

MitoTox™

Assays for Identifying Inhibition of Mitochondrial Biogenesis

*Important tools for developers of
antibacterial and antiviral drugs*



Introduction

The addition of “new” mitochondrial material within a cell, i.e. mitochondrial biogenesis, requires the coordinated synthesis of 13 proteins encoded on the organelle’s own DNA and made on mitochondrial ribosomes, along with thousands of nuclear DNA-encoded subunits, all of which are shipped to and transported into the growing organelle by way of now well-defined transport processes.

Mitochondria DNA and the organellar ribosomes differ from the chromosomal DNA and cytosolic ribosomes in several aspects, reflecting their prokaryotic origin in evolution. The similarity between mitochondrial biogenesis and bacterial/viral replication represents a significant challenge for drug developers designing novel antibacterials and antivirals, as many such drugs can cause serious mitochondrial toxicity.

This problem is now sufficiently well-recognized that the FDA in 2006 issued a guidance memo stating that new antiviral drugs should be screened for their potential mitochondrial toxicity [1]. The Agency’s position was reinforced in 2009 with the release of a guidance memo on Drug-Induced Liver Injury, in which the antiviral FIAU was cited as an example of the potential for antivirals to cause organ damage as a result of their toxic effects on mitochondria [2].

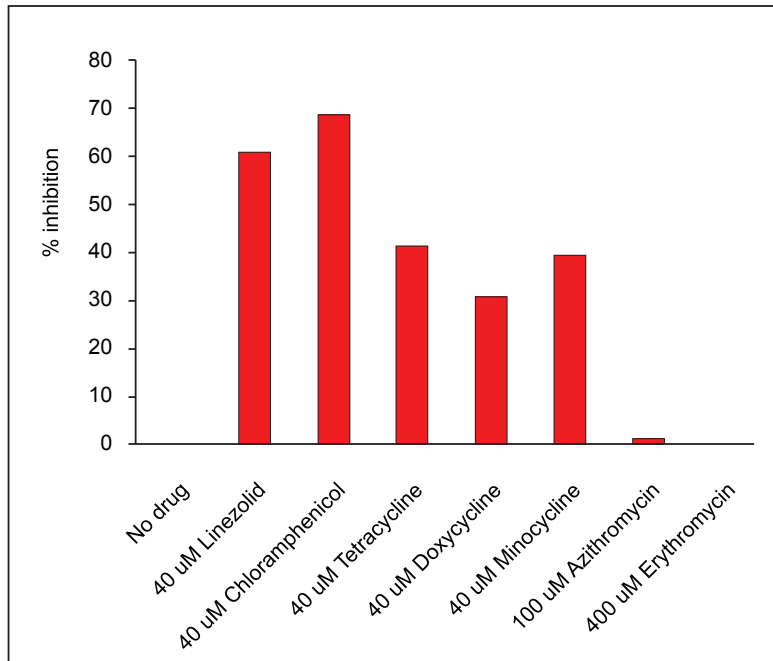


Figure 1. Rapid screening to identify antibiotics that inhibit mitochondrial biogenesis *in vitro*. Human HepG2 cells were grown in the indicated antibiotics for 5 population doublings and then effects on biogenesis were measured. Antibiotics that interfere with protein synthesis on mitochondrial ribosomes (Linezolid, Chloramphenicol, Tetracycline, Doxycycline and Minocycline) strongly inhibit mitochondrial biogenesis while other antibiotics do not.

Measuring RNA levels using reverse transcription qPCR is occasionally discussed as a useful high-throughput technique for identifying effects on mitochondrial biogenesis [3], but this approach has a fundamental limitation, that being its inability to identify inhibition of mitochondrial ribosomes. If such an effect is occurring then RNA levels might in fact increase in the cell, since mitochondrial translation is unable to process transcription products. The only way to easily and simultaneously identify inhibition of both transcription and translation is to ratio the levels of an mtDNA-encoded protein with total cell protein and/or a nDNA-encoded protein. This can be done by Western blotting or by ICC microscopy, but neither technique is suitably quantitative, nor are they amenable to high-throughput nor clinical applications.

MitoSciences offers the only solution for measuring drug-induced effects on mitochondrial biogenesis early in the safety screening process. Our MitoBiogenesis™ In-Cell ELISA is a true duplexing 96/384-well assay that ratios both an mtDNA- and an nDNA-encoded protein in cultured or primary cells, and which requires very little sample prep and few overall steps.

