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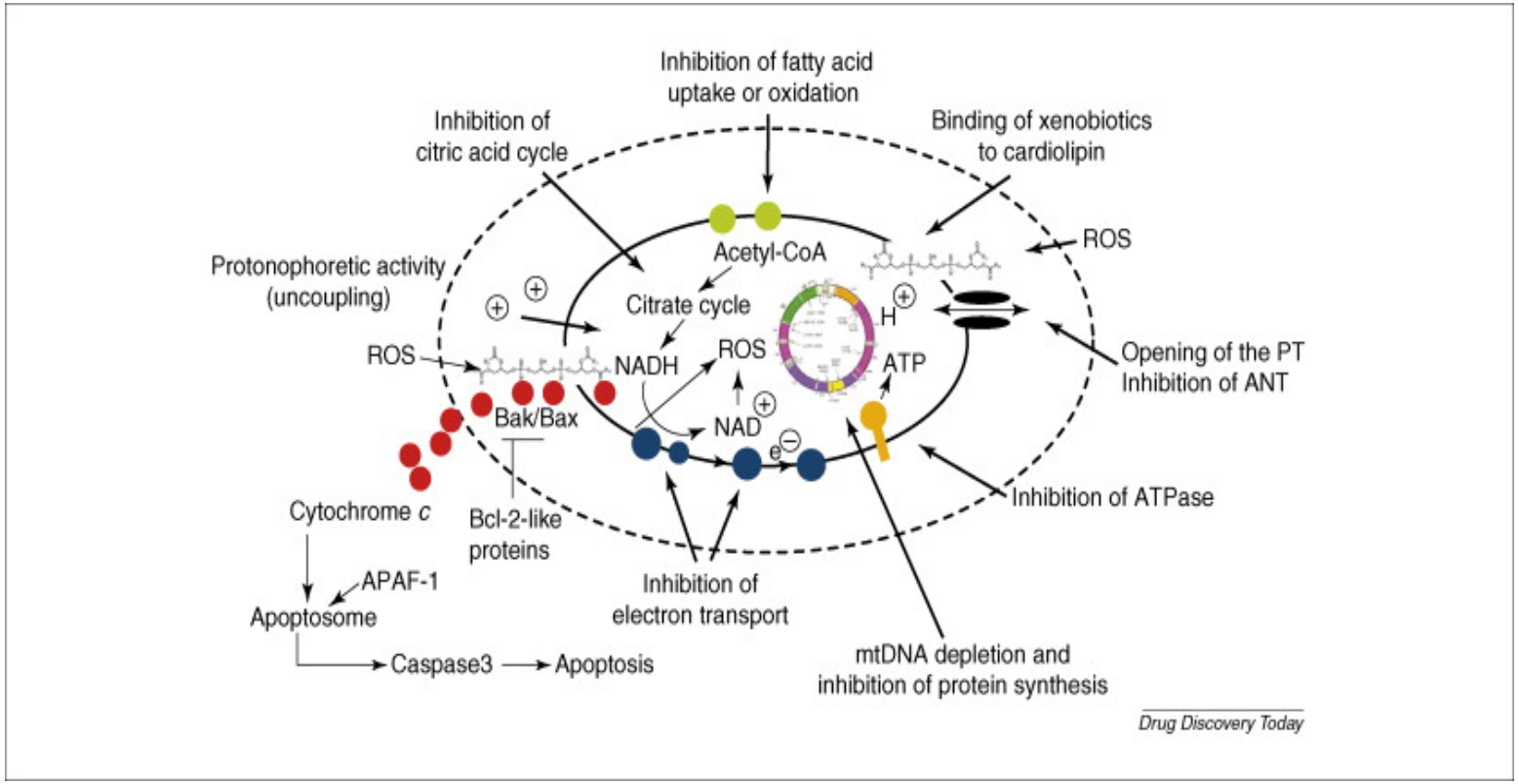
Development of a High Throughput High Content Imaging based Mitobiogenesis Assay

Drug-Induced Mitochondrial Toxicity

- More than half of the drugs that carry black box warnings for hepato- and cardiotoxicity have been shown to act as mitochondrial toxins
- Assays exist to measure the acute effects of drugs on the mitochondria
  - changes in membrane potential
  - mitochondrial integrity
  - reactive oxygen species generation
- There are few assays that can assess more long term effects such as inhibiting mitochondrial DNA transcription and inhibition of mitochondrial protein synthesis



Dykens & Will, Drug Discovery Today, 12:777-785, 2007

mt DNA and protein synthesis

Proteins localized in mitochondria are:

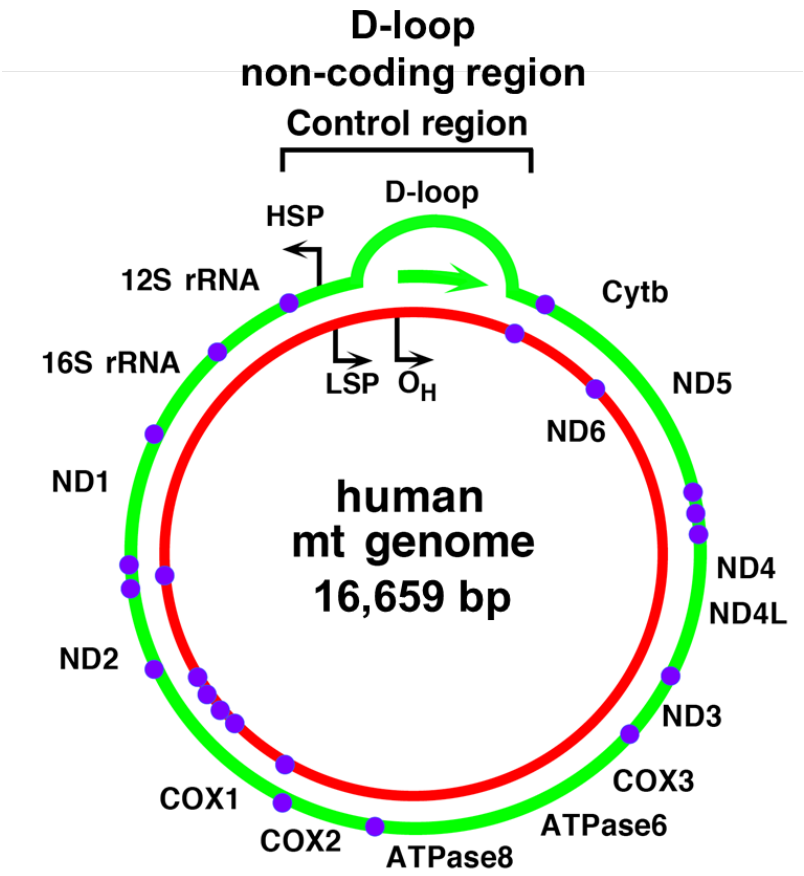
- **encoded by two genomes-**
  - **nuclear DNA (nDNA)**
  - **mitochondrial DNA (mtDNA)**

