

Development of *In Vitro* Screening Tools to Test for Drug-Induced Mitochondrial Toxicities

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Exploratory Safety Differentiation

Pfizer PGRD

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Dist. Prof of Biochemistry
(OSU)
SOT president 1991-1992

Glutathione status and chemical
induced Toxicity



Mentors provide their expertise to less experienced individuals in order to help them advance their careers, enhance their education, and build their networks.

Most scientists and governments agree that animal testing should cause as little suffering as possible, and that alternatives to animal testing need to be developed. The "three Rs", first described by Russell and Burch (1959), are guiding principles for the use of animals in research in many countries:



Reduction

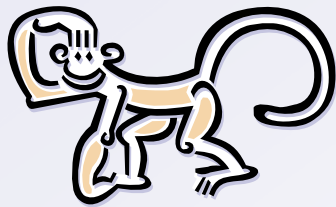
refers to methods that enable researchers to obtain comparable levels of information from fewer animals, or to obtain more information from the same number of animals.

Refinement

refers to methods that alleviate or minimize potential pain, suffering or distress, and enhance animal welfare for the animals still used.

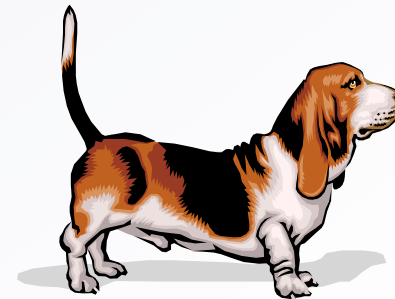
Replacement

refers to the preferred use of non-animal methods over animal methods whenever it is possible to achieve the same scientific aim.



The two major alternatives to in vivo animal testing are:

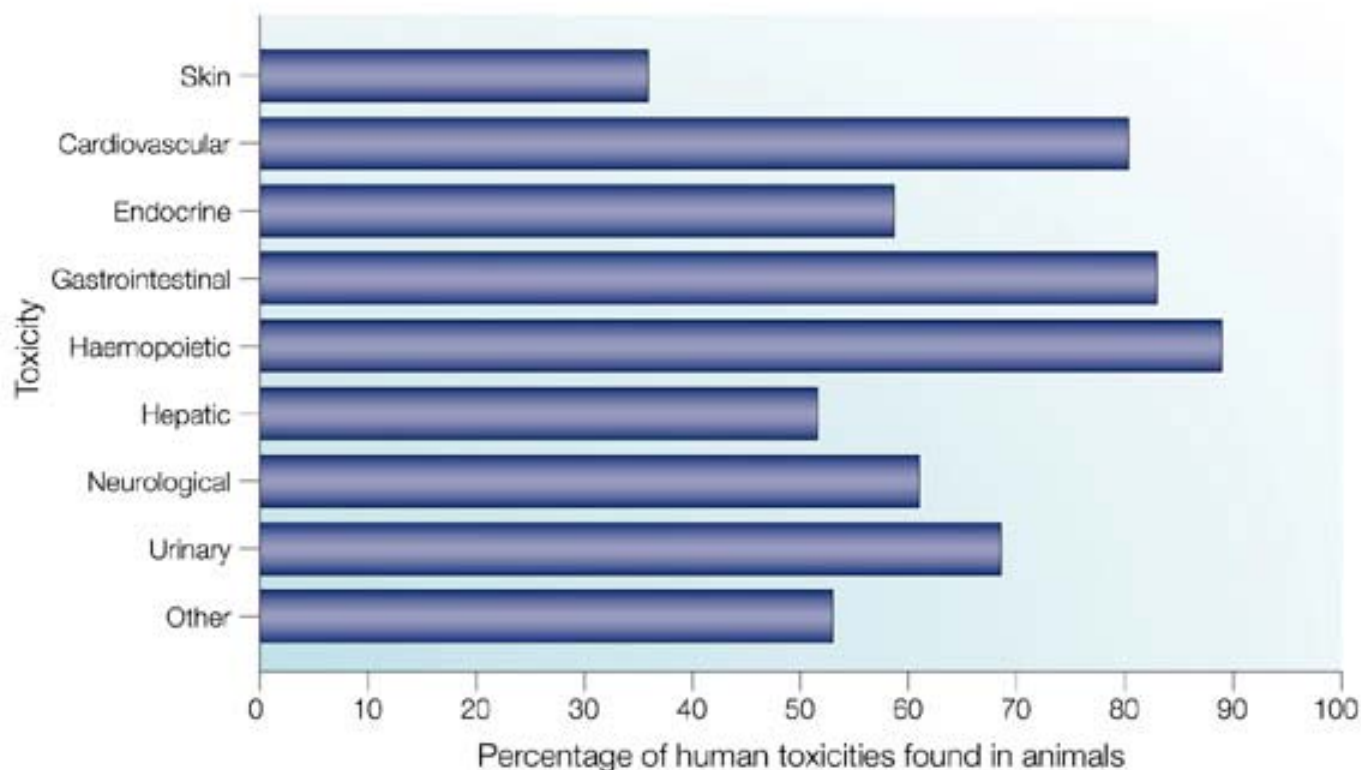
In vitro cell culture techniques and in silico computer simulation.



Some Differences between Animals and Humans Critical to Prediction of Toxicity

	Animals	Man
Subjects		
Number	Large groups	Individuals
Age	Young adult	All ages
State of health	Healthy	Usually sick
Genetic background	Homogeneous	Heterogeneous
Doses		
Magnitude	Therapeutic to toxic	Therapeutic
Schedule	Usually once daily	Therapeutic optimum
Environment		
Housing	Uniform, optimal	Variable
Nutrition	Uniform, optimal	Variable
Concomitant therapy	Never	Frequent
Diagnostic procedures		
Verbal contact	None	Intensive
Physical exam	Limited	Extensive
Clinical lab	Limited, standardized	Individualized
Timing	Predetermined	Individualized
Autopsy	Always	Exceptional
Histopathology	Extensive	Exceptional

Percentage Concordance Between Animal and Human Toxicities, Grouped by Organ



Nature Reviews | Drug Discovery

Similarly to data on anticancer drugs, correlation is better for toxicities in the gastrointestinal tract, and haemopoietic and cardiovascular systems. Modified, with permission, from Olson, H. *et al.* Concordance of the toxicity of pharmaceuticals in humans and animals. *Regul. Toxicol. Pharmacol.* **32**, 56–67 (2000). © (2002) Elsevier Science.

Many Drug Classes cause Mitochondrial Toxicity

Effect on Mitochondria	Example Drugs
Inhibitors of ETC	Fibrates, Glitazones, Statins
Uncouplers	Sulfonamides, Glitazones, NSAID
Oxidizing agents/ Redox cyclers	Doxorubicin, Acetaminophen
Inhibitors of FA synthesis	Valproic Acid, Tetracyclin Salicylates
Inhibitors of mitochondrial protein synthesis	Antibiotics (Macrolides)
Depletion of mtDNA	AZT, Abacavir, Efavirenz
Induction of MPT	Bile Acids, Anti Cancer Drugs

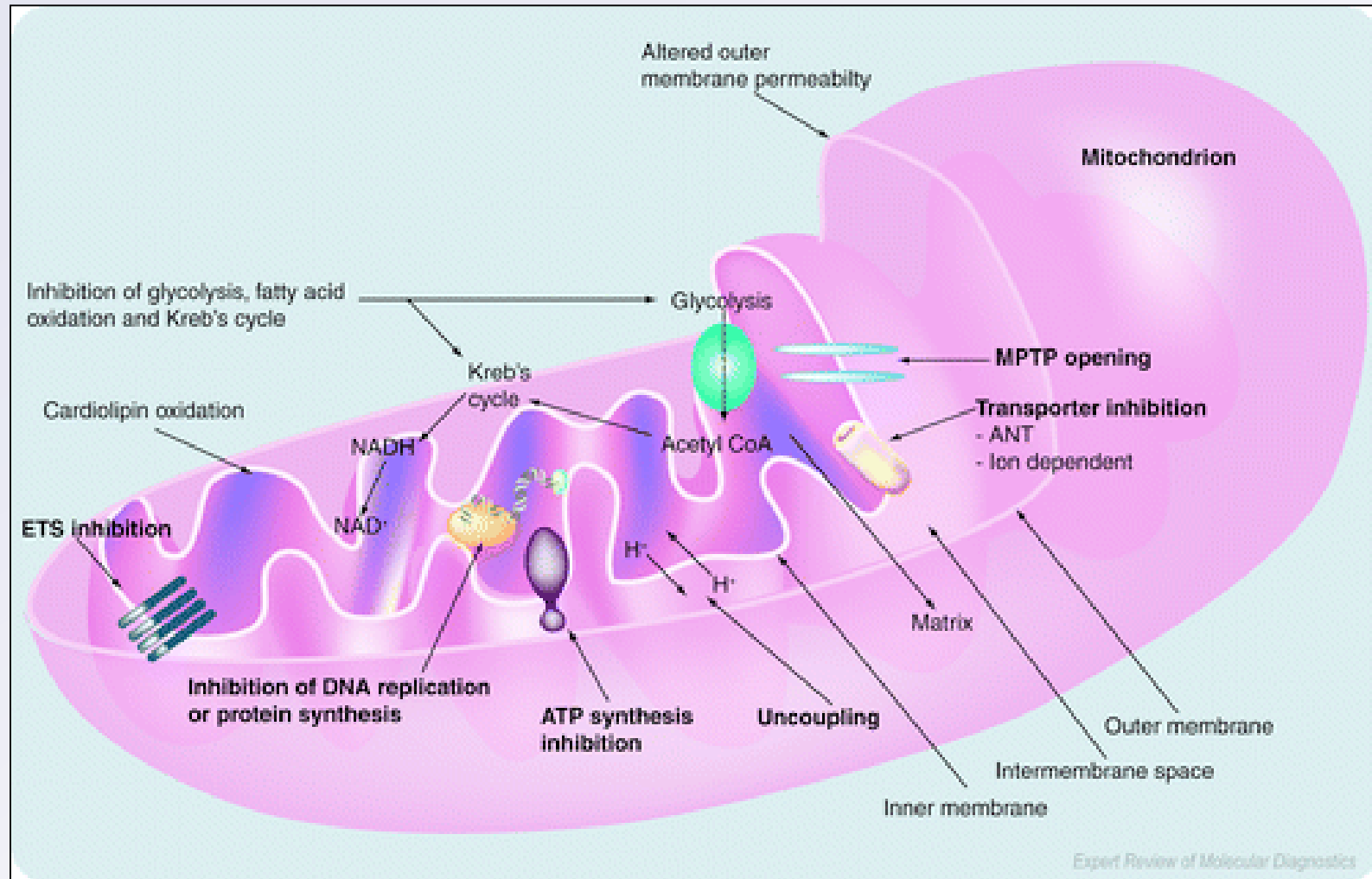
Mitochondria: Bioenergetics, Oxidative Pathology and Cellular Viability Converge

- Cytoplasmic Organelles
- **Generate > 90% of cellular energy**
- **Generate 90% of radicals**
- **Gatekeepers of cell death (apoptosis & necrosis)**
- Steroid synthesis; β -oxidation...
- Endosymbionts co-evolved from
- ancient bacteria
- Mitochondrial DNA = the only non-nuclear genome in all animals
- Replication independent of cell replication



