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## Introduction & Purpose

Mitochondria plays a crucial role in living organisms, one of which being the synthesis of ATP. Mitochondrial toxicity is widely tested as part of the compound's toxicity screening and within FDA's registered drug with a 'black box warning', roughly 80% of drugs are reported with mitochondrial toxicity. The aim of this research is to devise a simple method to evaluate mitochondrial toxicity across the different toxicological mechanism using human liver derived cell.

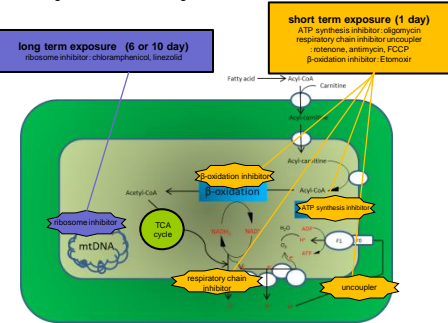


Figure 1 The mechanism of mitochondrial toxicity

## Method

### <Culturing and seeding method of HepG2>

HepG2 cells were purchased from ATCC. HepG2 cells were cultured and kept with Dulbecco's modified Eagle's medium (DMEM) (Cat. No.: 11960) supplied with 10% FBS and glutamine and pyruvate.

### <Exposure method of test compounds>

HepG2 cells were seeded [Glucose medium] in Collagen I Coated 96-well plates (catalog No. NCO3903, Corning) and incubated in a CO<sub>2</sub> incubator (37 °C, CO<sub>2</sub>: 5%) for more than 5 hours to allow the cells to attach to the bottom. After that, the medium in each well was replaced with any one of the exposure medium described below using incubation conditions with three cell culture types, glucose, galactose, and a medium that uses fatty acid as its sole energy source, and mitochondrial toxicity was evaluated separately from other cell toxicity using the Crabtree effect.

Glucose medium: 25mM glucose, pyruvate, glutamate and 10% FBS  
Galactose medium: 10mM galactose, pyruvate, glutamate and 10% FBS  
PA medium: 10mM galactose, palmitate, carnitine and 10% FBS

### <Measurement of ATP>

ATP was measured after exposure of 1 day (short term) or 10 day (long term) according to the CellTiter-Glo Luminescent Cell Viability Assay manual. (Promega) This assay was performed in duplicate.

### <Measurement of oxygen consumption>

An Seahorse XF96 Analyzer (Agilent Technologies Inc., Santa Clara, California) was used to measure oxygen consumption using HepG2 cells in real time.

HepG2 cells were seeded to XF96 plates and incubated at 5% CO<sub>2</sub>. The medium was then changed to the unbuffered medium and incubated at 37 °C without CO<sub>2</sub> until the measurement of oxygen consumption.

This assay was performed in duplicate.  
OCR: oxygen consumption rate

Figure 2 calculation method of basal respiration and ATP production

### <Calculation of EC<sub>50</sub> value>

The EC<sub>50</sub> value was calculated using the equation shown below from the relationship between percentage of control and the Typical inhibitor concentration.

$$\% \text{ of control} = \frac{[EC_{50}^h / (C^h + EC_{50}^h)] \times 100}{C \cdot \text{Typical inhibitor concentration} (\mu\text{M}) + h \cdot \text{hill coefficient}}$$

## Results

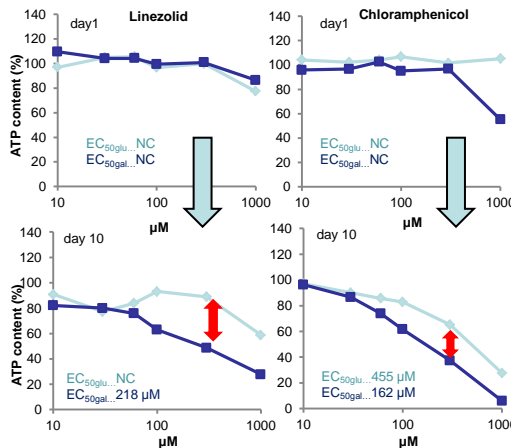


Figure 3 Concentration-dependent toxicity using long exposure method. Mitochondrial toxicity caused by linezolid and chloramphenicol (ribosome inhibitor) was not observed with short term exposure (for 1 day) but in both cases, it was observed after long term exposure (for 10 days). NC: not calculated

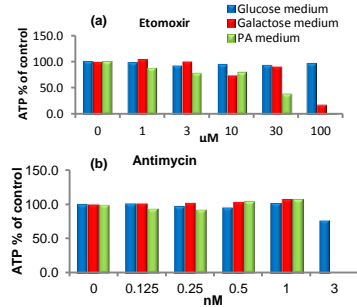


Figure 4 Concentration-dependent toxicity using three cell culture type. Concentration-dependent toxicity of (a) etomoxir (β-oxidation inhibitor) and (b) antimycin (respiratory chain inhibitor) using three cell culture types. Cytotoxic response of etomoxir in PA medium was most potently observed but there were no differences observed between PA medium and galactose medium in the antimycin group.