- **produced by two different protein synthesis machineries**
  - **cytosolic**
  - **mitochondrial**

- **majority of mitochondrial proteins are encoded by nuclear genes, which are synthesized in cytosol and post-translationally imported into mitochondria.**

- **13 mitochondrial proteins are encoded by mtDNA, which are transcribed by the mitochondrial RNA polymerase and translated in the mitochondrial matrix.**

Organization of the mitochondrial genome



Characteristics of animal mtDNAs:

- Circular
- Small in size ~16 kb in man
- 5-10 copies of mtDNA / mitochondrion
- ~1,000 mitochondria / cell
- ~1% of cellular DNA
- Encode:
  - 13 proteins
  - large and small rRNA
  - tRNAs
- NO INTRONS- polycistronic mRNAs
- Mitochondrial genetic code has different genetic code as compared to that in nucleus
  - UGA = tryptophan not STOP
  - AGA = STOP not arginine
  - AUA = methionine not isoleucine

mt DNA Depletion and inhibition of protein synthesis

- There are few assays that can assess more long term effects such as mitochondrial DNA (mtDNA) depletion and inhibition of mitochondrial protein synthesis.
- These two mechanisms of mitochondrial related toxicity are associated with serious and potentially fatal side effects.
- A number of drugs including AIDS therapies, antibiotics, and chemotherapeutic agents are associated with toxic side effects related to mtDNA depletion and inhibition of mitochondrial protein synthesis.
- There are few commercially available assays that directly measure mitochondrial protein synthesis utilize qPCR or inCell ELISAs
- Therefore, we are developing a new assay for use with high content screening systems, to directly measure mtDNA content and assess mitochondrial protein synthesis. The assay was run in HepG2 cells against compounds known to cause mtDNA depletion and protein synthesis inhibition.

Drugs inhibit mtDNA translation and protein synthesis

Pharmacologic Category	drug	Proposed mechanism	Adverse effects related to mitochondrial toxicity
Antibiotics	Gentamicin Tetracycline Minocycline Chloramphenicol Aminoglycosides Linezolid	Reduces mt protein synthesis Impair mtDNA translation	Deafness, renal failure, myopathy Hearing loss cardiac toxicity
Anti-retroviral	Nucleoside reverse transcriptase inhibitors Zidovudine Didanosine Lamivudine Abacavir	Impairs mtDNA replication which causes mtDNA depletion which then affects all functions	Carnitine deficiency; lactic acidosis; lipodystrophy; neuropathy; myopathy; hepatic dysfunction
Anti-cancer medicines	Doxorubicin Cisplatin Cis-platinum	mtDNA mutation	Cardiomyopathy
Anti-viral I	Interferon	Impairs mtDNA transcription	

Methods

- HepG2 cells are seeded on 384-well plates
- Compounds are serially diluted in DMSO and dosed in medium
- Cells are refed media with compound every 3 days
- At the end of the incubation period, cells are fixed, stained, and analyzed via HCS
  - Nuclei staining:
    - Hoechst 33342
  - Conjugated antibodies used:
    - α-SDHA Alexa Fluor 647 (nuclear coded mitochondrial protein)
    - α-COX1 Alexa Fluor 488 (mitochondrial coded protein)

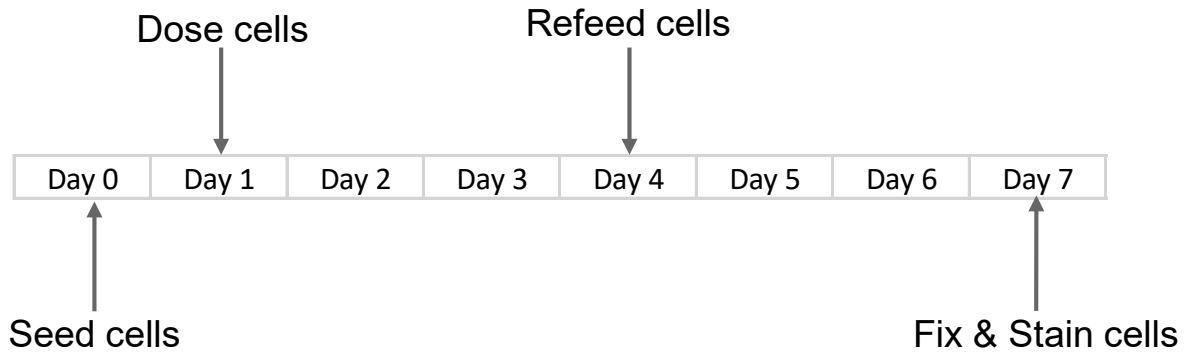
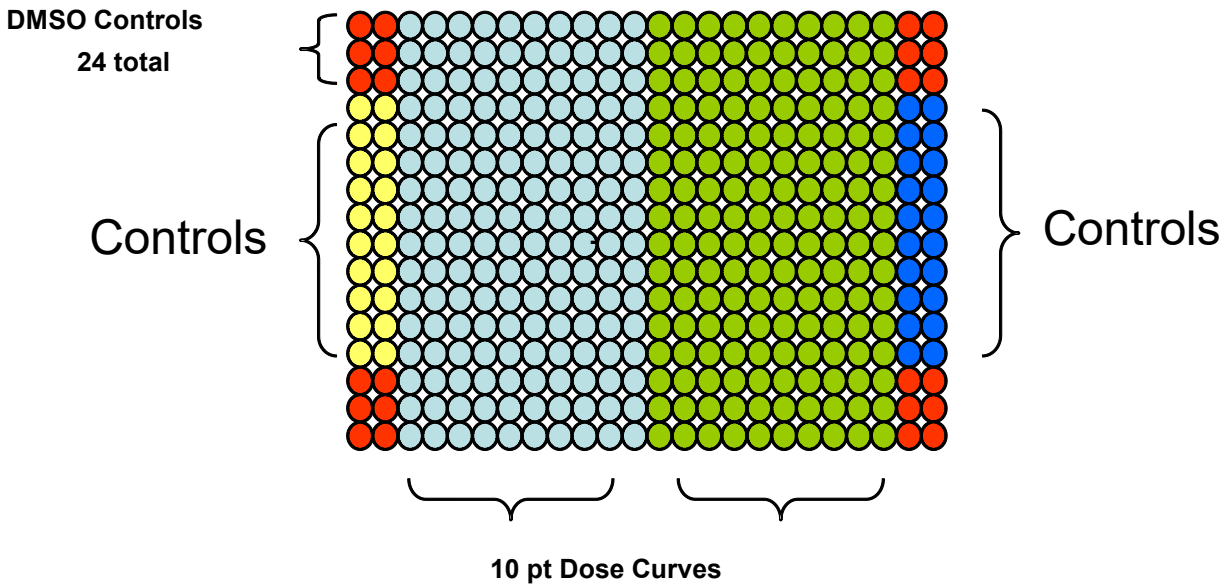