Frey & Perkins, SDSU

Multiple Mechanisms lead to Mitochondrial Toxicity



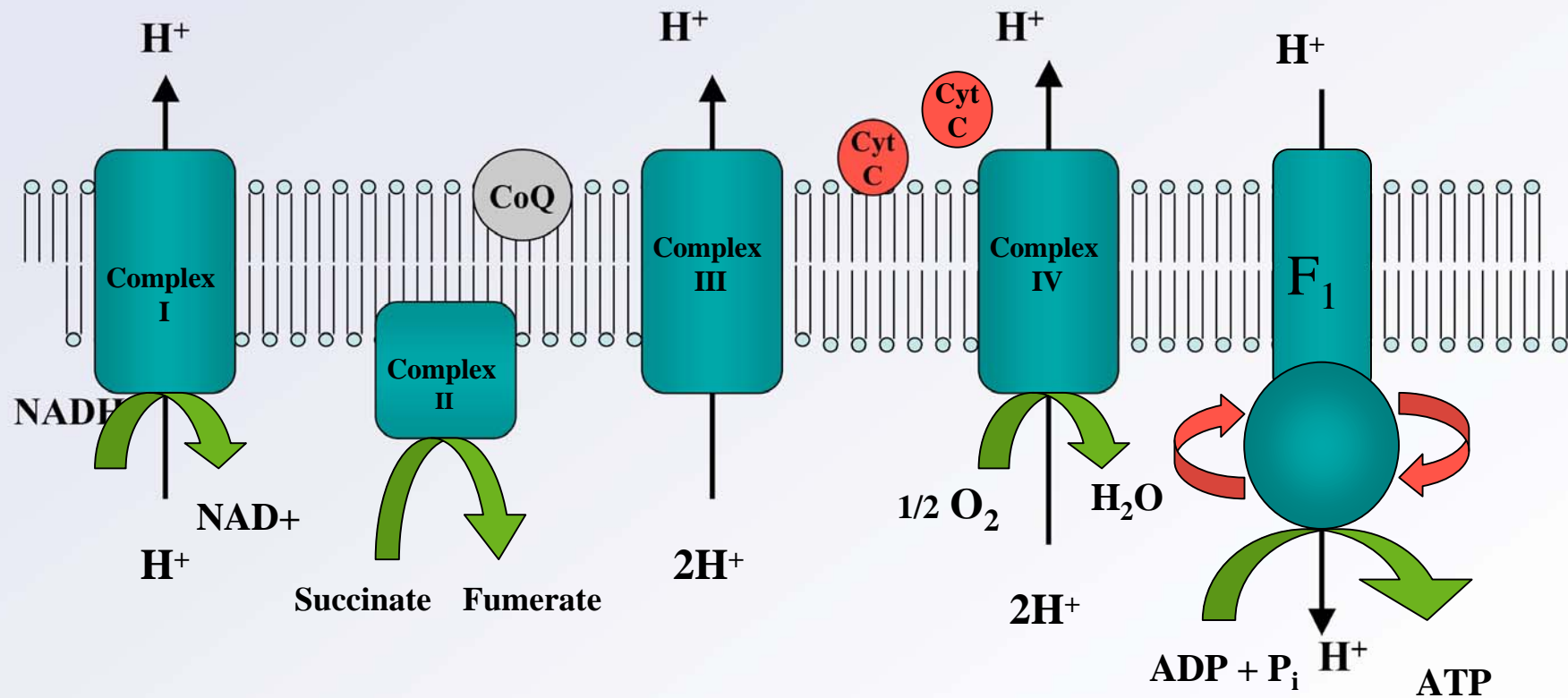
Dykens JA, Marroquin LD, Will Y. Strategies to reduce late-stage drug attrition due to mitochondrial toxicity. *Expert Rev Mol Diagnostics* 7,161-75 (2007).

Outline

- **Function of mitochondria-possible sites of xenobiotic interference**
- Techniques to measure mitochondrial function
 - **Isolated Mitochondria**
 - **Oxygen Consumption (Thiazolidones)**
 - Respiratory Screening Technology
 - **Target Identification (Thiazolidones)**
 - Immunocapture technology
 - **Cells**
 - **Metabolic Profiling (Formins)**
 - Oxygen and pH (Formins)
 - **Glucose/Galactose Model**
 - **Biogenesis (Antibiotics)**
 - Immunohistochemistry and Dipsticks
- Summary

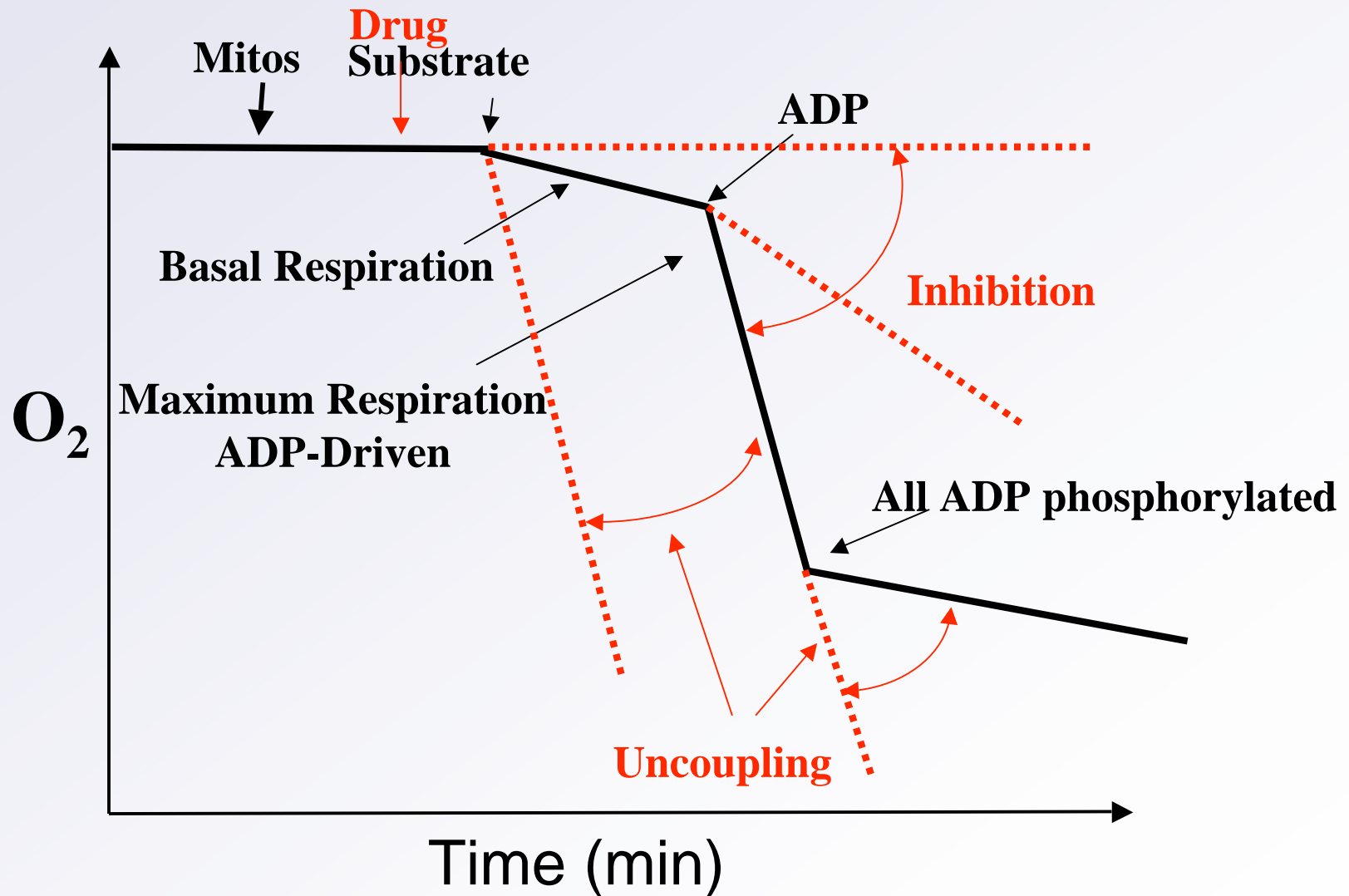
Early mitochondrial assessment allows the identification of compounds with the desired efficacy profile, but without ancillary liabilities.

Electron Transport Chain



- Complex I: NADH:CoQ oxidoreductase
- Complex II: Succinate:CoQ oxidoreductase
- Complex III: CoQ:CytC reductase
- Complex IV: CytC oxidase
- Complex V: F₁-F₀-ATP synthase

Polarographic Mitochondrial Respiration

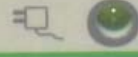
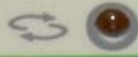
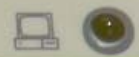




Hanatech
Oxytherm
Electrode Unit

BIOCHEM/TOX

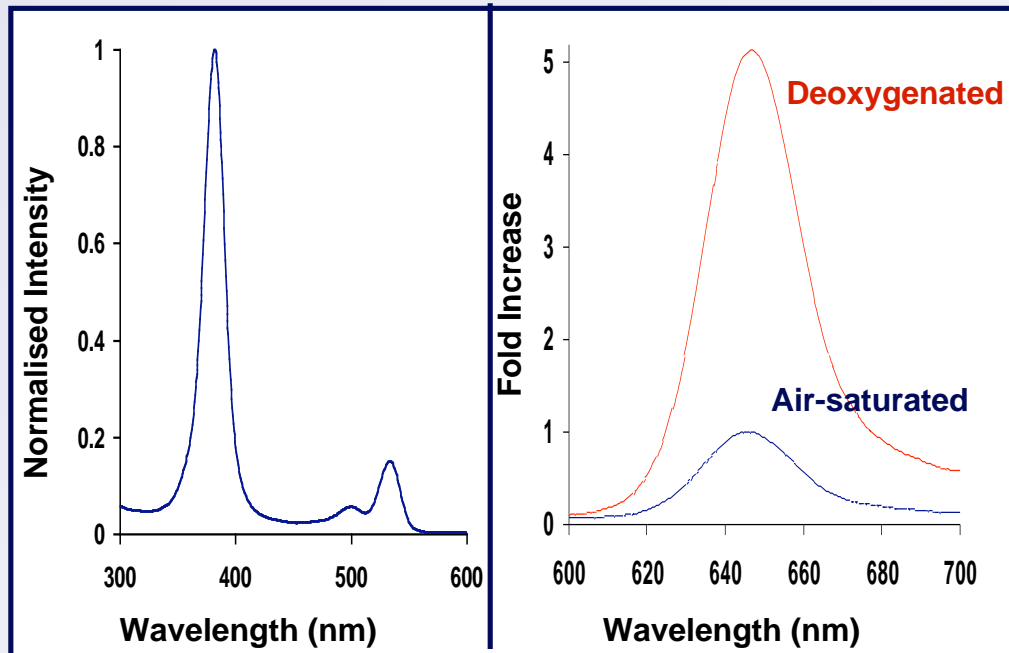
Hanatech **Oxytherm**



Outline

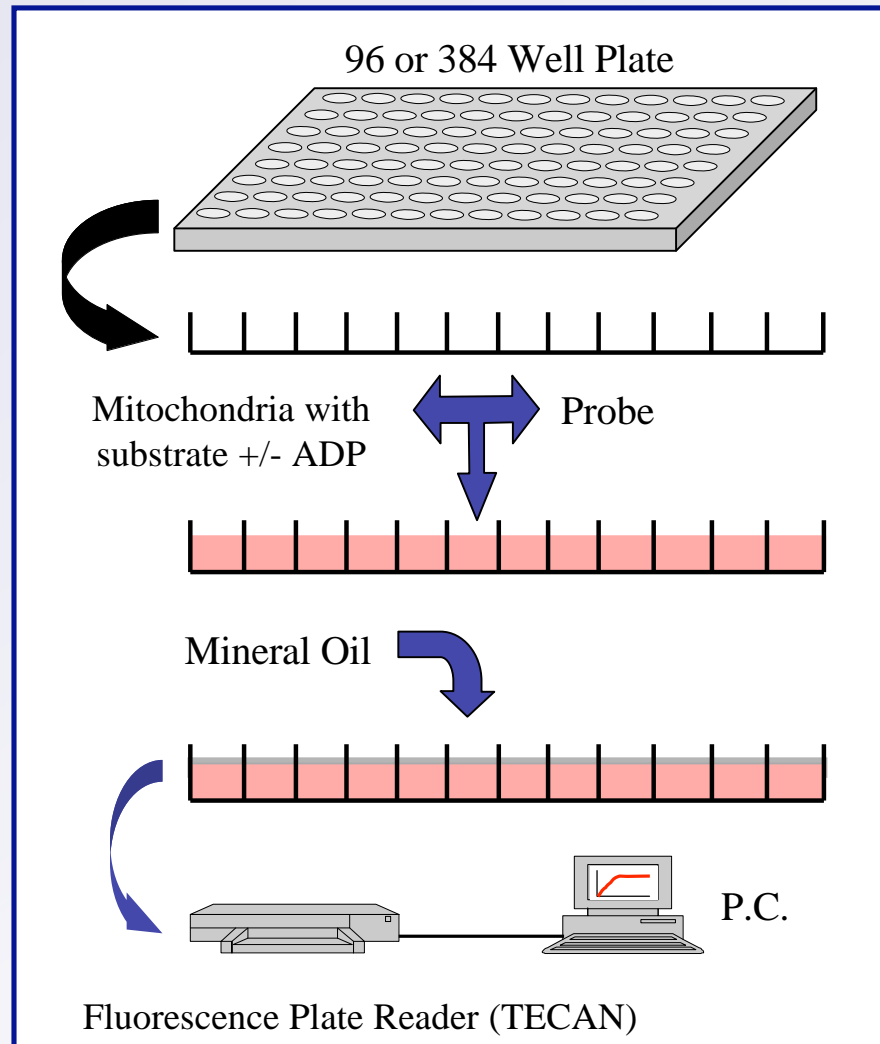
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Screen 1: Oxygen-Sensitive Probes

