P-103 A simple separated evaluation method for mitochondrial toxicity using Crabtree effect.

Ryo Fujino, **Toshimasa Ito, Kenta Hashizume, Shinsuke Aoyama, Shin-ichi Ninomiya**
Drug Development Solutions Division, Sekisui Medical Co., Ltd. 2117 Muramatsu, Tokai, Ibaraki 319-1182, Japan

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.

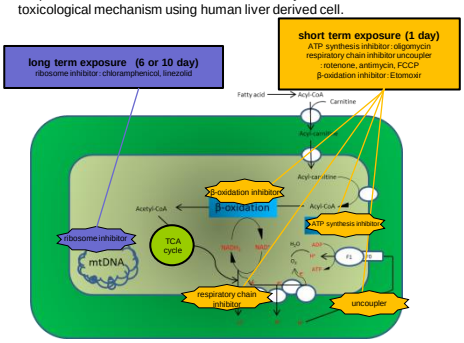

Figure 1 The mechanism of mitochondrial toxicity

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_2 incubator (37 ˚C, CO_2: 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 XFe96 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_2. The medium was then changed to the unbuffered medium and incubated at 37˚C without CO_2 until the measurement of oxygen consumption . This assay was performed in duplicate.
OCR: oxygen consumption rate

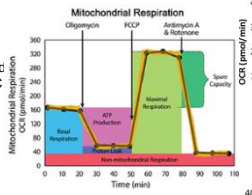

Figure 2 calculation method of basal respiration and ATP production

<Caluclation of EC_{50} value>
The EC_{50} value was calculated using the equation shown below from the relationship between percentage of control and the Typical inhibitor concentration.
% of control = $[EC_{50}{}^h/(C^h + EC_{50}{}^h)] \times 100$
C:Typical inhibitor concentration (μM)
h:hill coefficinet

Results

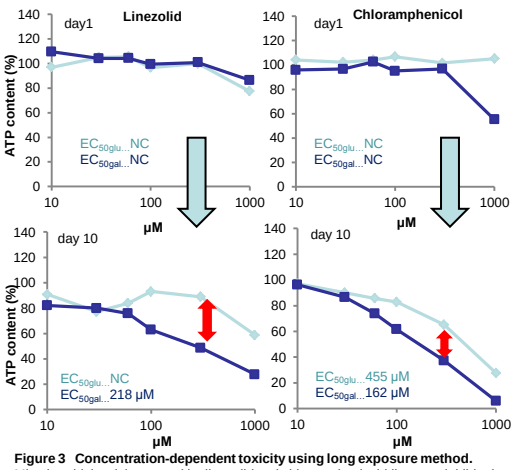

Figure 3 Concentration-dependent toxicity using long exposure method.
Mitochondrial toxicity caused by linezolid and chloramphenicol (ribosome inhibitor) was not observed with short term exposure (for 1 day) but in both cases, it was observed after long term exposure (for 10 days).
NC: not calculated

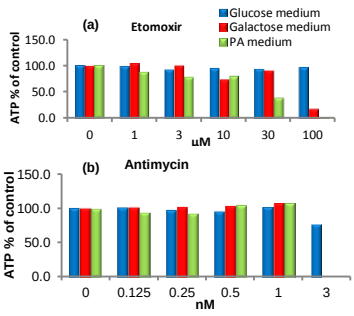

Figure 4 Concentration-dependent toxicity using three cell culture type.
Concentration-dependent toxicity of (a) etomoxir (β-oxidation inhibitor) and (b) antimycin (respiratory chain inhibitor) using three cell culture types.
Cytotoxic response of etomoxir in PA medium was most potently observed but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

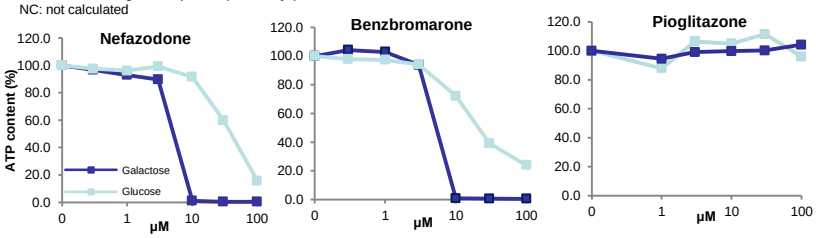

Figure 5 Concentration-dependent toxicity of nefazodone, benzbromarone and pioglitazone.
Mitochondrial toxicity of nefazodone and benzbromarone was observed. Conversely, the mitochondrial toxicity of pioglitazone was not observed or negligible.

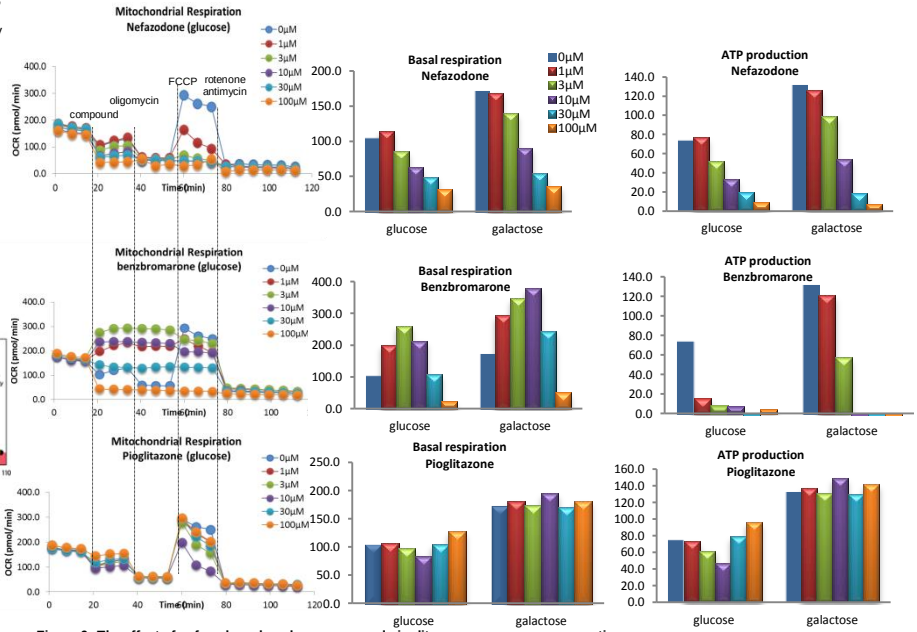

Figure 6 The effect of nefazodone, benzbromarone and pioglitazone on oxygen consumption.
Oxygen consumption was measured by XF96 Analyzer. For nefazodone, oxygen consumption decreased with increase in concentration. For benzbromarone, oxygen consumption increased with increase in concentration at the 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

1) Lisa D. Marroquin et al., TOXICOLOGICAL SCIENCES 97(2), 539–547 (2007)
2) J. Gerry Kenna et al., J Pharmacol Exp Ther. Feb;352(2), 281-290 (2015)
3) William Dott et al., Redox Biology 2 224–233 (2014)
4) Zhdanov AV et al.,Biochim Biophys Acta Jan;1837(1) 51-62 (2014)

Correspondence : Ryo Fujino,
ryo.fujino@sekisui.com

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 these result, it is possible that nefazodone has the potential for respiratory chain inhibition and benzbromarone has the potential for uncoupling effect. (Figure6)

ACKNOWLEDGMENTS
We gratefully acknowledge Primetech corp. for providing XF96 Analyzer