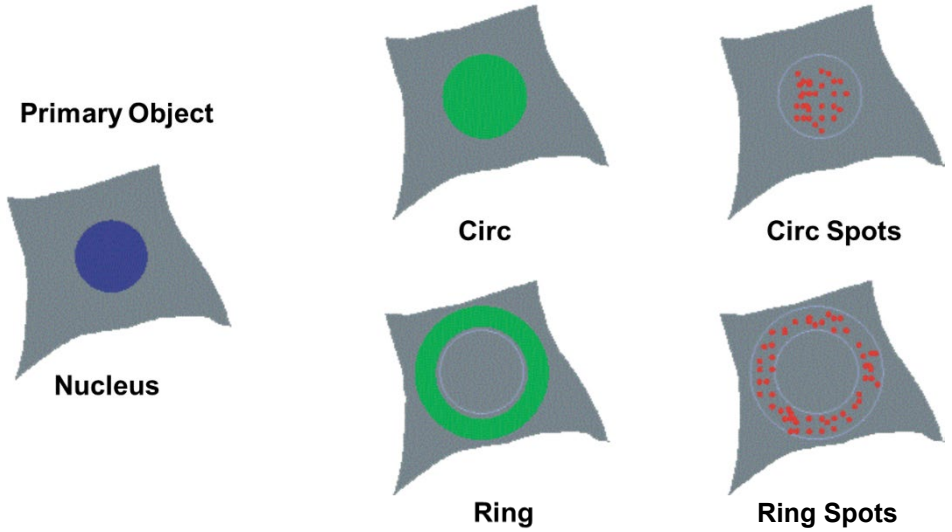
Plate Layout



Automation and Throughput

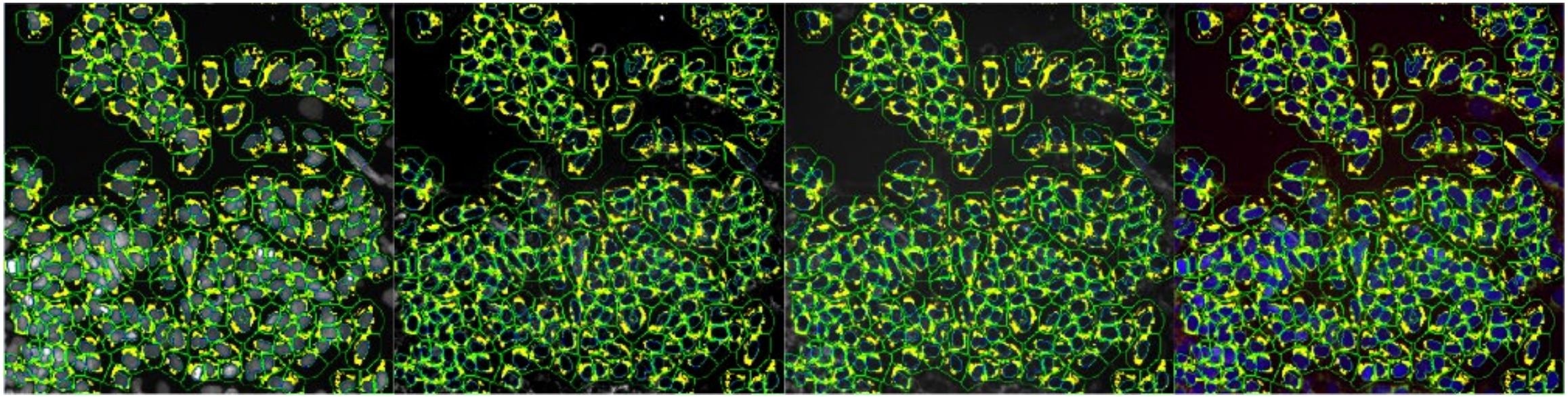
Object Segmentation and Identification

- The key to successful HCS is proper segmentation of cells and the identification of the primary object in the cell (usually the nucleus)
  - This is accomplished through the front end image analysis software that is included with the instruments.
  - Proper settings also allow for measurement of the size of the nucleus, as well as any changes that occur, which is an important indicator of toxicity
  - This also allows for quantification of nuclear and cytoplasmic stains with overall intensities as well as spot intensities for organelles such as mitochondria