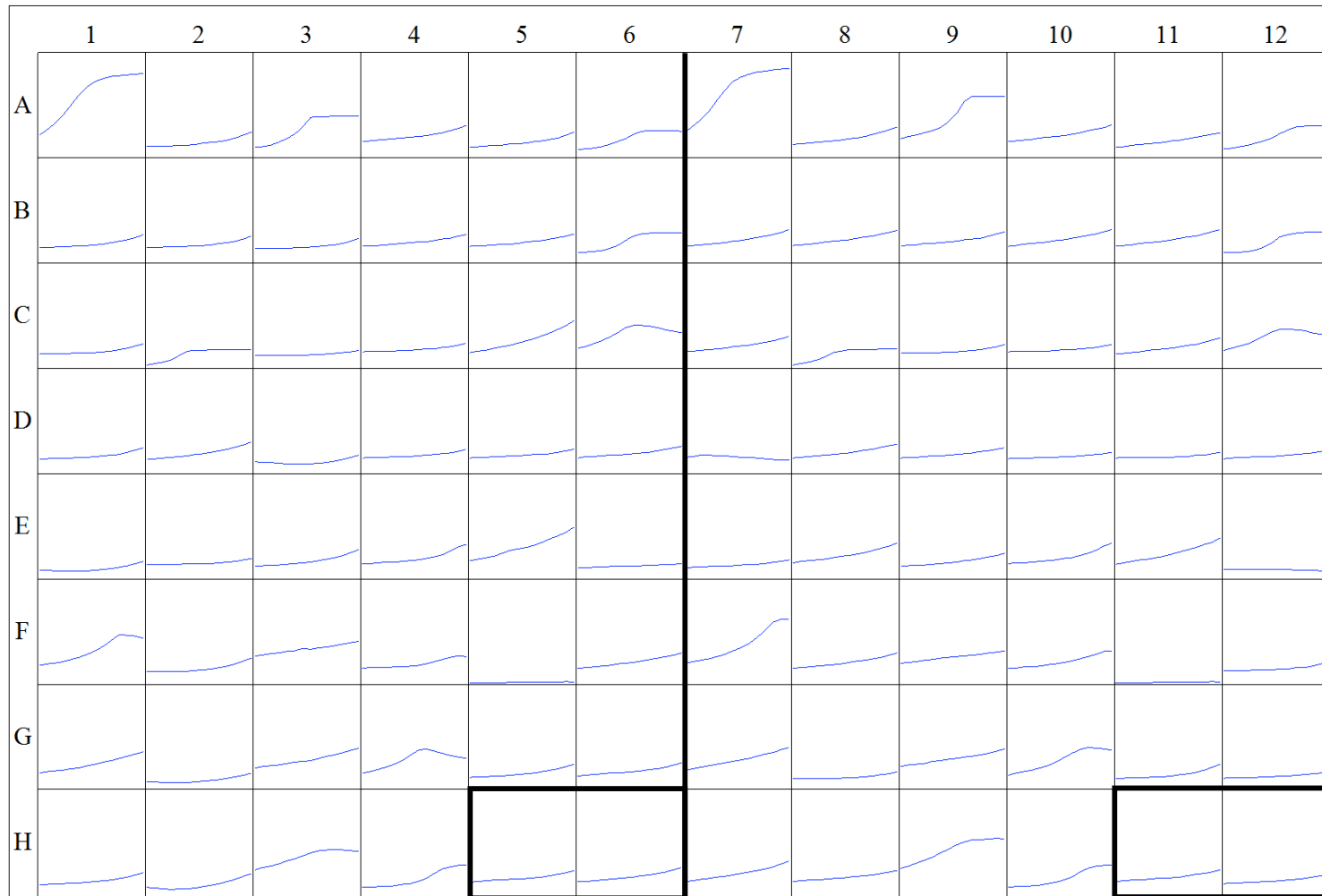


- Phosphorescent
- Water-soluble,
- Cell non-invasive, non-cytotoxic
- Stable
- Time resolved or prompt
- Compatible with any reader
- Large stoke shift allows for high signal to noise ratio
- multiplex with “green dyes”

Measurement

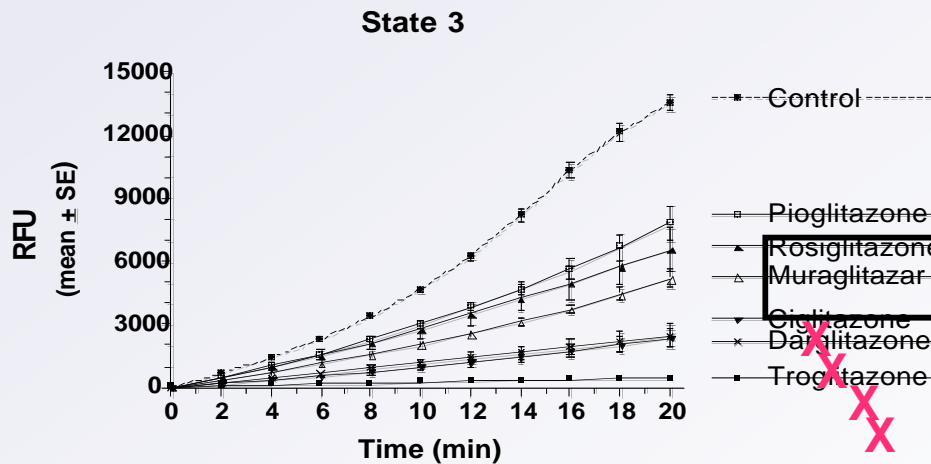
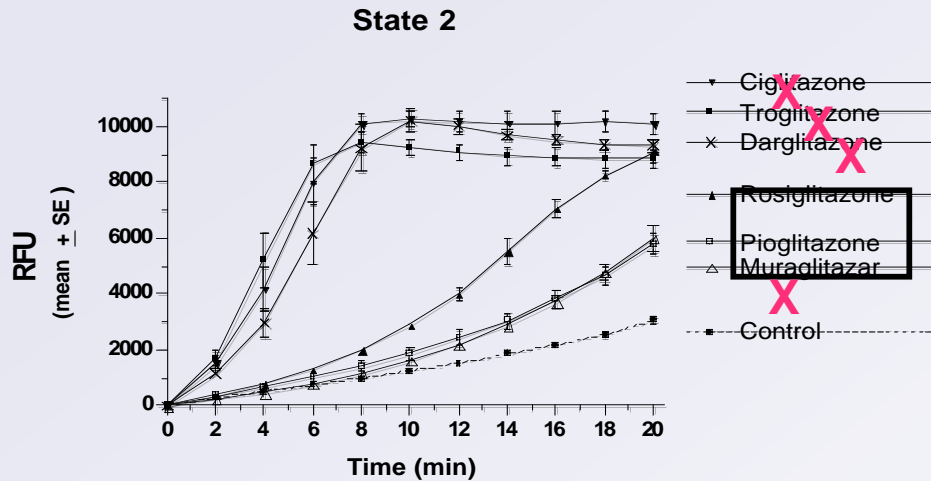


Screen 1: Mitochondrial respiration in 96 well format.



Will et al., Nature Protocols 1: 2563 (2007).

Mitochondrial Effects of Thiozolidinediones Vary



In addition to these acute effects & PPAR binding:

Pioglitazone photoaffinity probe pulls down MitoNEET, an atypical 2Fe-2S protein integral to outer membrane.

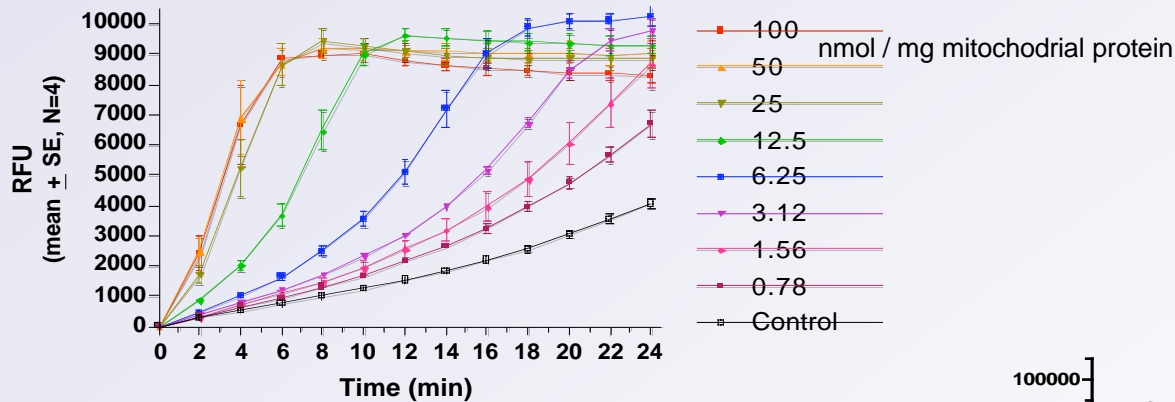
- Likely involved in Fe-S import &/or metabolism, and regulating maximal respiration
- Redox active
- Pioglitazone stabilizes & forestalls pH-dependent loss of Fe-S cluster

- Paddock et al. PNAS, 104:14342, 2007.
- Wiley et al., PNAS, 104:5318, 2007.
- Wiley et al, JBC, 282:23745, 2007.
- Colca et al., Am J Physiol Endocrinol Metab. 286:E252, 2004
- Nadanaciva et al., 2007

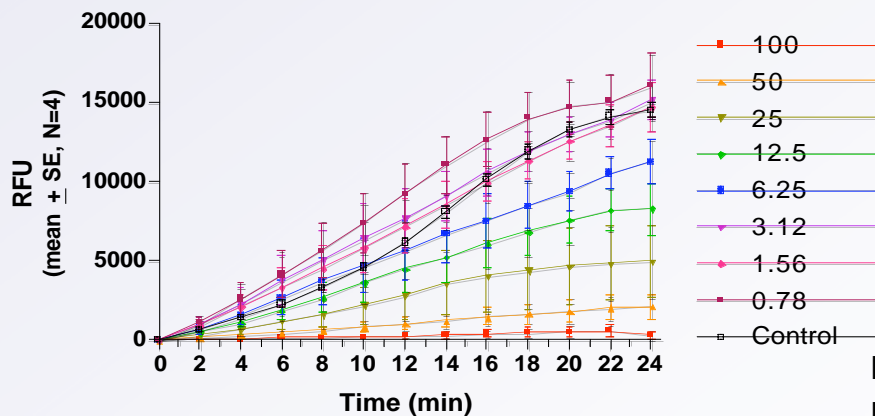
Drugs present at 25nmol/mg mitochondrial protein. N=4, except for controls N=48.

