DILIsym Services

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DILIsym User Training - In Vitro Data Collection Considerations: Assessment of Mitochondrial Function and Assessment of Oxidative Stress

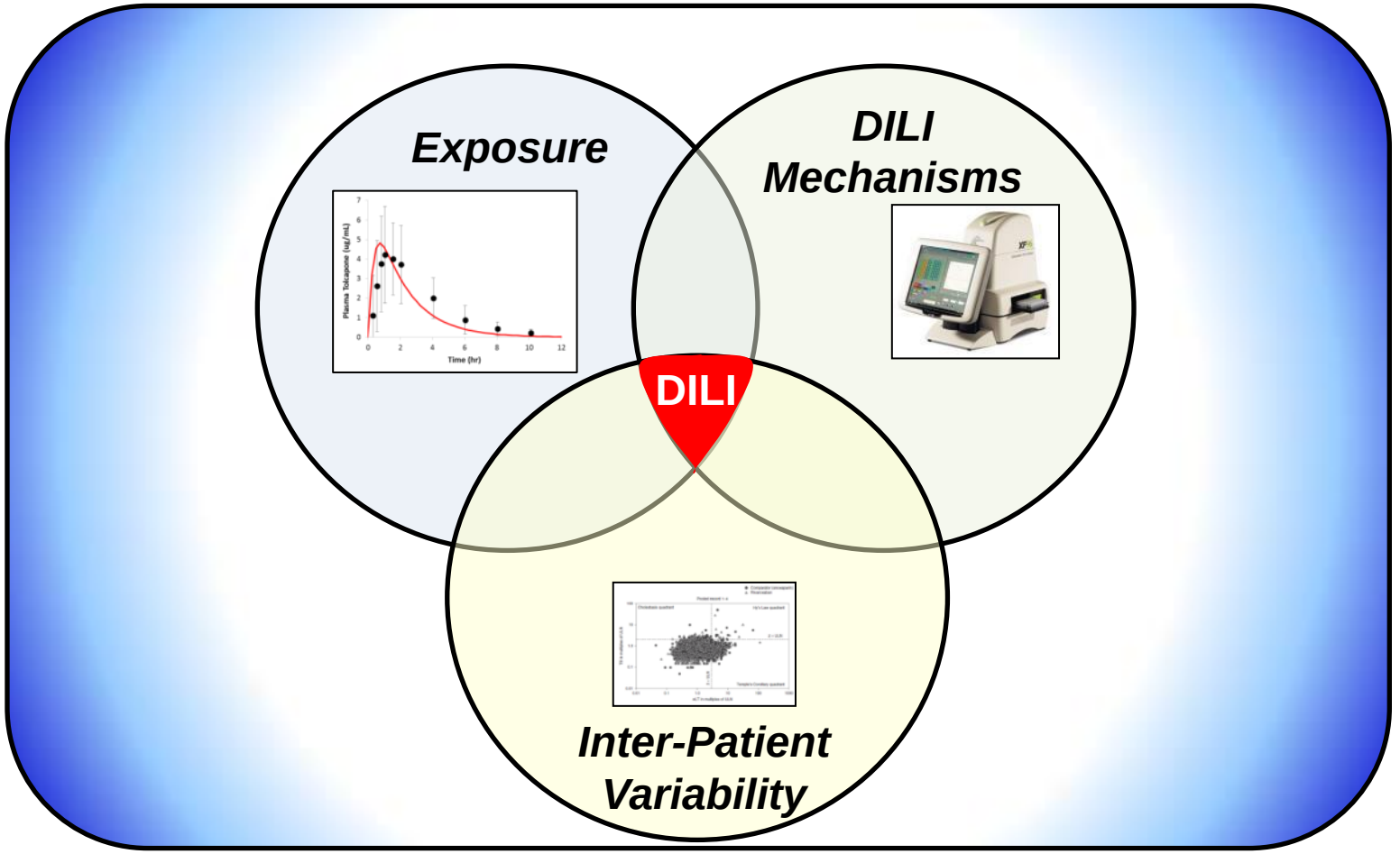
DILI-sim Team

Goals for This Training Session

Participants should understand the following general concepts:

- Methods and tips related to gathering data in the area of mitochondrial toxicity for use within DILIsym
- Methods and tips related to gathering data in the area of oxidative stress for use within DILIsym: updated recommendations on cell type

DILIsym Predicts DILI via the Intersection Between Exposure, Mechanisms, and Inter-Patient Variability