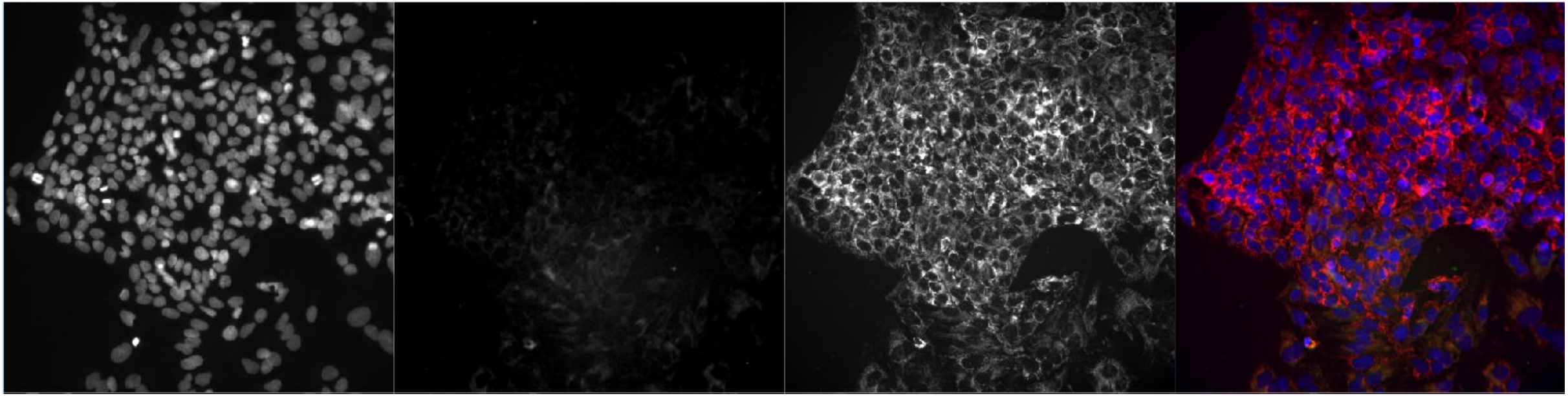
HCS Object Identification and Quantification

Nuclei (Hoechst) | Cox1 (Mt coded) | SDH-A (Nuc Coded) | Overlay

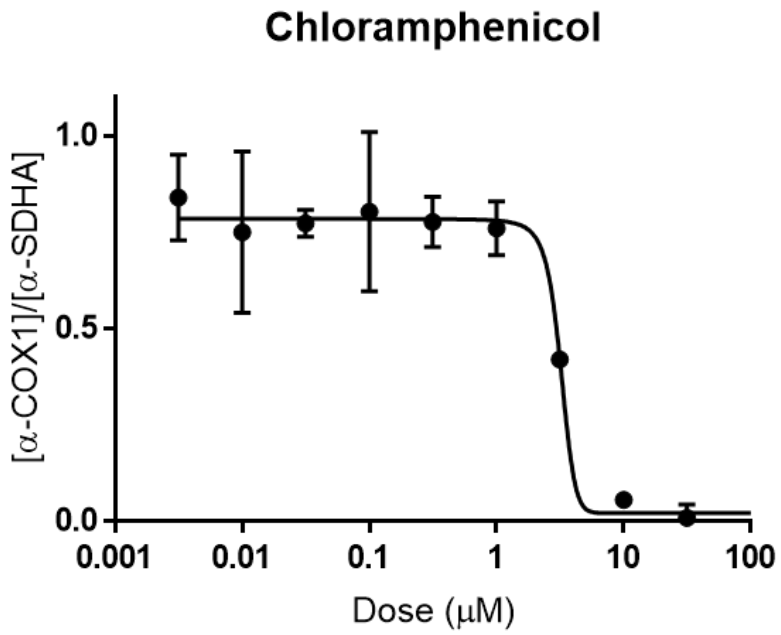


Chloramphenicol Images

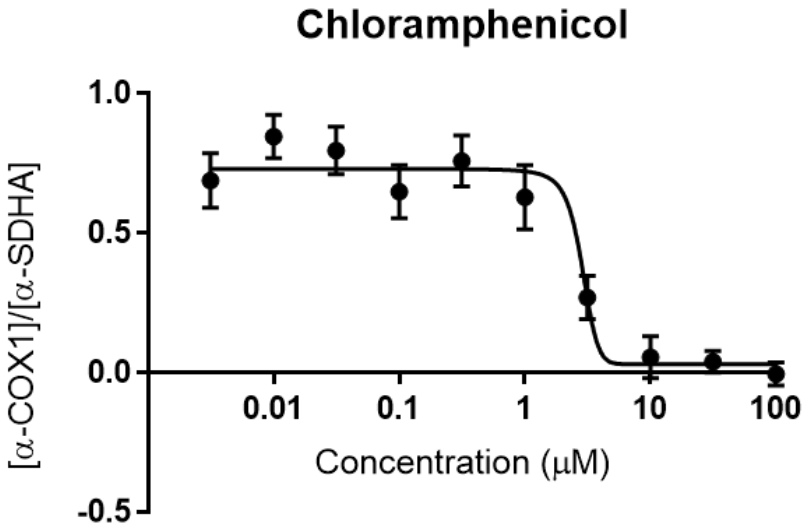
Nuclei (Hoechst) | Cox1 (Mt coded) | SDH-A (Nuc Coded) | Overlay