Troglitazone Impairs Mitochondrial Function: IC₅₀ values are Readily Determined

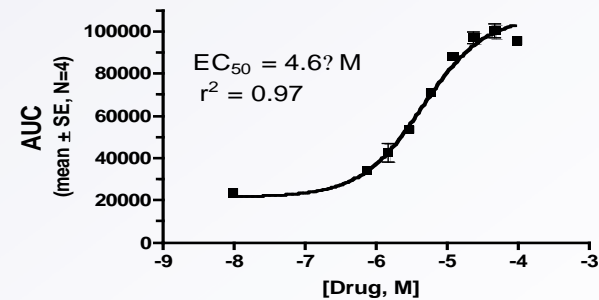
Troglitazone Dose Response
State 2



Troglitazone Dose Response
State 3



Troglitazone
State 2



Nadanaciva S, Dykens JA, Bernal A, Capaldi RA, Will Y. Mitochondrial impairment by PPAR agonists and statins identified via immunocaptured OXPHOS complex activities and respiration. Toxicol Appl Pharmacol. 2007

Summary Luxcel RST

- easy to use, accurate and reproducible
 - HTS format allows for implementation in lead development and even series selection
 - Information on mechanism if performed using different substrates
 - Rank order compounds, generation of IC50 values for comparison with other parameters
 - Useful for SAR
 - Early derisking of chemical series/programs
- BUT:
- Some targets are hard to distinguish (ANT vs ATPase)
 - Application in cells (intracellular probes) under development
 - Potentially overpredict

**Measurement of respiration in
HTS format identifies
compounds with “GENERIC”
mitochondrial toxicity such as
Uncouplers and Inhibitors**

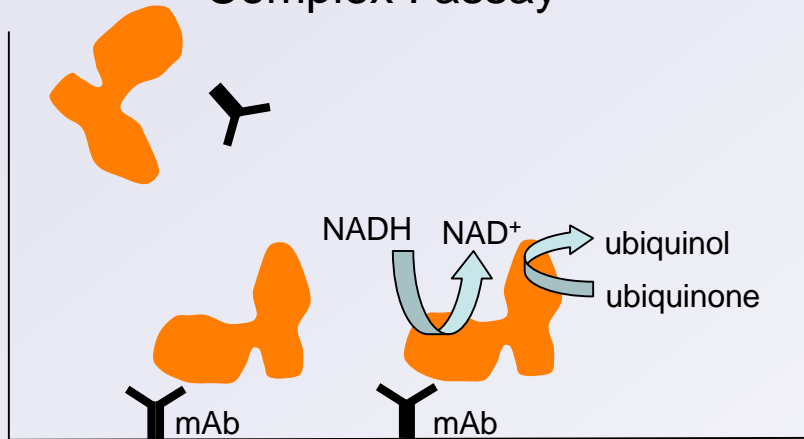
Screen 2: The MitoProfile® approach

Dissecting out the Site(s) of
mitochondrial Toxicity using
immunocapture

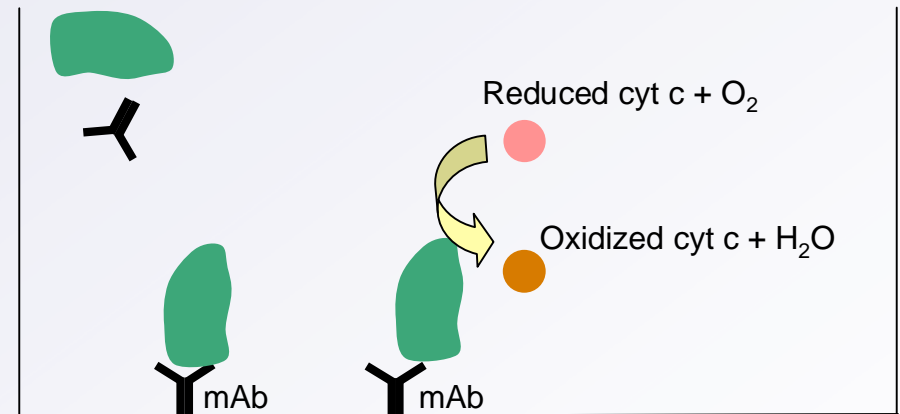


Complexes I, IV and V Activity assays

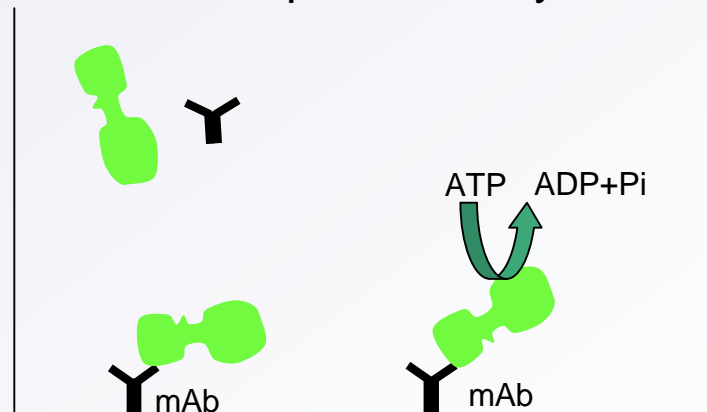
Complex I assay



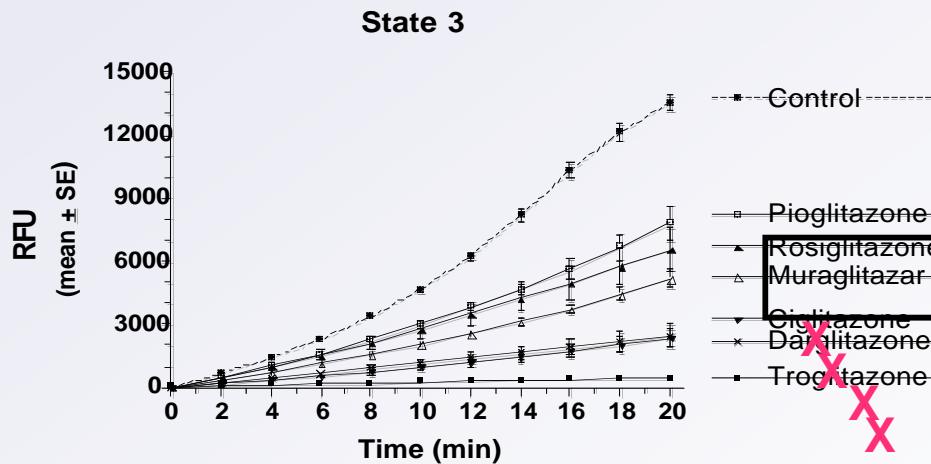
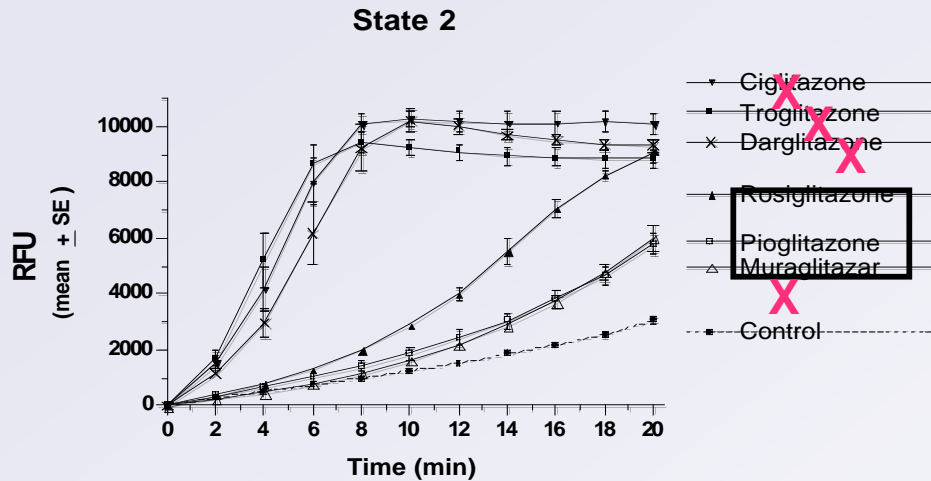
Complex IV assay



Complex V assay



Mitochondrial Effects of Thiozolidinediones Vary



In addition to these acute effects & PPAR binding:

Pioglitazone photoaffinity probe pulls down MitoNEET, an atypical 2Fe-2S protein integral to outer membrane.

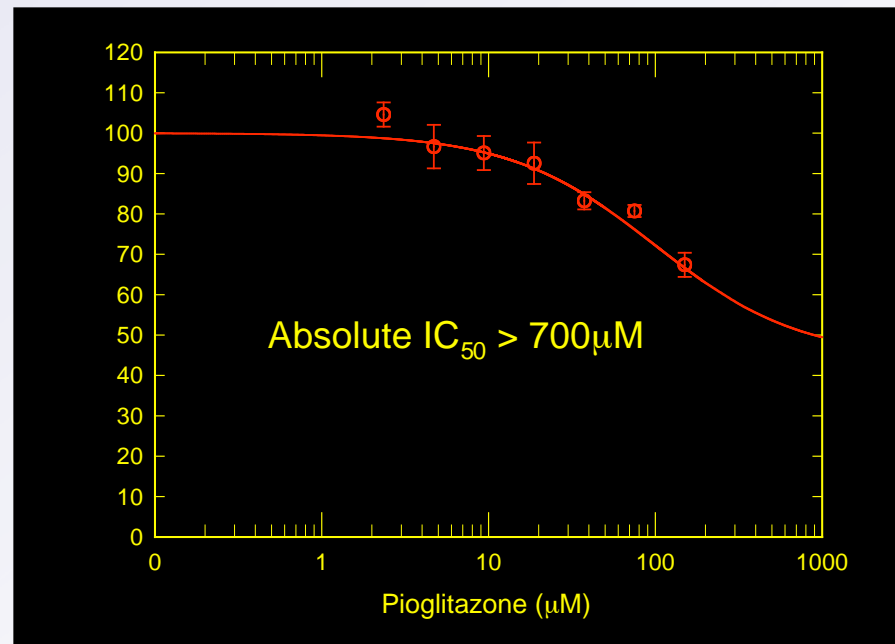
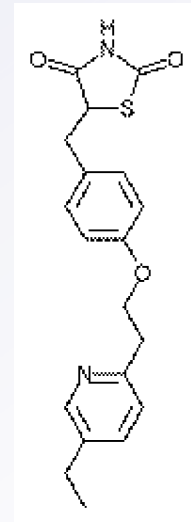
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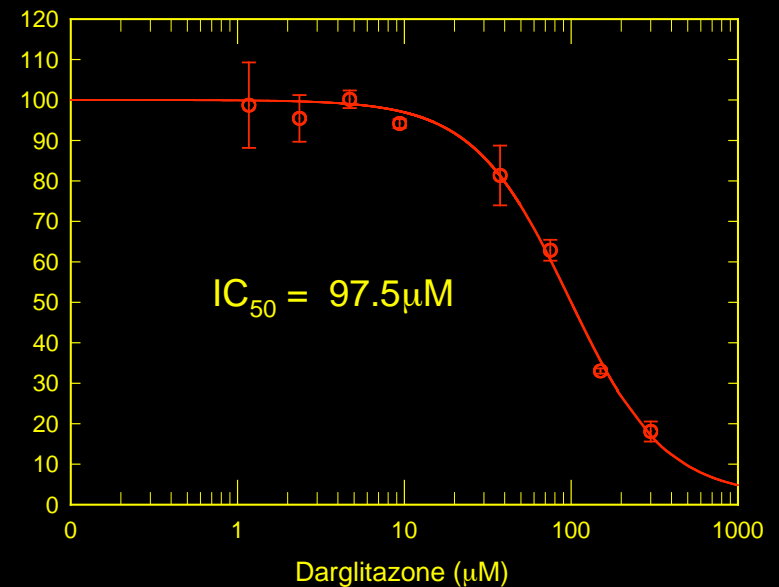
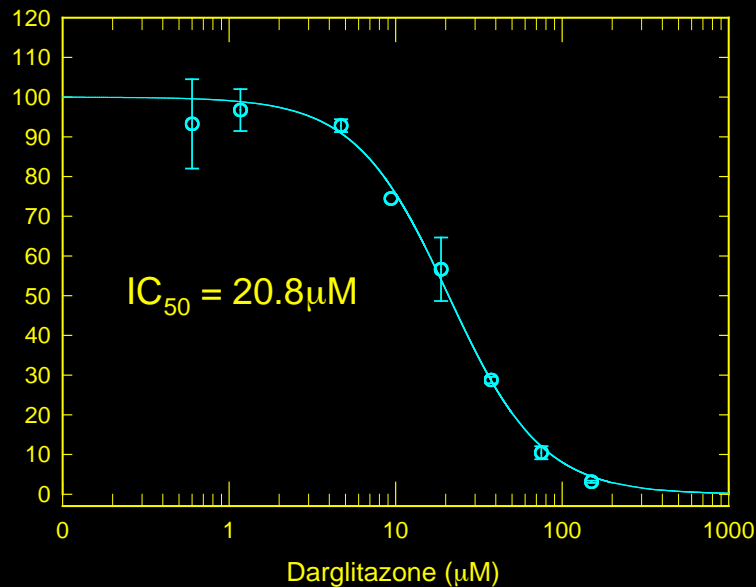
Pioglitazone

- Complex I Activity: Not inhibited at 150 μM
- Complex II/III Activity: Not inhibited at 150 μM
- Complex IV Activity: Not inhibited at 150 μM .
- Complex V Activity: $\text{IC}_{50} > 700 \mu\text{M}$.