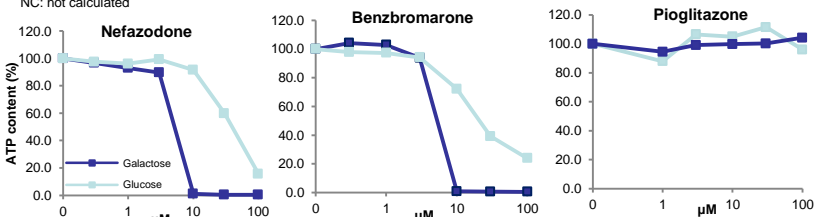


Figure 5 Concentration-dependent toxicity of nefazodone, benzbromarone and pioglitazone. Mitochondrial toxicity of nefazodone and benzbromarone was observed. Conversely, the mitochondrial toxicity of pioglitazone was not observed or negligible.

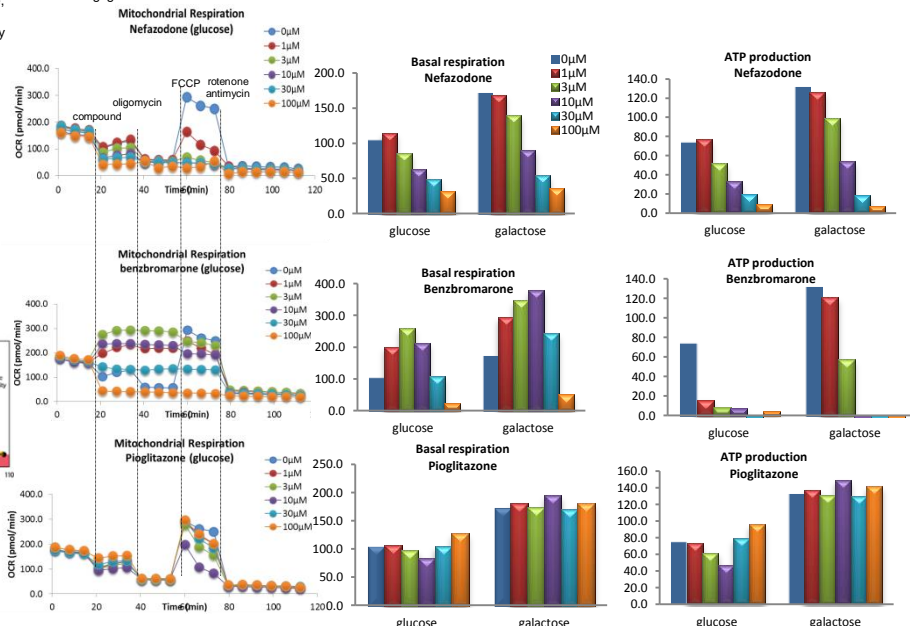


Figure 6 The effect of nefazodone, benzbromarone and pioglitazone on oxygen consumption. Oxygen consumption was measured by XF96 Analyzer. For nefazodone, oxygen consumption decreased with increase in concentration. For benzbromarone, oxygen consumption increased with increase in concentration at the low concentration range. However, oxygen consumption decreased with increase in concentration at the high concentration range. For pioglitazone, no correlation was found between oxygen consumption and increase in concentration.

## References

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## Discussion

- We were able to distinguish the β-oxidation inhibitor from the respiratory chain inhibitor by using crabtree effect and energy source restricted medium (Figure4)
- Using the long term exposure method, we were able to evaluate the mechanism of mitochondrial ribosome inhibition which was not possible by the short term exposure. The short term method was used to evaluate the ATP synthesis inhibition, respiratory chain inhibition and uncoupling effect. (data not shown. Reference to leaflet)
- Mitochondrial toxicity derived from nefazodone and benzbromarone can be observed. Conversely pioglitazone has no effect on mitochondria at the measured concentration. (Figure5)
- By monitoring the oxygen consumption using XF96 Analyzer, it was able to distinguish the difference in the mechanism for mitochondrial toxicity. According to this result, it is possible that nefazodone has the potential for respiratory chain inhibition and benzbromarone has the potential for uncoupling effect. (Figure6)

## ACKNOWLEDGMENTS

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