Chloramphenicol Dose Curves

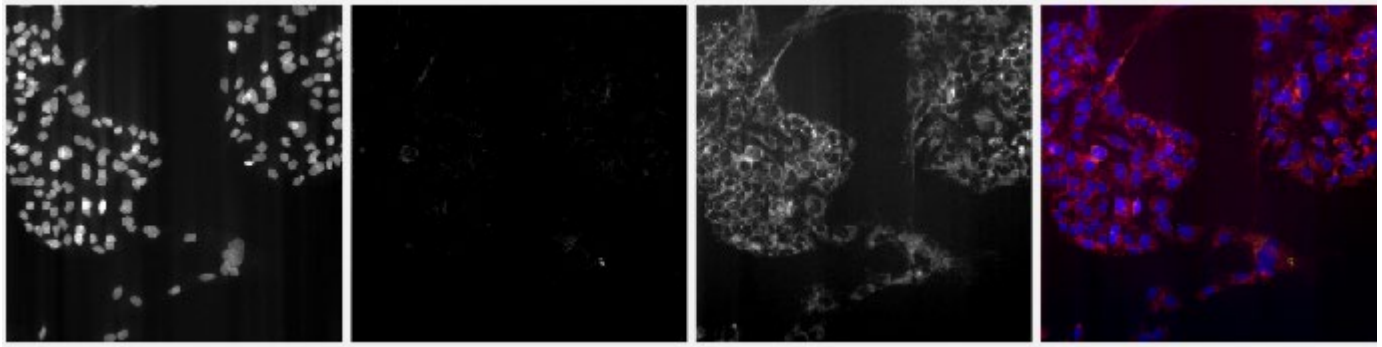


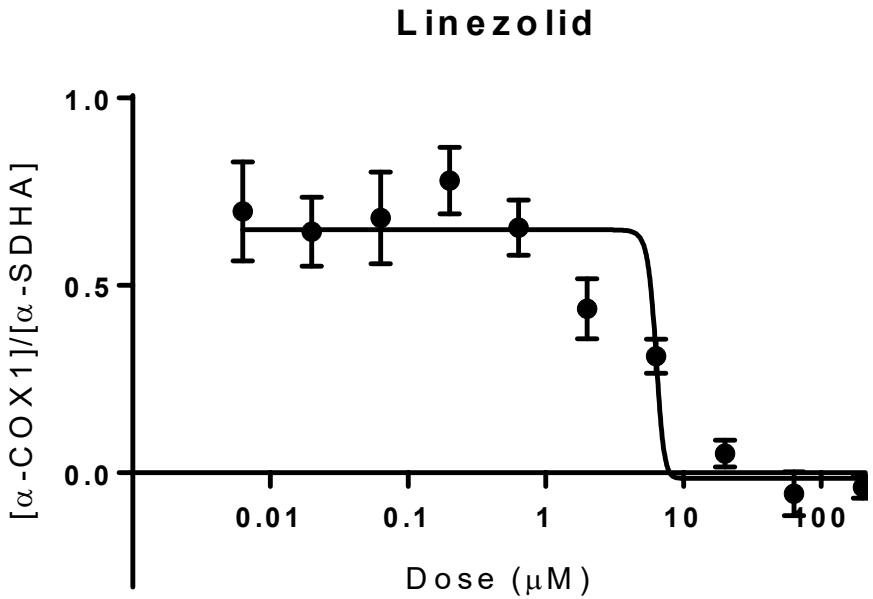
EC50 3.196µM



EC50 2.859 µM

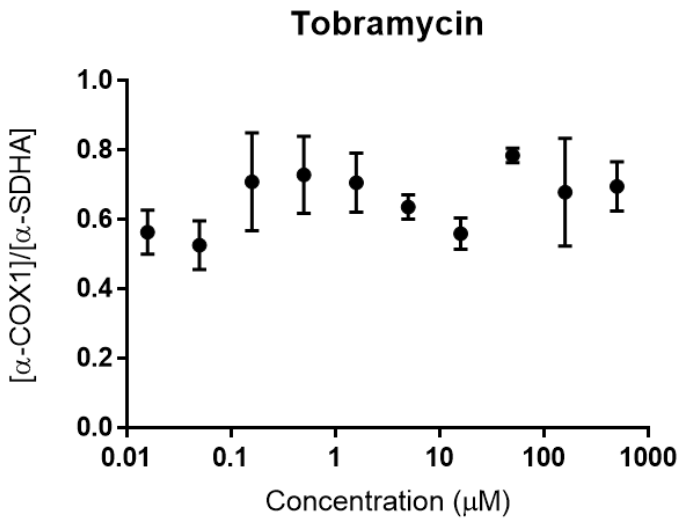
Linezolid Dose Curve

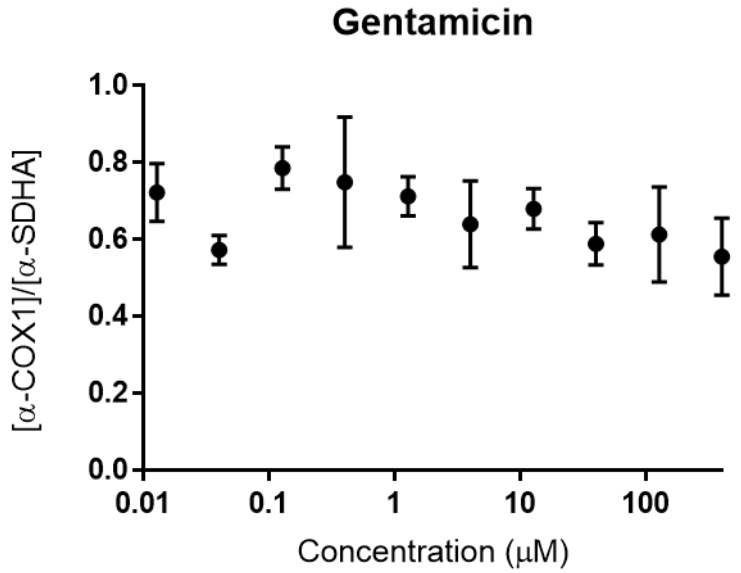




EC50 6.303μM

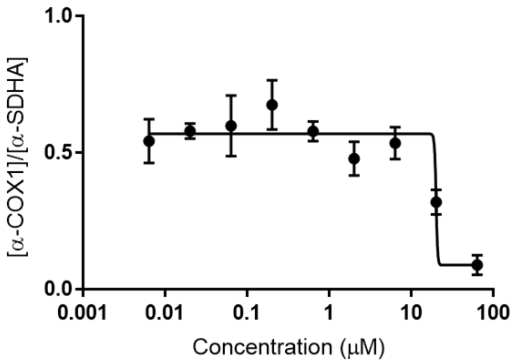
Negative Antibacterials





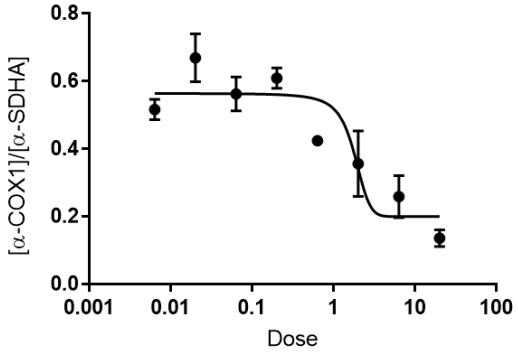