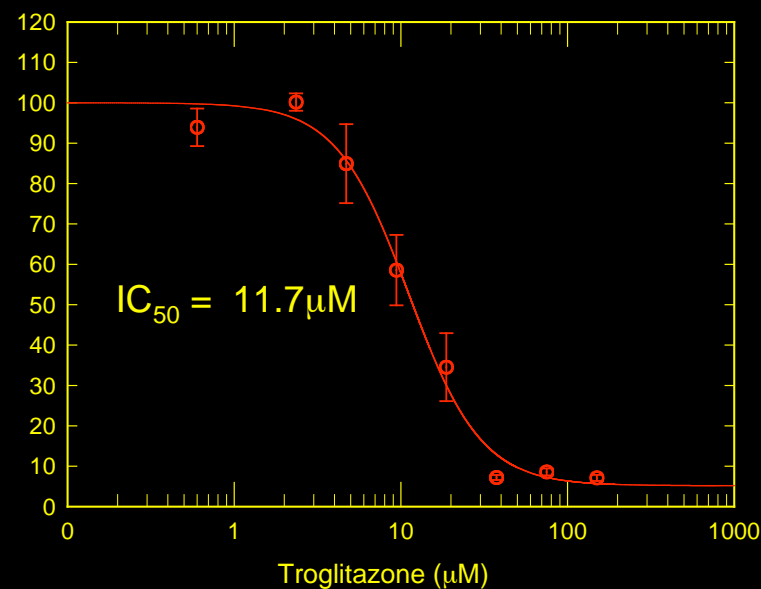
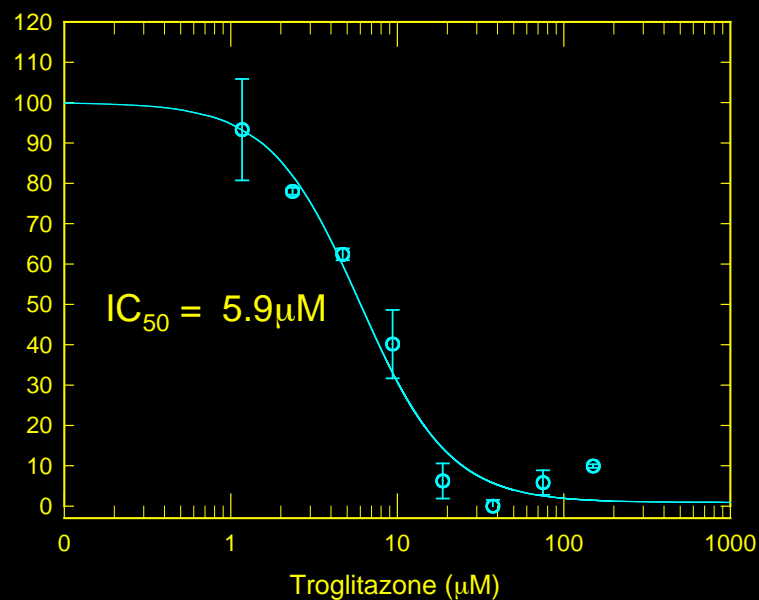
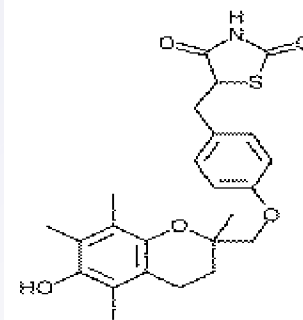
Darglitazone

- Complex I Activity: Not inhibited at 150 μM .
- Complex II/III Activity: Not inhibited at 150 μM
- Complex IV Activity: IC_{50} 20.8 μM
- Complex V Activity: IC_{50} 97.5 μM



Troglitazone

- Complex I Activity: Not inhibited at 150 μM .
- Complex II/III Activity: Not inhibited at 150 μM
- Complex IV Activity: IC_{50} 5.9 μM
- Complex V Activity: IC_{50} 11.7 μM



Glitazones inhibit Complex IV and V

*(and this is their oxphos fingerprint or
biomarker)*

**Rank Order of Effects
parallels Human Toxicity**

Summary Mitosciences

➤ In Vitro

- easy to use, accurate and reproducible
- HTS format allows for implementation in lead development and even series selection
- Can be used for SAR
- Rank order compounds, generation of IC50 values for comparison with other parameters
- Early derisking of chemical series/programs
- Target Identification provides mechanistic info for *in vivo* monitoring
- Application in cells “only” for biogenesis

➤ BUT:

- Free access of compounds
- Uncoupler/ANT/MPT insensitive
- Reconfirm with RST after SAR

What do we know?

TOXICITY is a function of C_{\max}/IC_{50}

What do we need to consider?

Metabolism

Species differences

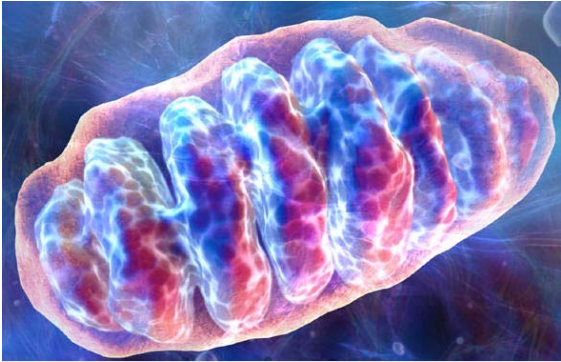
Organ specificity

Genetic background (haplotypes)

Combination therapies

Multiple dosing/accumulation (tissue/organelle)

In vitro/in vivo correlations



**Dipstick Technology
Other non invasive tests**

**RST
ImmunoCapture**



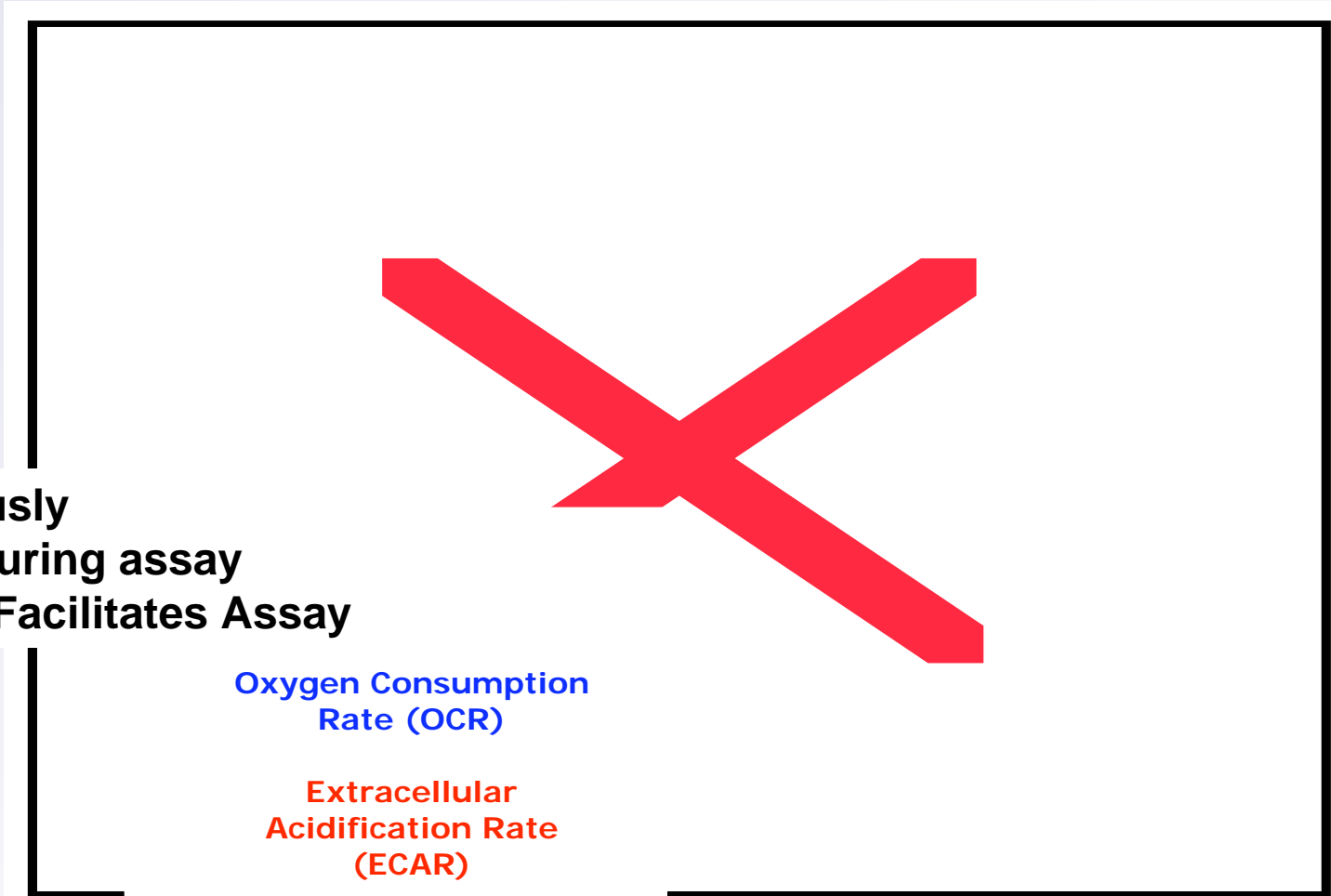
**Aerobically Poised Cell Models
oxygen & pH sensors
Histo/Immunohistochemistry
Dipsticks**