Applications

Cell-Based Measurement of Mitochondrial Biogenesis

Overview

Screening new drug candidates early in the safety screening process requires an assay that is adequately quantitative, specific, and reproducible, and which is flexible enough to be suitable for a variety of cell types. It must also have a minimum of sample steps and, ideally, be adaptable to automation. MitoSciences' MitoBiogenesis™ In-Cell ELISA provides the toxicologist with a solution that meets all of these requirements.

Assay Principle

Cells (human, rat or mouse) are seeded in 96- or 384-well microplates, and after exposure to experimental compounds for several cell doublings, the levels of two mitochondrial proteins are measured simultaneously in each well. The two proteins are each subunits of a different oxidative phosphorylation enzyme complex, one protein being subunit I of Complex IV (COX-I), which is mtDNA-encoded, and the other being the 70kDa subunit of Complex II (SDH-A), which is nDNA-encoded. Complex IV includes several proteins which are encoded in the mitochondrion, while the proteins of Complex II are entirely encoded in the nucleus. Optionally, total level protein can also be measured.

This assay employs In-Cell ELISA technology, which utilizes antibodies and fluorescent dyes or enzyme-linked detection to perform quantitative immunocytochemistry on fixed cells grown in 96- or 384-well microplates. The resulting data has specificity similar to Western blotting, but with much greater quantitative precision and reproducibility. Because the assay is able to measure two proteins simultaneously, and because it can be run using either 96- or 384-well microplates, it also offers unparalleled throughput potential.

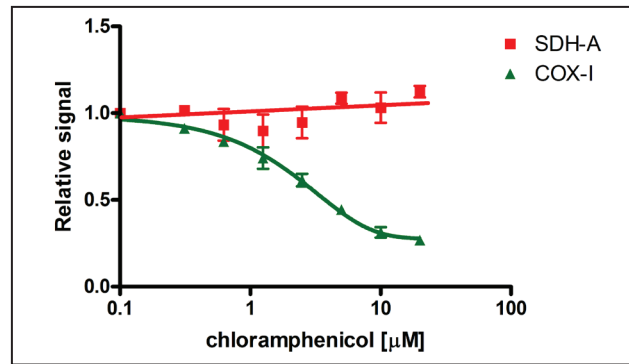


Figure 2. Inhibition of mitochondrial biogenesis by chloramphenicol The IC₅₀ of a drug's effect on mitochondrial protein translation can be determined quickly using the MitoBiogenesis™ In-Cell ELISA Kit (IR). In this example, cells were seeded at 3000 cells/well, allowed to grow for 3 cell doublings in a drug dilution series and then the relative amounts of COX-I, and SDH-A were measured in each well. Chloramphenicol inhibits mtDNA-encoded COX-I protein synthesis relative to nuclear DNA-encoded SDH-A protein synthesis by 50% at 3.5 μM.

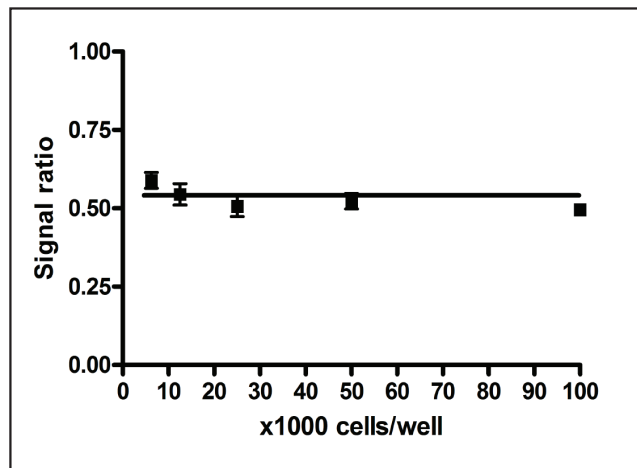
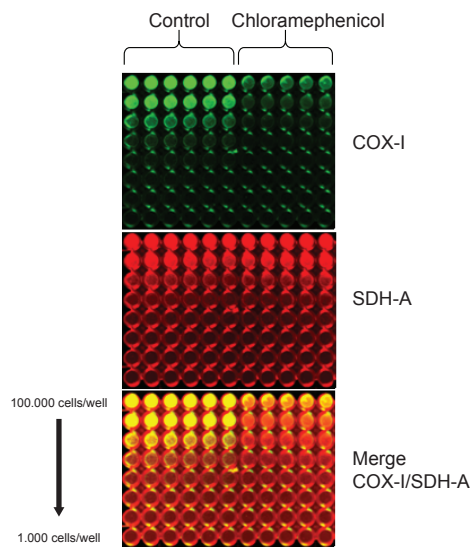