Additional Positive Antibiotics

Tetracycline



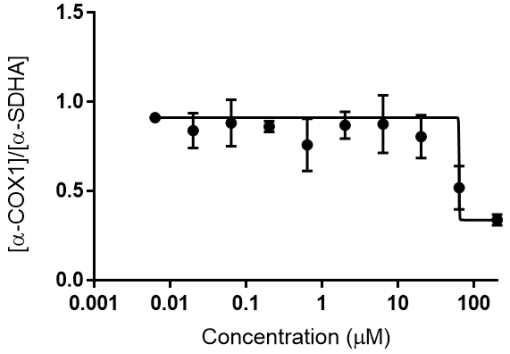
EC50 20.35

Doxycycline



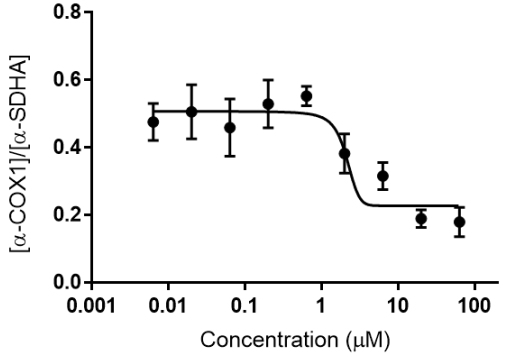
EC50 1.796μM

Erthromycin



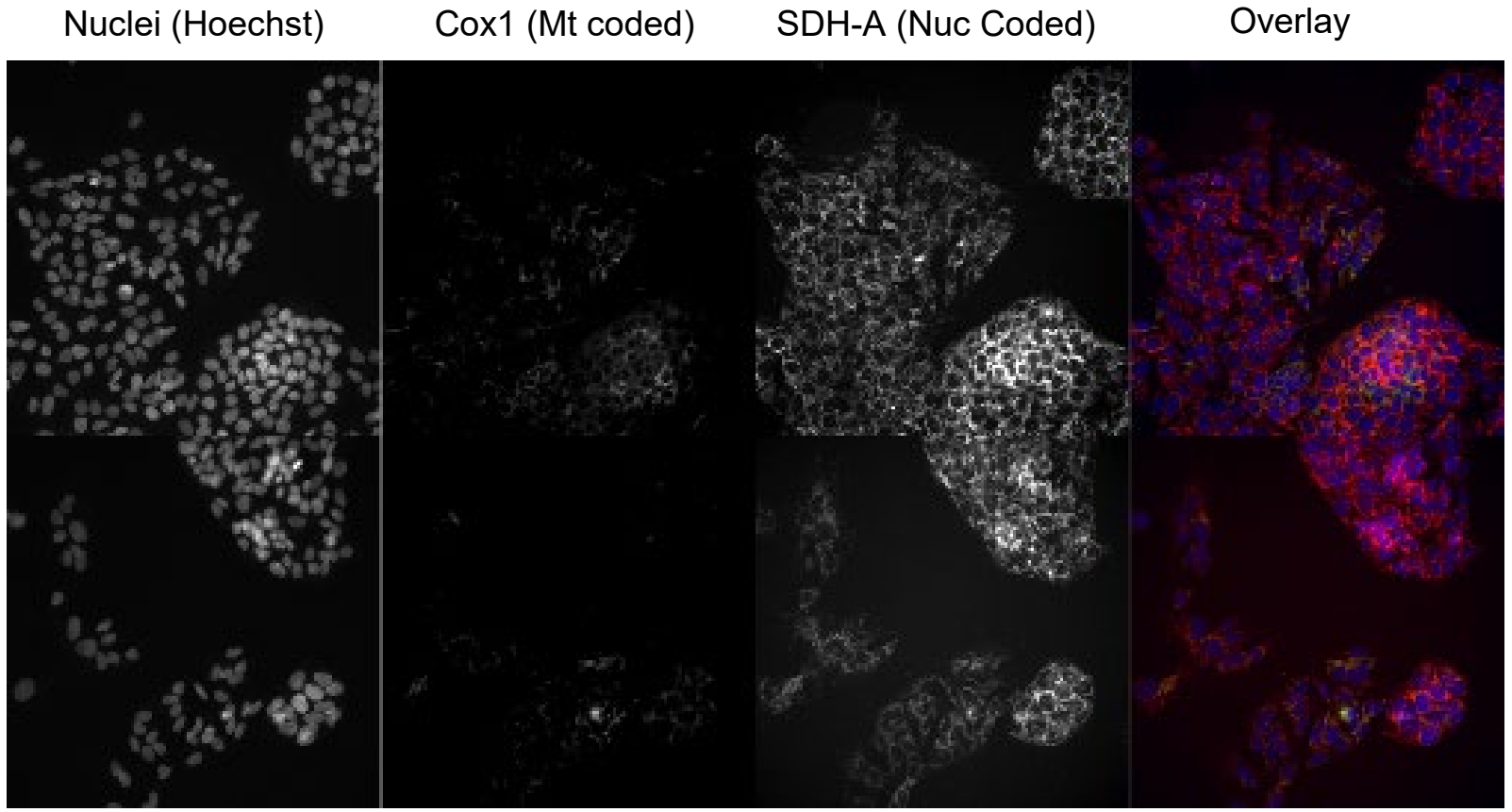
EC50 63.31μM

Minocycline

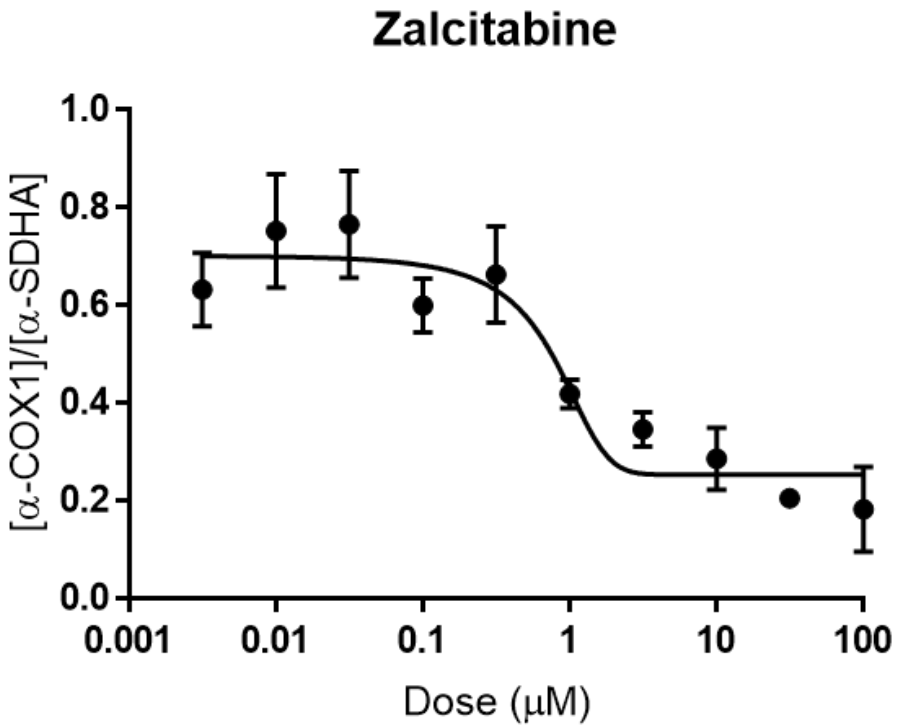


EC50 2.12μM

Antivirals – Zalcitabine (ddC)