Outline

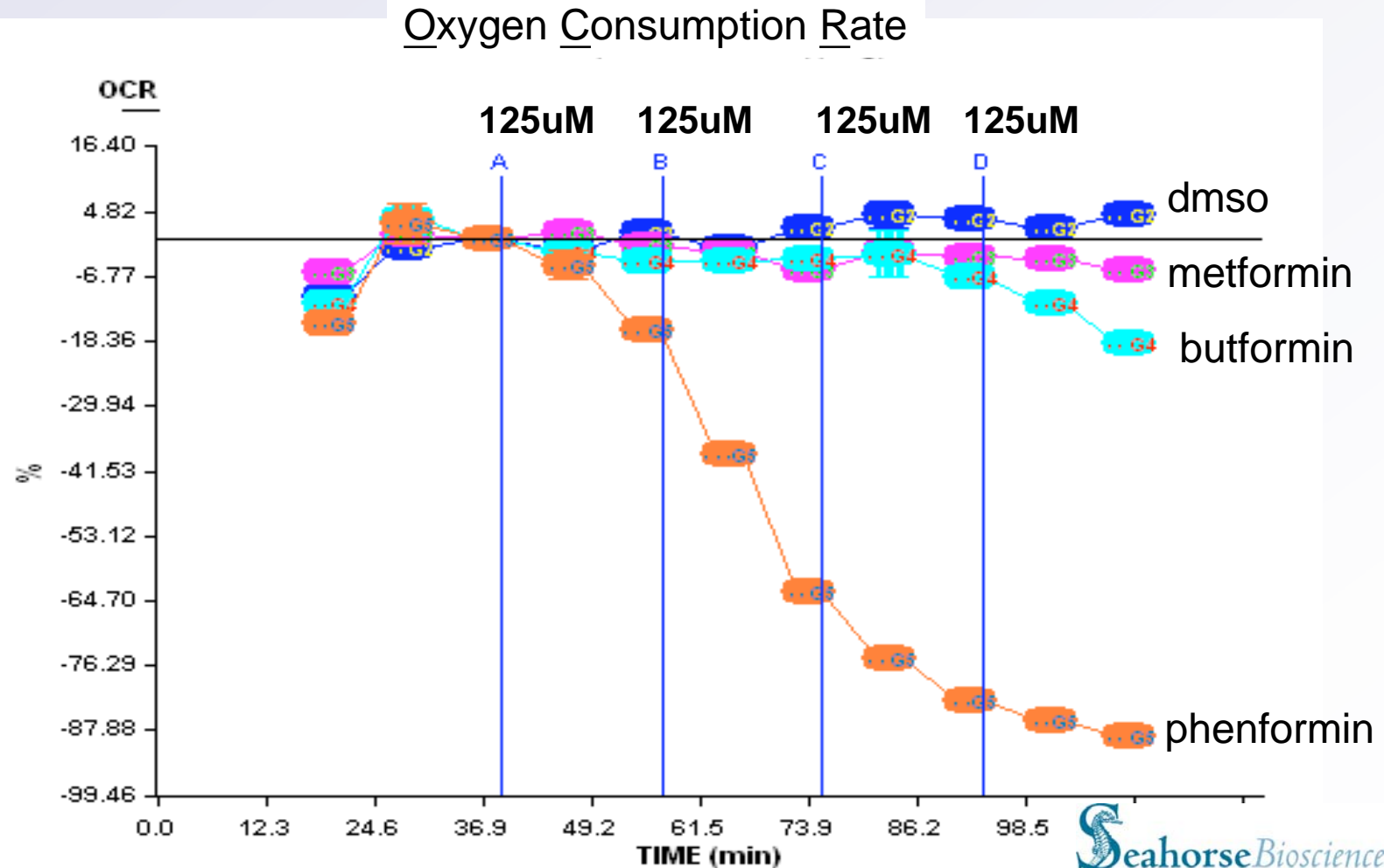
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 - Oxygen and pH (Formins)
 - **Glucose/Galactose Model**
 - **Biogenesis (Antibiotics)**
 - Immunohistochemistry and Dipsticks
 - **In vitro-In vivo Correlations**
 - Immunohistochemistry and Dipsticks
- Summary

Screen 3: Metabolic Profiling to Detect Drug-Induced Mitochondrial Toxicity



- pH simultaneously
- Ability to add during assay
- Microchamber Facilitates Assay

Screen 3: Metabolic Profiling to Detect Mitochondrial Toxicity



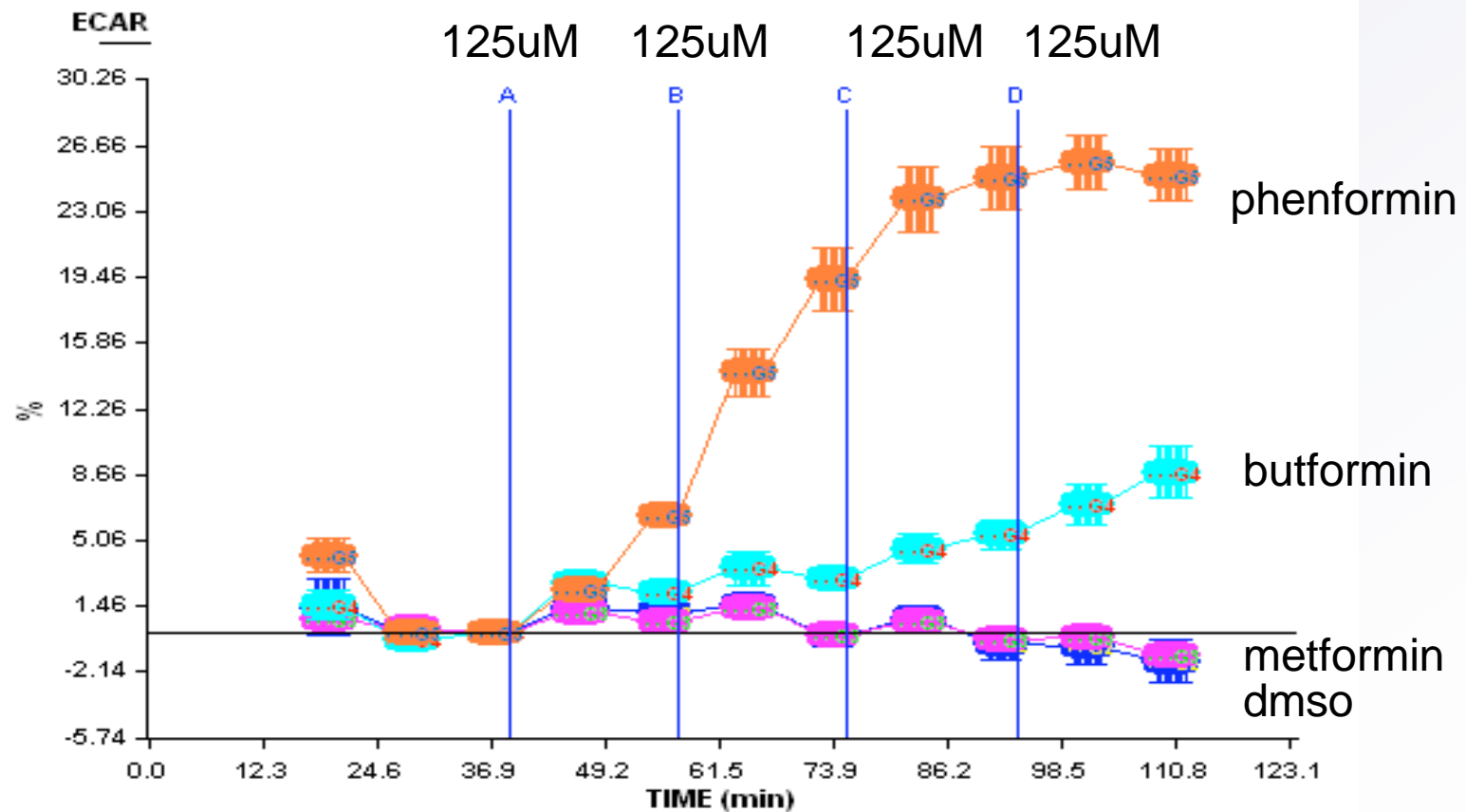
HepG2 cells

SeahorseBioscience

Data from Lisa Marroquin

Metabolic Profiling to Detect Mitochondrial Toxicity

Extracellular Acidification Rate



HepG2 cells



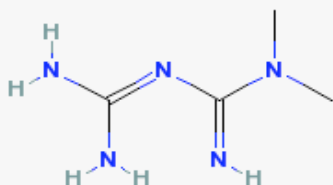
Data from Lisa Marroquin

Metabolic Profiling Parallels EC50 for *in vivo* Lactic Acidosis

Metformin

EC50 (μ M) for lactic acidosis*

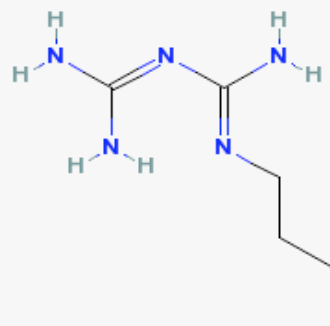
734 \pm 168



Molecular Weight: 129.164 g/mol
Molecular Formula: C₄H₁₁N₅
LogP: -0.267
Hydrogen Bond Donor Count: 3
Hydrogen Bond Acceptor Count: 5
Rotatable Bond Count: 2
Tautomer Count: 3

Buformin

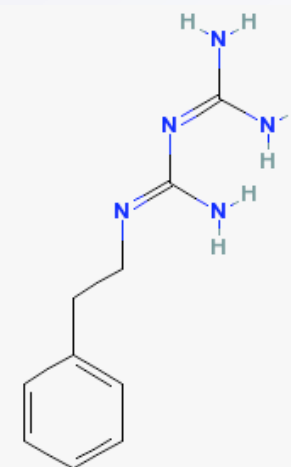
119 \pm 18



Molecular Weight: 157.217 g/mol
Molecular Formula: C₆H₁₅N₅
LogP: 0.243
Hydrogen Bond Donor Count: 3
Hydrogen Bond Acceptor Count: 5
Rotatable Bond Count: 4
Tautomer Count: 5 formin

Phenformin

4.97 \pm 0.87



Molecular Weight: 205.26 g/mol
Molecular Formula: C₁₀H₁₅N₅
XLogP: 0.759
Hydrogen Bond Donor Count: 3
Hydrogen Bond Acceptor Count: 5
Rotatable Bond Count: 4
Tautomer Count: 5

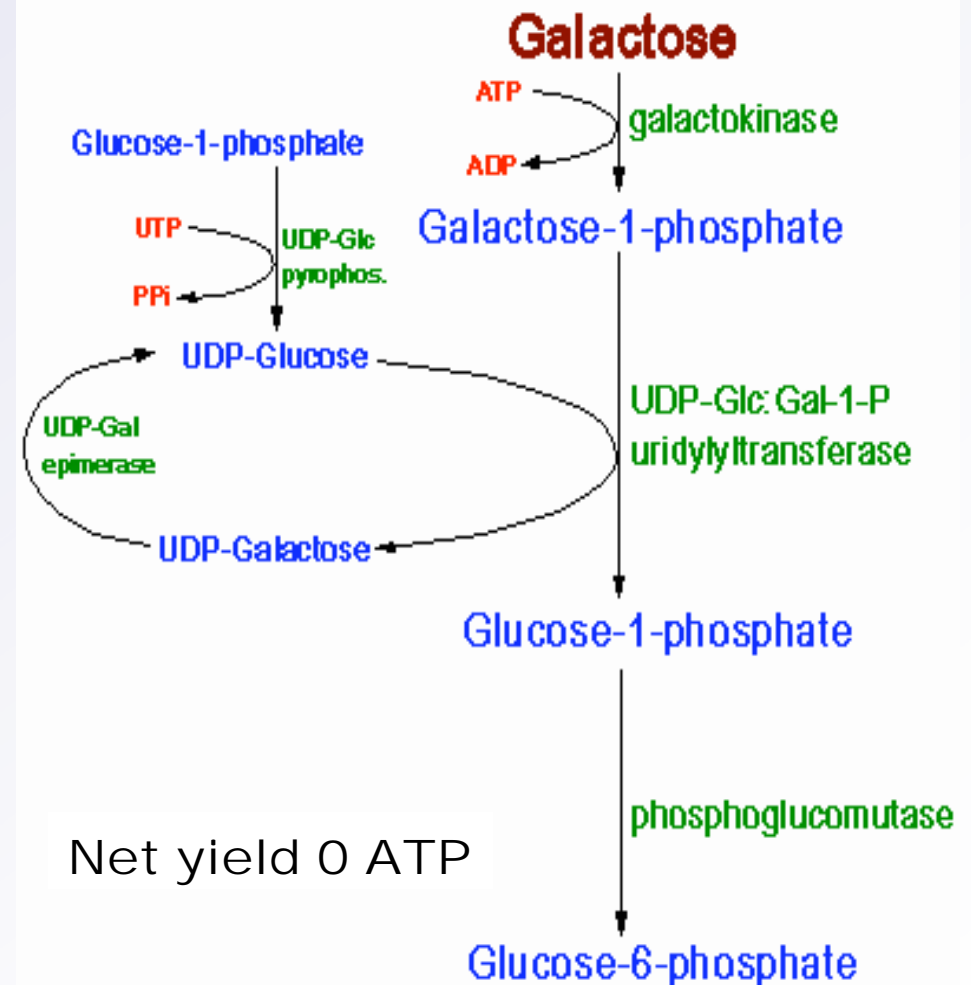
* Wang et al., Mol Pharmacol, 63:844, 2003