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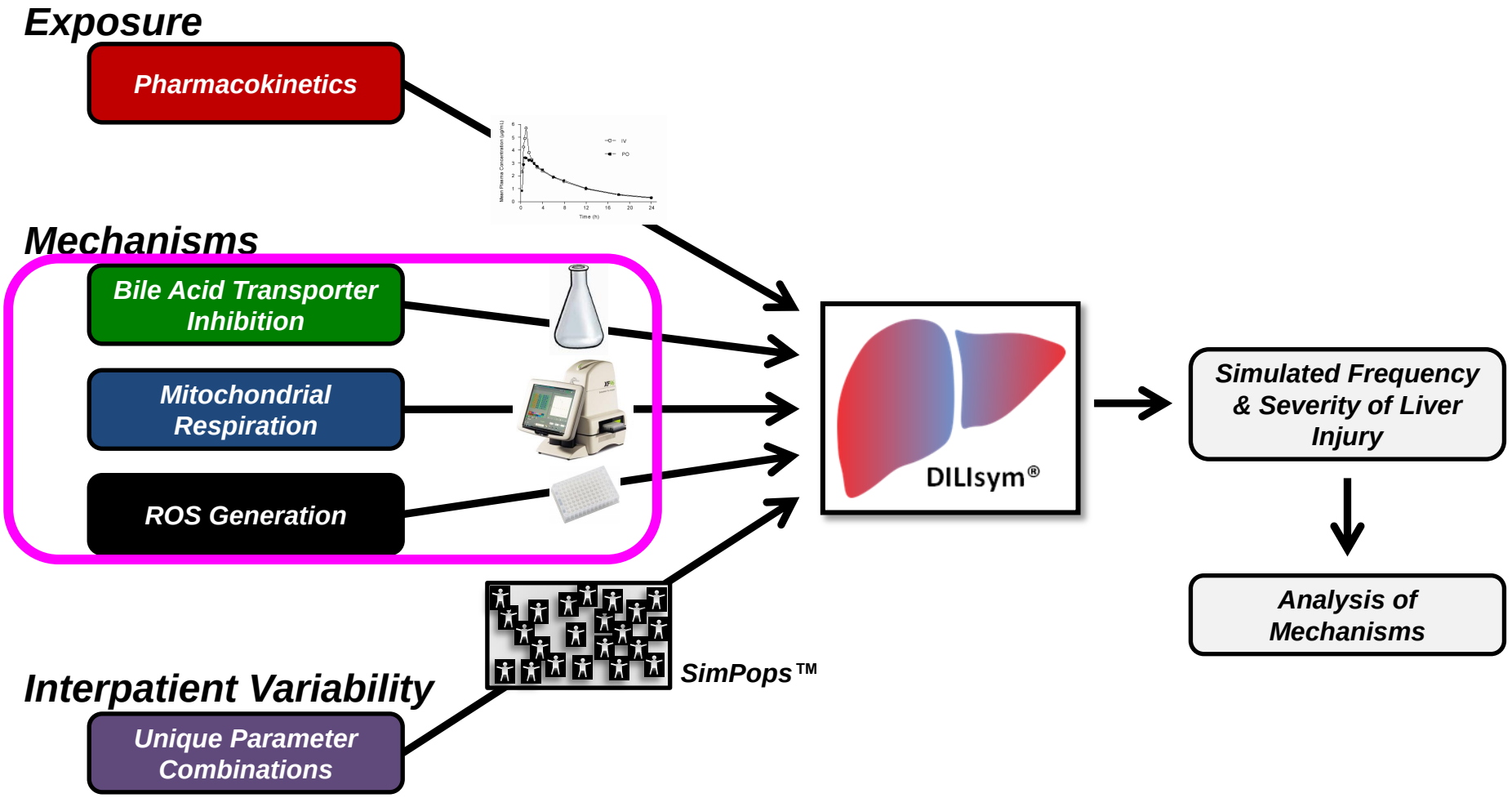
DILIsym Integrates Multiple Inputs to Simulate/Predict Hepatotoxicity



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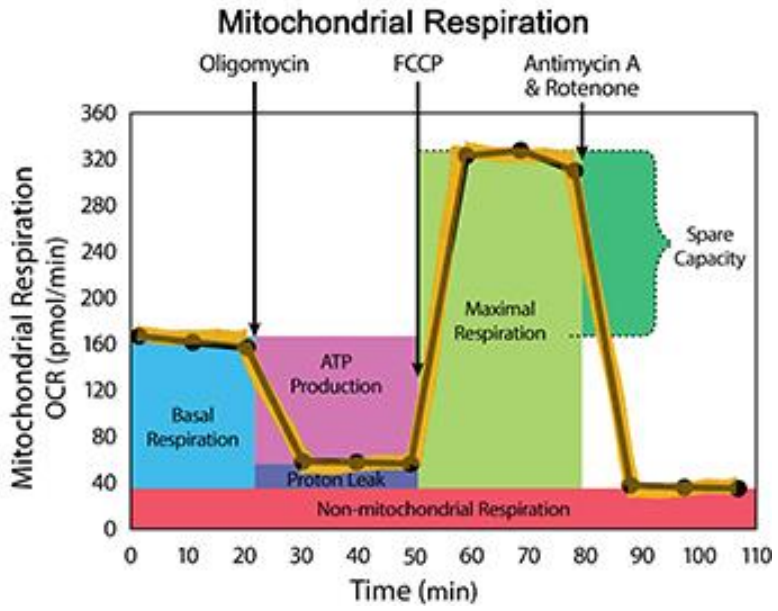
Agilent/Seahorse XF Analyzer Collects Cellular Respiration Data

