic acid production. Drugs that inhibit mitochondrial redox reactions of the Krebs's cycle or electron transport system (ETS) lower OCR, whereas those that uncouple electron flow from oxidative phosphorylation, increase OCR. Simultaneously monitoring ECAR, as an indicator of glycolysis, quantifies the acceleration of glycolytic flux to compensate for loss of mitochondrial ATP production.

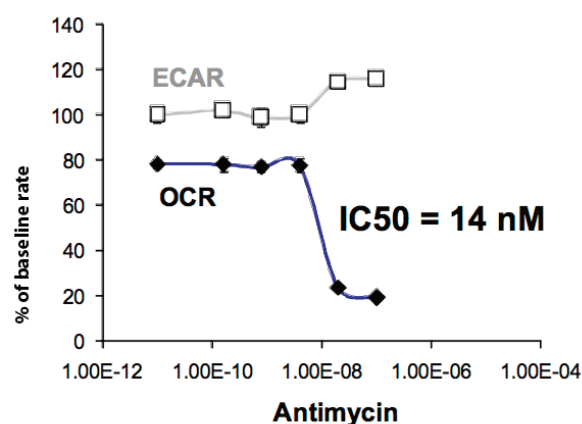


Figure 1: Dose-dependent inhibition of mitochondrial function in HepG2 cells.

The human liver cell line, HepG2, was seeded in XF24 microtiter plates at 30,000 cells/well and incubated overnight. Escalating doses of Antimycin known to inhibit Complex III was automatically injected into wells and the OCR and ECAR were subsequently measured. The IC_{50} value was calculated for OCR using GraphPad Prism 4.

Materials & Methods

Cell Culture.

Human hepatocellular carcinoma cell line, HepG2, was obtained from ATCC and cultured in Eagle's MEM supplemented with 10% FBS and 100 µg/mL Penicillin-Streptomycin. Cells were seeded at 30,000 cells per well in 24-well XF plates and incubated overnight in 37°C at 5% CO₂ incubator. Primary human cryopreserved hepatocytes were obtained from Celsis In Vitro Technologies (Baltimore, MD). The cells were rapidly thawed and plated using In Vitro Thaw Media and rates were measured 24 hours post thaw.

Test Compounds.

Antimycin, oligomycin and dantrolene were obtained from Sigma (St. Louis, MO).

References

Ferrick, DA et al. (2008). Advances in measuring cellular bioenergetics using extracellular flux. *Drug Discov Today*, **13**:268-274.

Ferrick, DA et al. (2008). Mitochondrial dysfunction; quantitatively assessed in real-time by measuring the extracellular flux of oxygen and protons. In Editors Will, Y. & Dykens, J.A., *Mitochondrial Dysfunction in Drug-induced Toxicity*. New York, NY. John Wiley & Sons, in press.

In this brief report we show two examples of how the XF24 can be used to not only identify compounds with mitochondrial liabilities but also elucidate their mechanisms of action.

Identifying Mitochondrial Toxicities

The four redox active complexes (I-IV) of the mitochondria and the ATP-synthase, Complex V, make up the ETS that drives oxidative phosphorylation. Compounds that impair redox reactions of the mitochondria, specifically the TCA cycle or ETS, will cause a decrease in oxygen consumption. For example, in Figure 1, using HepG2 liver cells we demonstrate the sensitivity and simplicity of identifying and determining the potency of compounds that diminish mitochondrial respiration using the well-characterized Complex III inhibitor, Antimycin. OCR reflects the inhibition in mitochondrial respiration and the calculated IC₅₀ value is indicated. The increased ECAR shows the ability of the cells to counter the aerobic insult. In contrast, if ECAR was unchanged or decreased it would imply another or additional mechanism of toxicity.

In Figure 2, we show OCR data for primary human hepatocytes exposed to dantrolene, a muscle relaxant known to exhibit clinical hepatotoxicity. Although the mechanistic basis for dantrolene's hepatotoxicity is not known, it does not exhibit significant cytotoxicity in primary hepatocytes *in vitro*.

Dantrolene bears structural similarity to the mitochondrial toxicant nitrofurantoin suggesting that its clinical toxicity is due to mitochondrial oxidative stress. It was found that an acute exposure of cryopreserved human hepatocytes to 50 µM dantrolene (dark bars) caused an immediate decrease in OCR that was sustained after 1 hour. Although the decrease was moderate, the absence of an oligomycin response (light bars) indicates that much of the respiration at 1 hour is uncoupled from ATP production. Although these results do not a priori suggest a mechanism for hepatotoxicity, the observation that dantrolene causes a sustained mitochondrial dysfunction in these cells would warrant further investigation into the agent's potential for organ toxicity.

In summary, compounds affecting mitochondrial function can be detected in minutes using the XF24 and their IC₅₀ values determined, rendering XF assays well-suited for identifying compounds with mitochondrial liabilities at early stages of drug discovery.

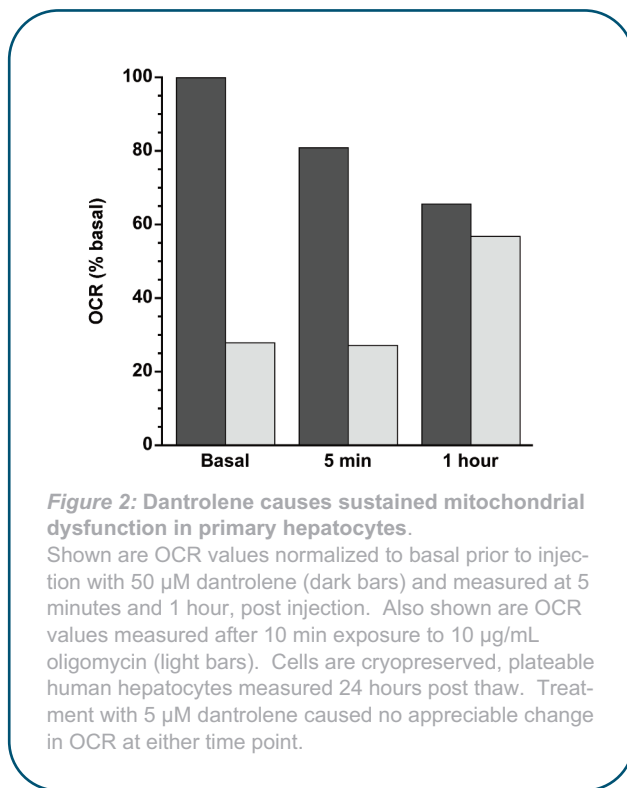


Figure 2: Dantrolene causes sustained mitochondrial dysfunction in primary hepatocytes.

Shown are OCR values normalized to basal prior to injection with 50 µM dantrolene (dark bars) and measured at 5 minutes and 1 hour, post injection. Also shown are OCR values measured after 10 min exposure to 10 µg/mL oligomycin (light bars). Cells are cryopreserved, plateable human hepatocytes measured 24 hours post thaw. Treatment with 5 µM dantrolene caused no appreciable change in OCR at either time point.