Screen 4: Circumventing the Crabtree Effect

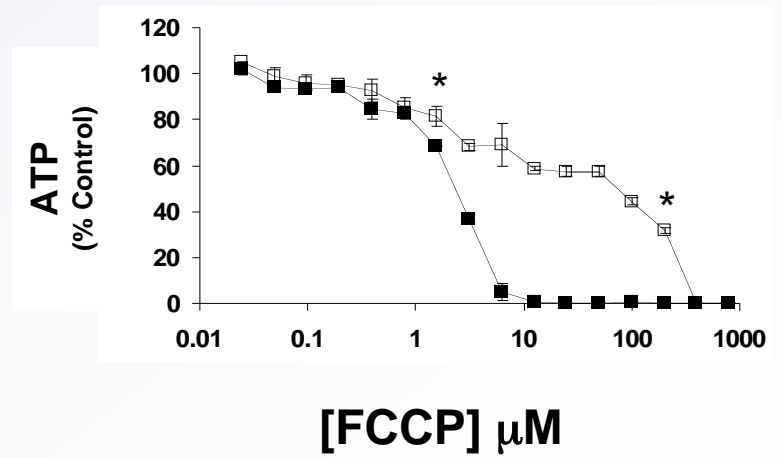
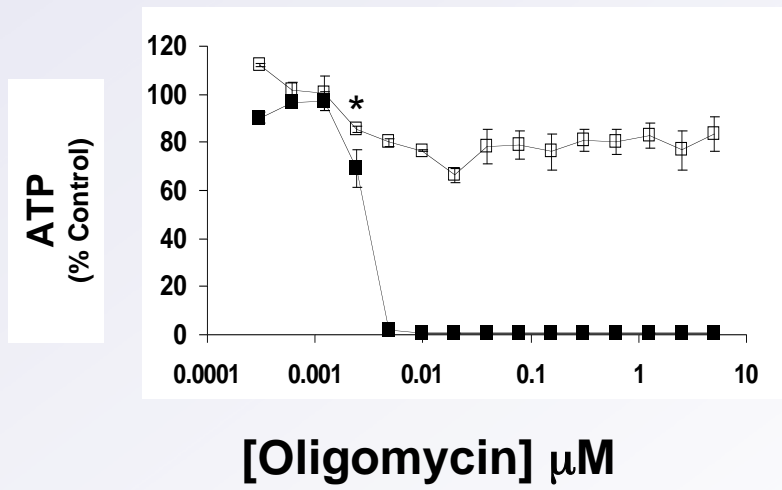
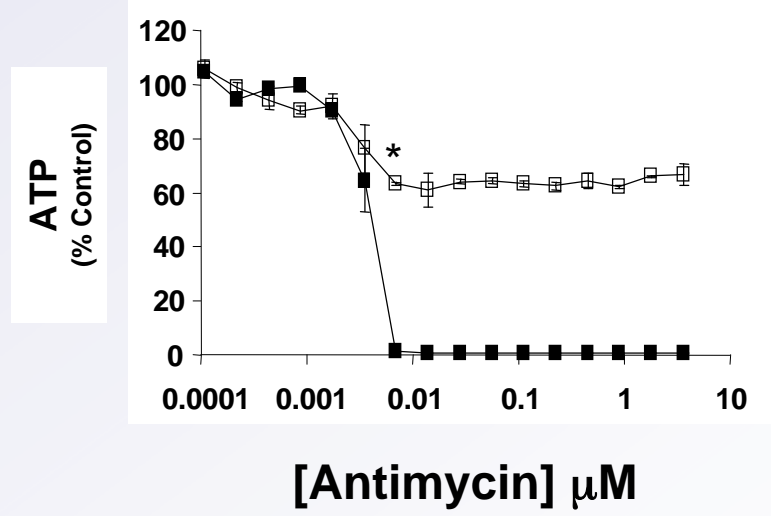
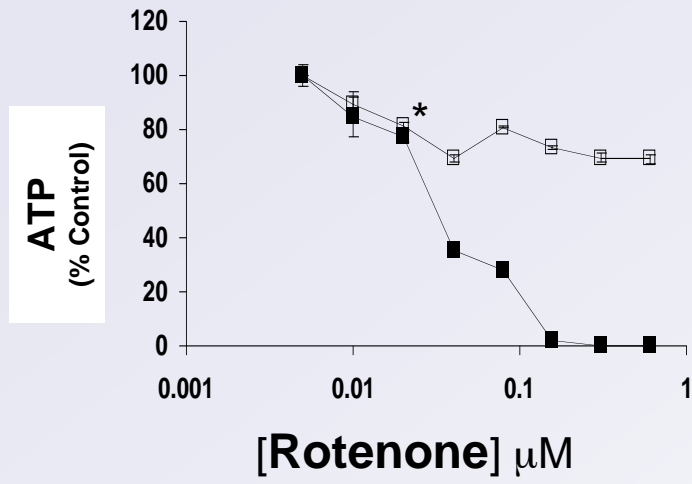
Crabtree Effect (1929):
inhibition of respiration by
glucose.

Warburg Effect (1929):
aerobic glycolysis yields
lactate despite competent
mitochondria.

Characterized by low rates
of O₂ consumption &
resistance to mitotoxics.



Cells Grown in Galactose Become Susceptible to Mitochondrial Inhibition



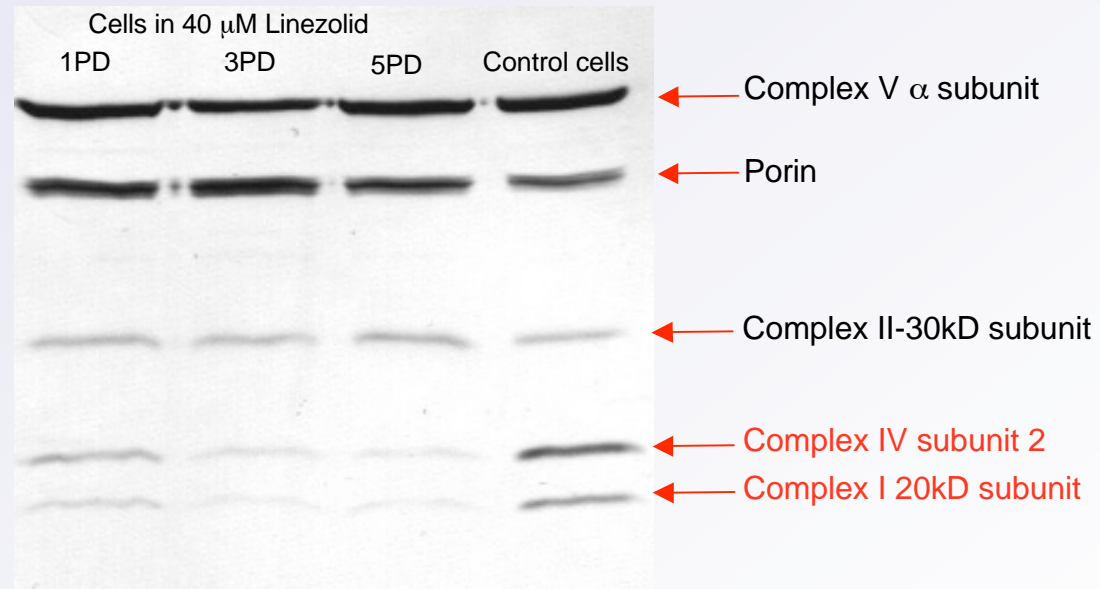
Screen 4: Summary

- Galactose grown cells are more susceptible to mitochondrial toxins
- BUT:
 - Lack of certain drug metabolizing enzymes
 - unresponsive to drugs that alter biogenesis
 - Develop for other organ specific cell lines

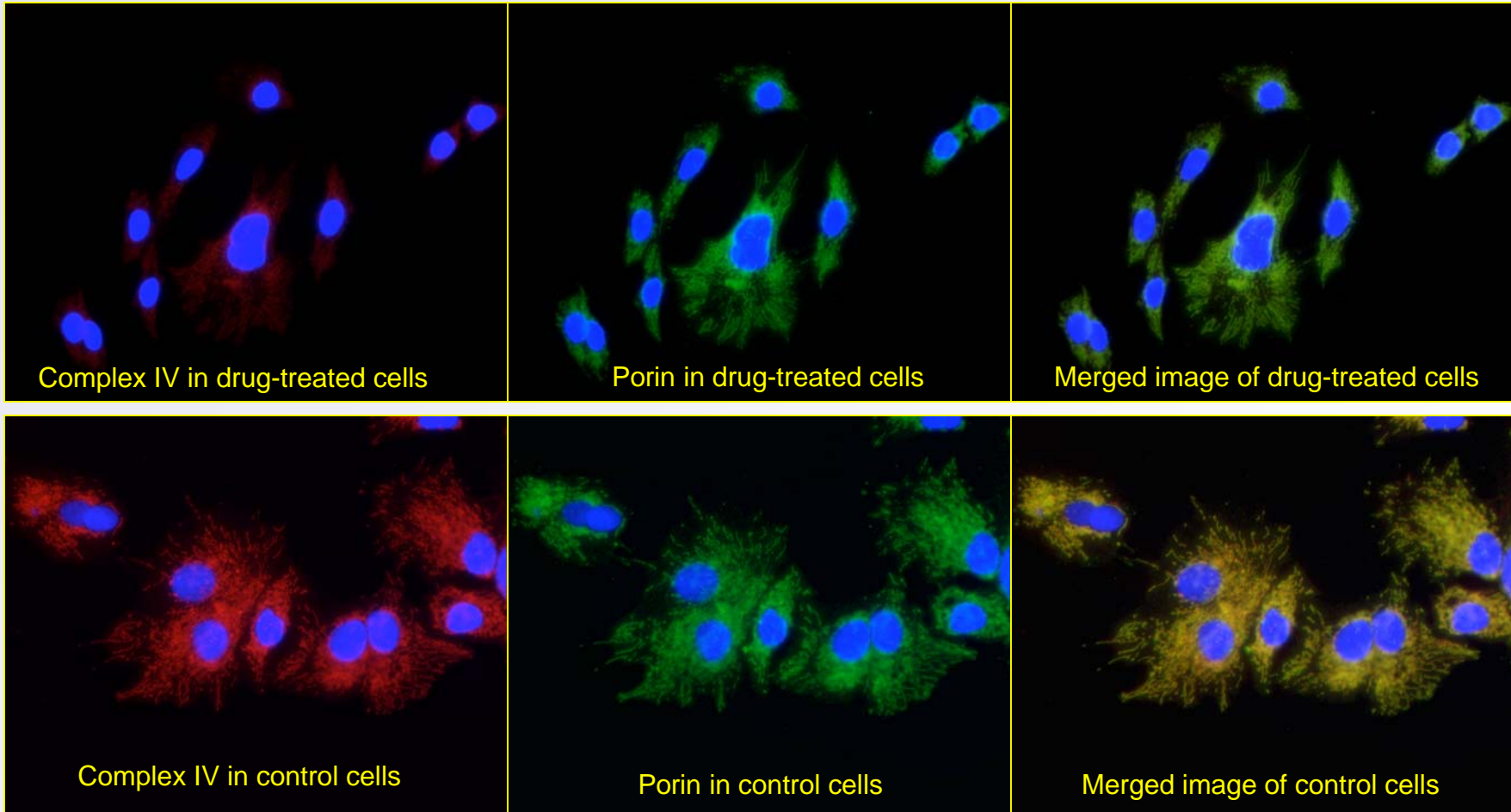
Screen 5 – Mitochondrial Toxicity of Antibiotics and Antivirals

- Antibiotics can potentially target mt DNA and protein synthesis (Oxaxolidines, Mycins, NRTIs)
- Mitosciences developed a non-radioactive screen to detect potential liabilities (Dipstick)
- Validation accomplished using western blots/imaging and dipstick for OXPHOS