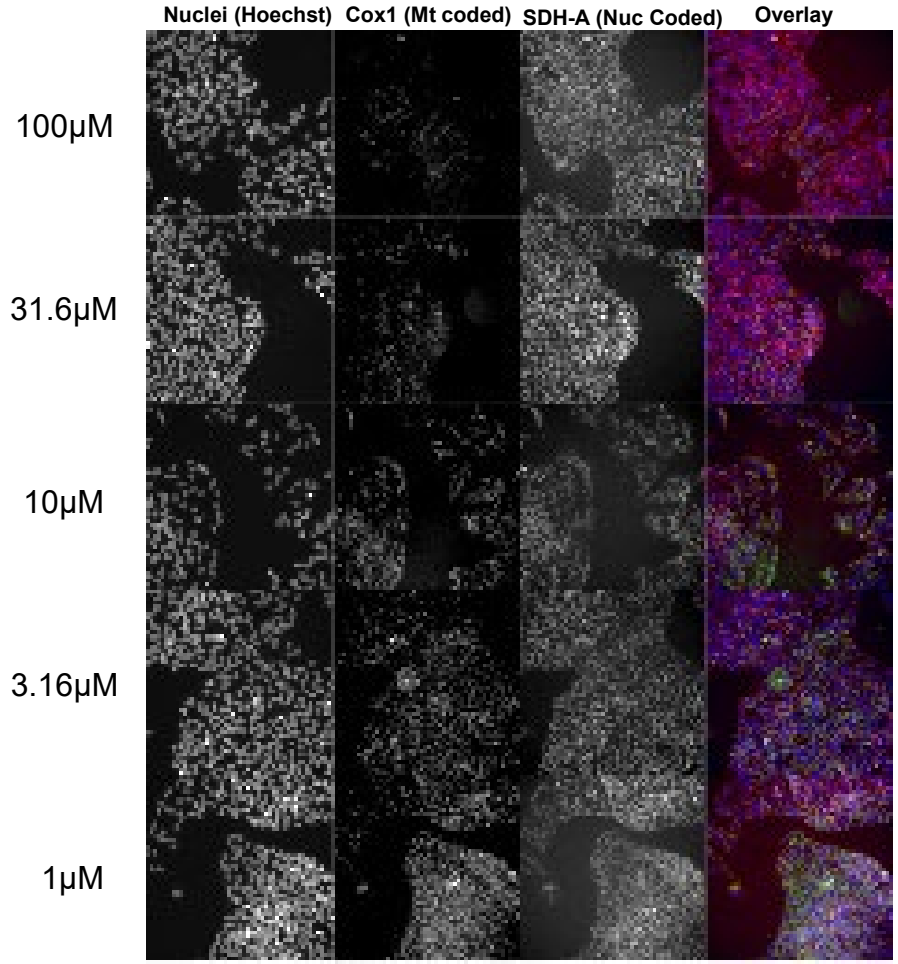


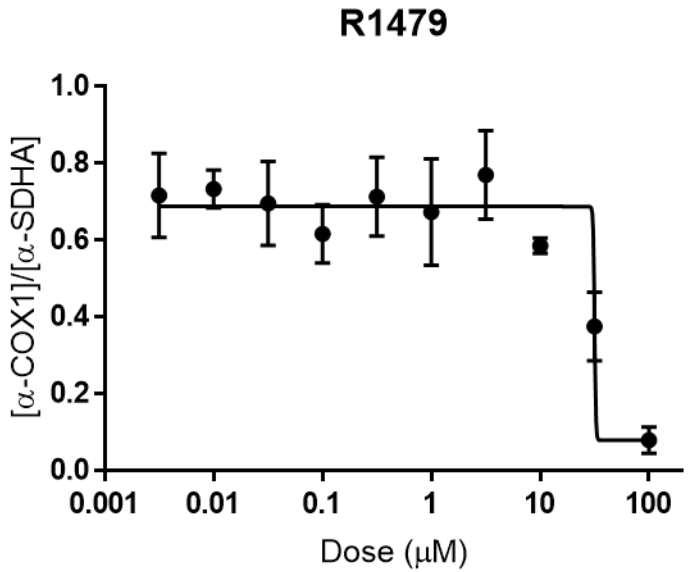
Zalcitabine Dose Curve



EC50 0.678μM

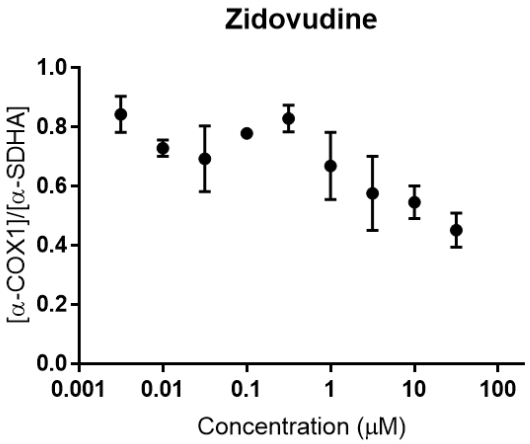
R1479 Dose Curve

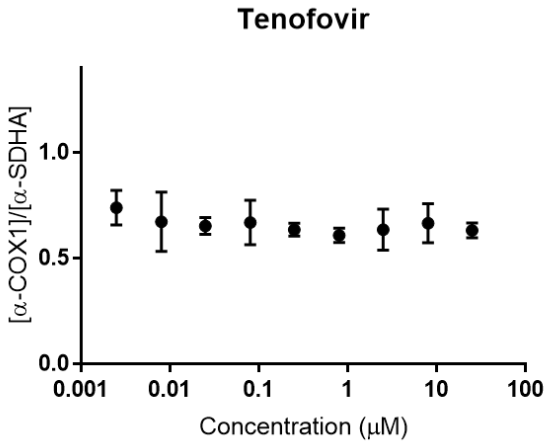


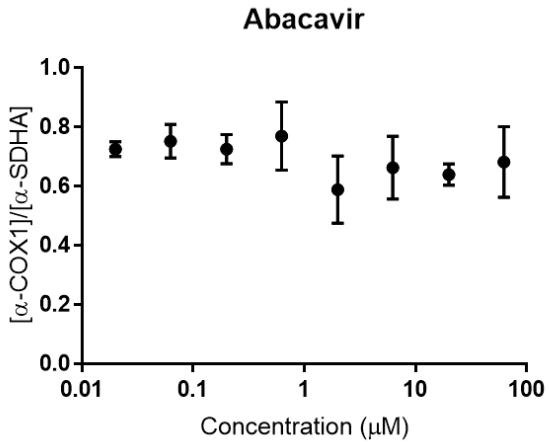


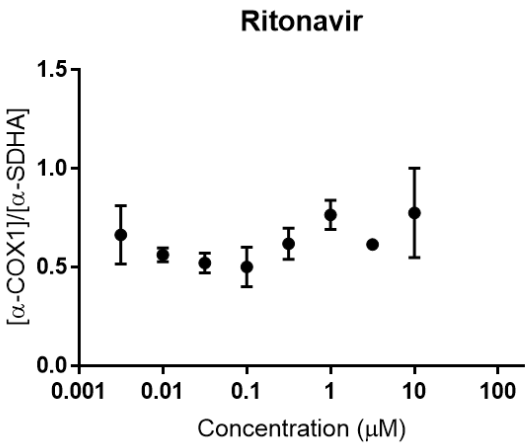
EC50 31.58µM

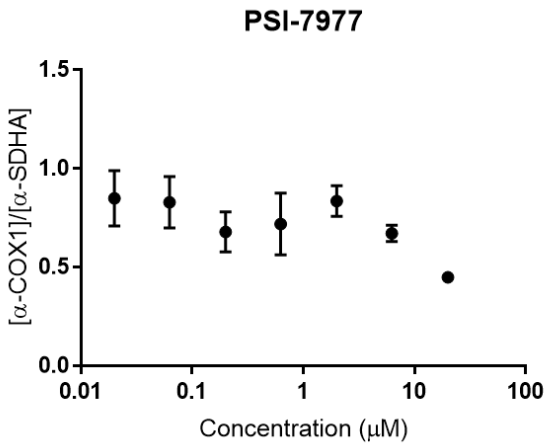
Negative Antivirals

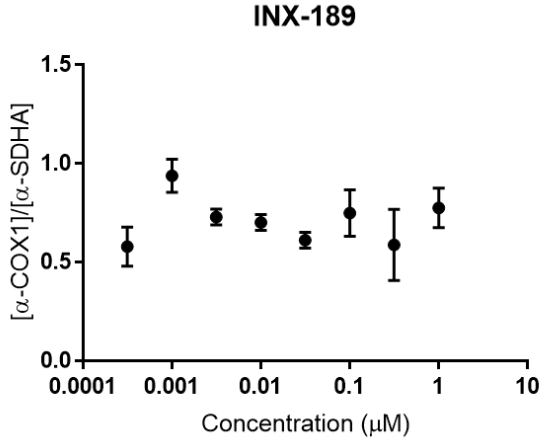












Conclusions

- Antibacterials and Nucleoside reverse transcriptase inhibitors (NRTIs) are known for their potential to induce mitochondrial dysfunction through depletion of mitochondrial coded proteins.
- Here we show development of a high content imaging assay which uses immunofluorescence to directly and simultaneously measure expression of the mitochondrial coded protein, COX-1, and the nuclear coded mitochondrial protein, SDH-A, on a per cell basis.
- We evaluate the effects of 8 NRTIs and 8 antibacterials in dose curve in a 384 well plate format
- Result show that we correctly identify the antivirals Zalcitabine (ddC) and 4’ Azidocytidine (R-1479) as well as the antibiotics chloramphenicol and linezolid at their expected concentrations
- In conclusion, this assay is quantitative and reproducible, with the added advantage that cell count is determined simultaneously and results can be directly verified by analysis of the cellular images.

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