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

Yvonne Will, PhD

Exploratory Safety Differentiation
Pfizer PGRD
Groton, CT
Mentors provide their expertise to less experienced individuals in order to help them advance their careers, enhance their education, and build their networks.

Donald J. Reed
Dist. Prof of Biochemistry (OSU)
SOT president 1991-1992
Glutathione status and chemical induced Toxicity
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**
- **In silico computer simulation**
## Some Differences between Animals and Humans Critical to Prediction of Toxicity

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

**CONCORDANCE OF THE TOXICITY OF PHARMACEUTICALS IN HUMANS AND IN ANIMALS**


**FIRST DOSE OF POTENTIAL NEW MEDICINES TO HUMANS: HOW ANIMALS HELP**

Many Drug Classes cause Mitochondrial Toxicity

<table>
<thead>
<tr>
<th>Effect on Mitochondria</th>
<th>Example Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitors of ETC</td>
<td>Fibrates, Glitazones, Statins</td>
</tr>
<tr>
<td>Uncouplers</td>
<td>Sulfonamides, Glitazones, NSAID</td>
</tr>
<tr>
<td>Oxidizing agents/Redox cyclers</td>
<td>Doxorubicin, Acetaminophen</td>
</tr>
<tr>
<td>Inhibitors of FA synthesis</td>
<td>Valproic Acid, Tetracyclin Salicilates</td>
</tr>
<tr>
<td>Inhibitors of mitochondrial protein synthesis</td>
<td>Antibiotics (Macrolides)</td>
</tr>
<tr>
<td>Depletion of mtDNA</td>
<td>AZT, Abacavir, Efavirenz</td>
</tr>
<tr>
<td>Induction of MPT</td>
<td>Bile Acids, Anti Cancer Drugs</td>
</tr>
</tbody>
</table>
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; b-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

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

- Basal Respiration
- Maximum Respiration ADP-Driven
- ADP
- Uncoupling
- All ADP phosphorylated
- Inhibition

Drug

Mitos

Substrate

Time (min)

$O_2$
Outline

➢ Function of mitochondria-possible sites of xenobiotic interference
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➢ Summary
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

96 or 384 Well Plate

Mitochondria with substrate +/- ADP

Probe

Mineral Oil

Fluorescence Plate Reader (TECAN)

P.C.

Screen 1: Mitochondrial respiration in 96 well format.

Mitochondrial Effects of Thiazolidinediones 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

* Colca et al., Am J Physiol Endocrinol Metab. 286:E252, 2004
* Nadanaciva et al., 2007
Troglitazone Impairs Mitochondrial Function: IC\textsubscript{50} values are Readily Determined

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
- NADH → NAD$^+$
- ubiquinol → ubiquinone

Complex IV assay
- Reduced cyt c + O$_2$ → Oxidized cyt c + H$_2$O

Complex V assay
- ATP → ADP + Pi

Nadanaciva et al., 2007 Toxicology In vitro, online
Mitochondrial Effects of Thiazolidinediones Vary

In addition to these acute effects & PPAR binding:

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• Nadanaciva et al., 2007

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: IC\textsubscript{50} > 700 μM.

![Graph showing the effect of pioglitazone on various complexes with IC\textsubscript{50} values.](image)

Absolute IC\textsubscript{50} > 700 μM
Darglitazone

- Complex I Activity: Not inhibited at 150 μM.
- Complex II/III Activity: Not inhibited at 150 μM
- Complex IV Activity: IC₅₀ 20.8 μM
- Complex V Activity: IC₅₀ 97.5 μM
Troglitazone

- Complex I Activity: Not inhibited at 150 μM.
- Complex II/III Activity: Not inhibited at 150 μM
- Complex IV Activity: IC₅₀ 5.9 μM
- Complex V Activity: IC₅₀ 11.7 μM

![Graphs showing IC₅₀ values for Troglitazone]
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 $\frac{C_{\text{max}}}{IC_{50}}$

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
RST ImmunoCapture

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

Dipstick Technology
- Other non invasive tests
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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

Oxygen Consumption Rate (OCR)

Extracellular Acidification Rate (ECAR)
Screen 3: Metabolic Profiling to Detect Mitochondrial Toxicity

Data from Lisa Marroquin
Metabolic Profiling to Detect Mitochondrial Toxicity

Data from Lisa Marroquin

HepG2 cells
Metabolic Profiling Parallels EC50 for \textit{in vivo} Lactic Acidosis

<table>
<thead>
<tr>
<th></th>
<th>Metformin</th>
<th>Buformin</th>
<th>Phenformin</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (µM) for lactic acidosis*</td>
<td>734 ± 168</td>
<td>119 ± 18</td>
<td>4.97 ± 0.87</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Molecular Weight: 129.164 g/mol</th>
<th>Molecular Weight: 157.217 g/mol</th>
<th>Molecular Weight: 205.26 g/mol</th>
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<tbody>
<tr>
<td>Molecular Formula:</td>
<td>C4H11N5</td>
<td>C6H15N5</td>
<td>C10H15N5</td>
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<tr>
<td>LogP:</td>
<td>-0.267</td>
<td>0.243</td>
<td>0.759</td>
</tr>
<tr>
<td>Hydrogen Bond Donor Count:</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Hydrogen Bond Acceptor Count:</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Rotatable Bond Count:</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Tautomer Count:</td>
<td>3</td>
<td>5 formin</td>
<td>5</td>
</tr>
</tbody>
</table>

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 $O_2$ consumption & resistance to mitotoxicants.

Net yield 0 ATP

Marroquin et al., Tox. Sci, 2007
Cells Grown in Galactose Become Susceptible to Mitochondrial Inhibition

Marroquin et al., Tox. Sci, 2007
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 μM Linezolid shows decreased levels of Complex I and Complex IV subunit Porin and Complex IV subunit 2. Control cells 1PD 3PD 5PD Cells in 40 μM Linezolid

- Complex V α subunit
- Porin
- Complex II-30kD subunit
- Complex IV subunit 2
- Complex I 20kD subunit
Fluorescence microscopy confirms loss of mtDNA-encoded protein in 40 mM linezolid-treated HepG2 cells
OXPHOS Dipstick Assays

Reference protein
Proteins of Interest
Linezolid inhibits mitochondrial protein synthesis

![Graph showing the inhibition of mtDNA-encoded protein synthesis by Linezolid and other compounds. The x-axis represents different treatments including Control, 40 uM Linezolid, 40 uM Chloramphenicol, 100 uM PF-2319817, and 100 uM PF-3962558. The y-axis represents the % Inhibition of mtDNA-encoded protein synthesis. Linezolid and Chloramphenicol show a significant inhibition compared to the Control.]
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- Summary
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

Fail Early – saves time and money

Cumulative Cost/compound (millions $$)

- Discovery (3 yrs)
  - Pre Clinical (1yr)
- Clinical Development (PI/II) (3 yrs)
- Clinical Development (PII/III) (8 yrs)
- Post Marketing

Billions

2.6
9.6
31.3
880

Cumulative Cost/compound (millions $$)
Acknowledgements

- Lisa Marroquin, BS
- Dr. James Dykens
- Dr. James Hynes
- Dr. Sashi Nandanaciva