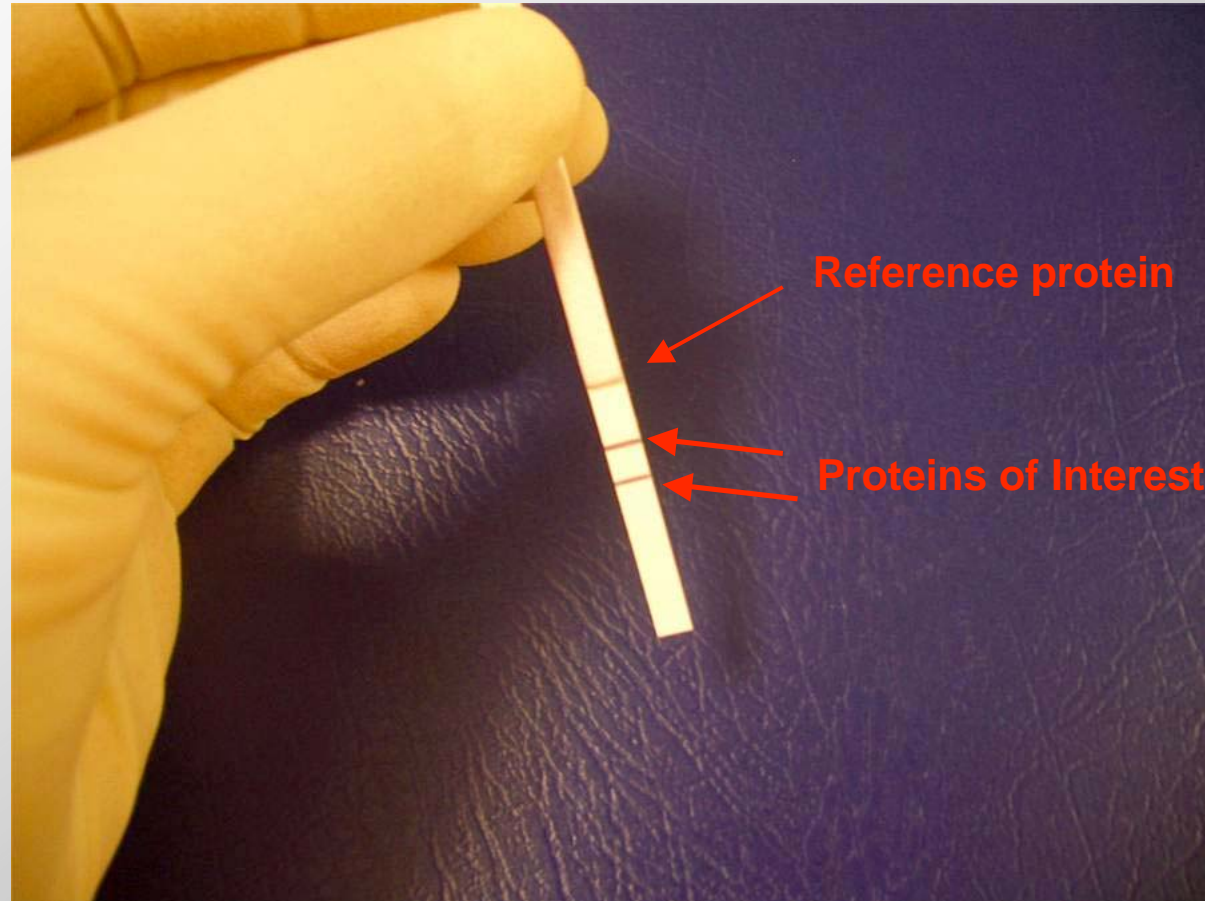
Western blot of HepG2 cells grown in 40 μ M Linezolid shows decreased levels of Complex I and Complex IV



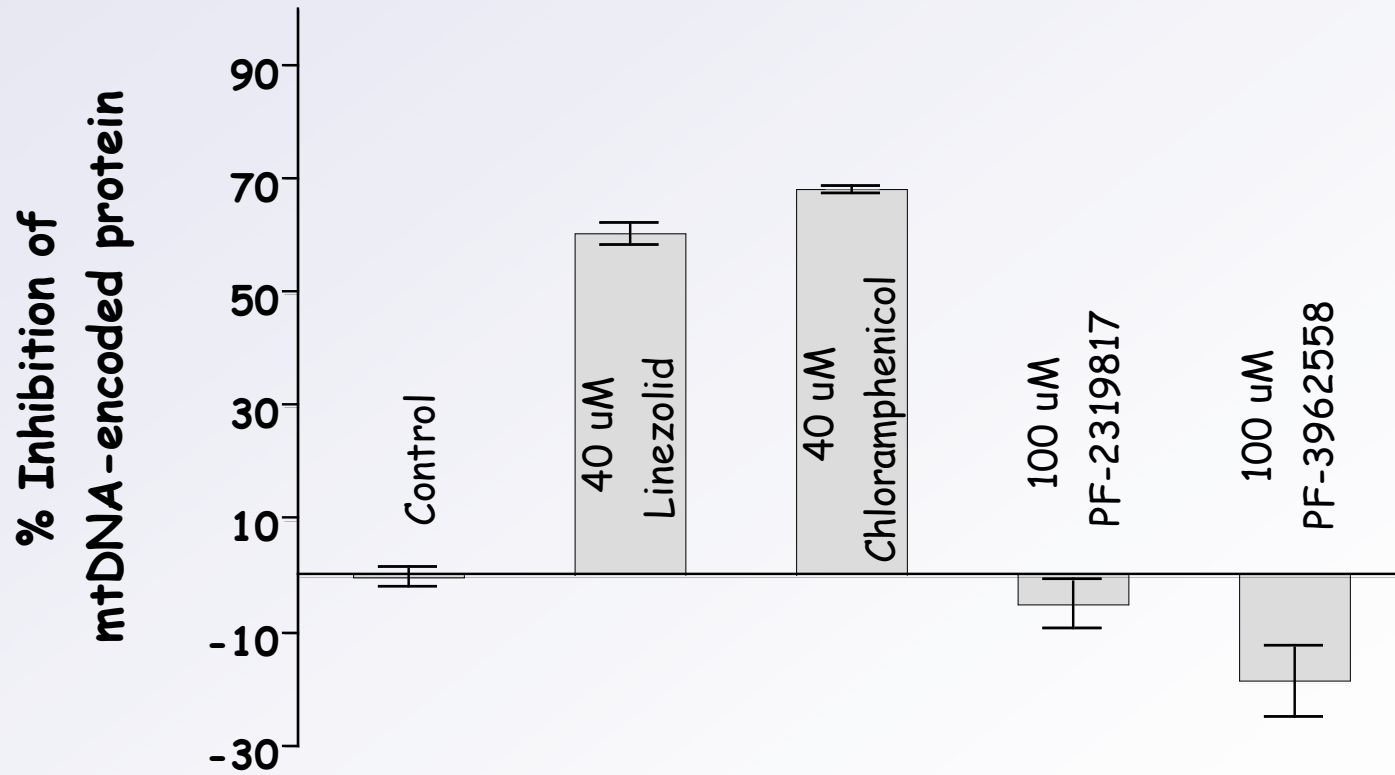
Fluorescence microscopy confirms loss of mtDNA-encoded protein in 40 mM linezolid-treated HepG2 cells



OXPHOS Dipstick Assays



Linezolid inhibits mitochondrial protein synthesis



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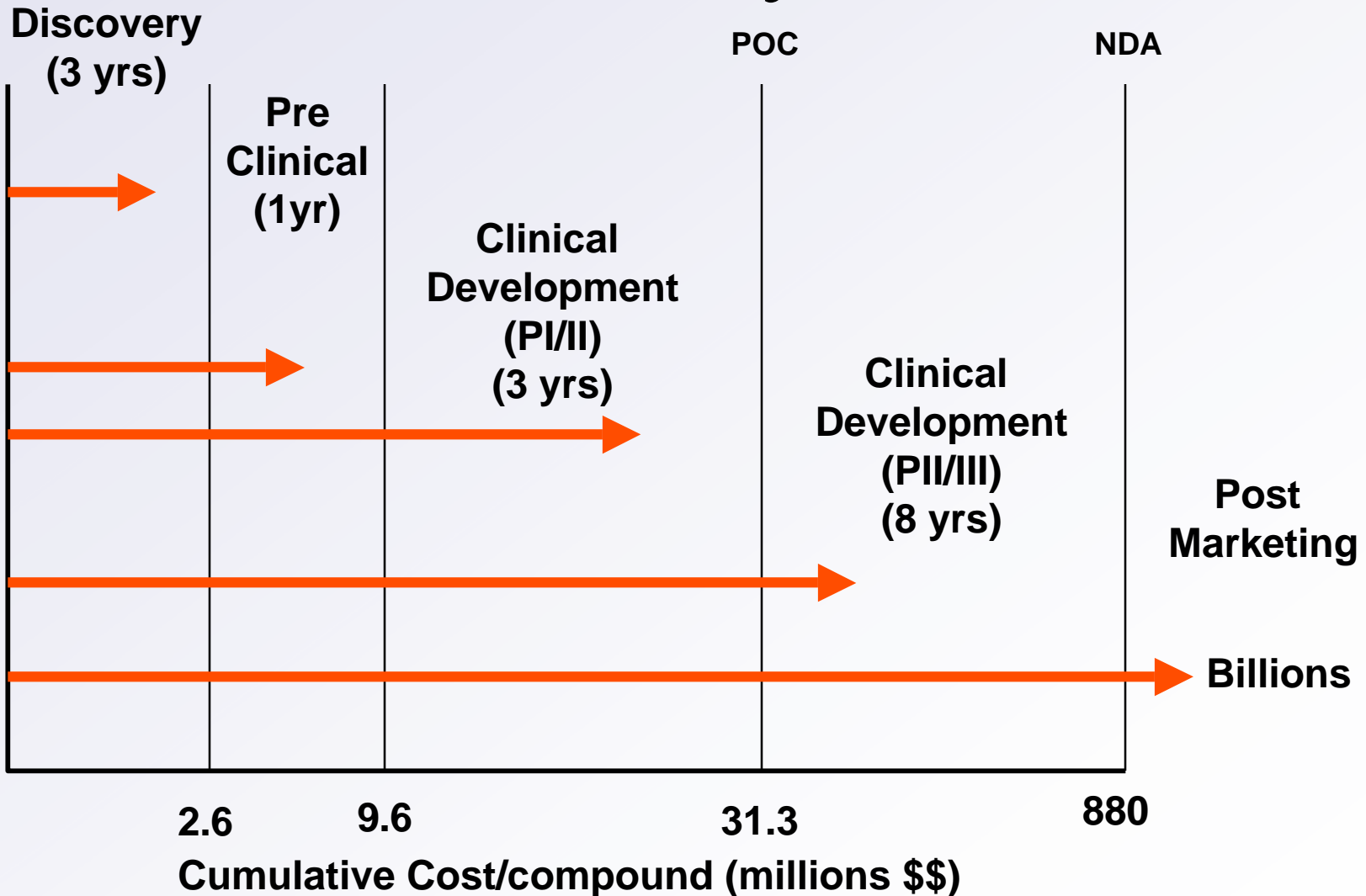
Drug-Induced Mitochondrial Toxicity

- Many, but not all, drugs with organ toxicity have mitochondrial liabilities.
 - **Elevated serum liver enzymes = hepatocyte death**
 - **Lactic acidosis is classic hallmark.**
- Depending on severity, if a drug has a mitochondrial liability, it will have deleterious consequences.
 - **Acute vs. Chronic Exposure**
 - **Bio-accumulation**
 - **Threshold effects**
 - **Combination therapies worse (cervistatin & gemfibrozil)**
 - **Idiosyncratic responses function of genetics and organ history.**
- “The first opportunity to prevent hepatotoxicity arises in the early stages of drug development...”

Navarro & Senior, NEJM, 354:731, 2006

Dykens et al., Expert Rev. Mol. Diagnostics, 7: 161 (2007)

Fail Early - saves time and money



Acknowledgements

➤ Lisa Marroquin, BS

➤ Dr. James Dykens



➤ Dr. James Hynes



➤ Dr. Sashi Nadanaciva

 MitoSciences

The logo for MitoSciences features a red, stylized graphic element resembling a mitochondrion or a cell, positioned to the left of the text "MitoSciences" in a bold, black, sans-serif font.