Figure 3. mtDNA-encoded protein expression is reduced specifically in rat H9C2 cardiomyocytes treated in vitro with the antibiotic chloramphenicol.

Figure 3a. Levels of mtDNA-encoded COX-I (green 800 channel), nuclear DNA-encoded SDH-A (red 700 channel) and relative ratios COX-I/SDH-A (merged) are shown in each microwell. Wells with normal levels of both proteins exhibit a yellow merged color due to approximately equal red and green signals. In contrast, wells with low levels of COX-I and normal levels of SDH-A exhibit an orange merged color due to the relative lack of green (COX-I) fluorescence.

Figure 3b. Quantitative measurement of the COX-I/SDH-A protein expression ratio. At all cell concentrations, a constant ratio of mtDNA-encoded protein expression (COX-I) to nuclear DNA-encoded mitochondrial protein expression (SDH-A) is observed in untreated cells. Therefore, normalizing COX-I levels to SDH-A levels simplifies data analysis and eliminates the need to perform all tests at the same cell concentration.

Total cell protein and/or number can be measured if desired by using the total cell stain Janus Green after recording the In-Cell ELISA immunofluorescence or colorimetric readings. Normalizing levels of both proteins to total cell protein can provide additional information regarding the effects of drugs or conditions on mitochondrial mass and biogenesis per cell.

Drugs that adversely affect mitochondrial biogenesis are identified easily with this assay (Figure 2), and dose response series' are easily run to quickly establish IC50's (Figure 3).

Availability

The MitoBiogenesis™ In-Cell ELISA is available in either kit form (catalog # MS642 or MS643), or as a service through MitoLab, MitoSciences' Laboratory Services division. The kit includes all necessary reagents and a detailed protocol for conducting drug screening, and versions are available for both IR and colorimetric detection.

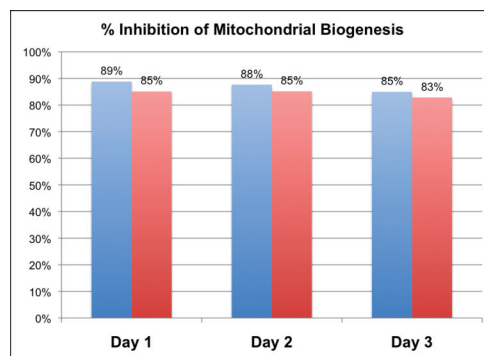


Figure 4. Assay reproducibility demonstrated by experiments conducted on three separate days. HepG2 cells were grown for 7 days in the presence of 10 μM chloramphenicol and then measured in duplicate on 3 different days using the MitoBiogenesis™ In-Cell ELISA Kit. The assay was able to record inhibition of mitochondrial biogenesis with an average intra-day CV of 2.3% and an average day-to-day CV of 1.9%.

Product Information:

Catalog #	Product Name	Amount	Reactivity
MS642	MitoBiogenesis™ In-Cell ELISA Kit (IR)	2 x 96 tests	human, rat, mouse, bovine
MS643	MitoBiogenesis™ In-Cell ELISA Kit (Colorimetric)	2 x 96 tests	human, rat, mouse, bovine

References:

1. Food and Drug Administration. *Guidance for Industry; Antiviral Product Development — Conducting and Submitting Virology Studies to the Agency*. <http://www.fda.gov/OHRMS/DOCKETS/98fr/05d-0183-gdl0002-01.pdf>
2. Food and Drug Administration. *Guidance for Industry; Drug-Induced Liver Injury: Premarketing Clinical Evaluation*. <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM174090.pdf>
3. Medeiros, DM (2008) *Methods*. 46(4):288-94. Assessing Mitochondria Biogenesis.



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