- XF Analyzer measures extracellular O_2 and H^+
- OCR is measure of cellular respiration
- ECAR, extracellular acidification, is index of glycolysis rate
  - Cellular H+ production largely dependent upon glycolysis rates
- Drug-induced alterations in mitochondria function can be measured
  - Use classic effectors of mitochondrial function
  - Reveal adaptive responses in associated pathways
  - Provide comparators for novel drugs
- Can characterize mitochondria and cellular function in different situations
  - Mitochondria stress test allows for assessment of different aspects of mitochondria function
  - Serial injection of oligomycin, FCCP, and antimycin/rotenone



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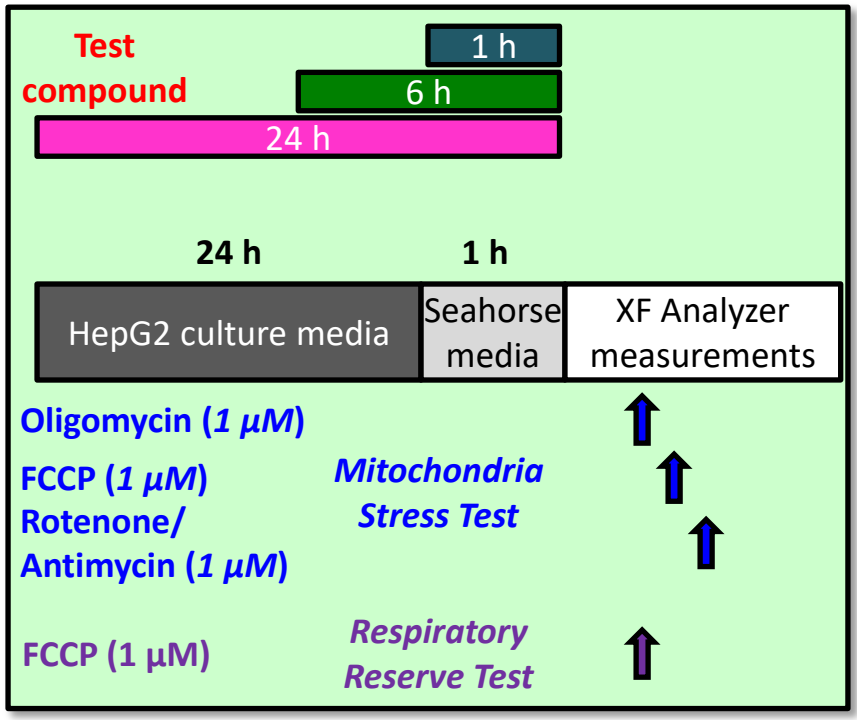
General XF Analyzer Protocol for DILIsym Input Panel Mitochondria Measurements

- HepG2 cells lack drug metabolism capacity
  - Exposure with parent, metabolites
  - Cells seeded into plates and cultured for 24-48 h
- Cells incubated in Seahorse media for 1 h
- Several possibilities for administering test compound
  - Direct injection, 1 h, 6 h, 24 h incubation
  - Depends on compound characteristics
  - Need to measure cell count with longer incubations
- Mitochondria Stress Test provides information on mitochondria toxicity mechanisms
- Respiratory Reserve Test can reveal modest ETC inhibition effects
- Measure intracellular compound concentrations coincident with OCR and ATP measures
  - Can estimate intracellular concentrations via *in silico* methods



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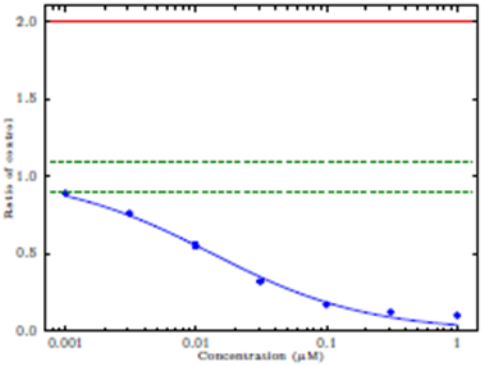
Observed ETC Inhibition Profile Has Provided Motivation to Update DILIsym Parameterization

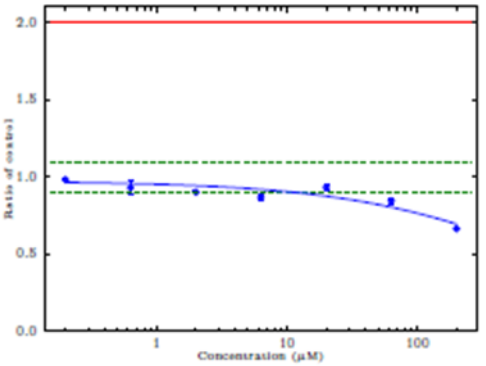
- The majority of compounds that have mitochondrial ETC inhibition properties have clear exposure-response inhibition profiles
  - Rotenone is an archetypical ETC inhibitor
  - Primary data provided by Cyprotex
- A smaller group of compounds appear to elicit a more subtle, exposure-independent ETC inhibition
  - Promethazine is an example
  - Primary data provided by Cyprotex
  - May represent inhibition of complex II in ETC
  - May also include less potent inhibition of complex I, III, or IV

Rotenone



Eakins 2016

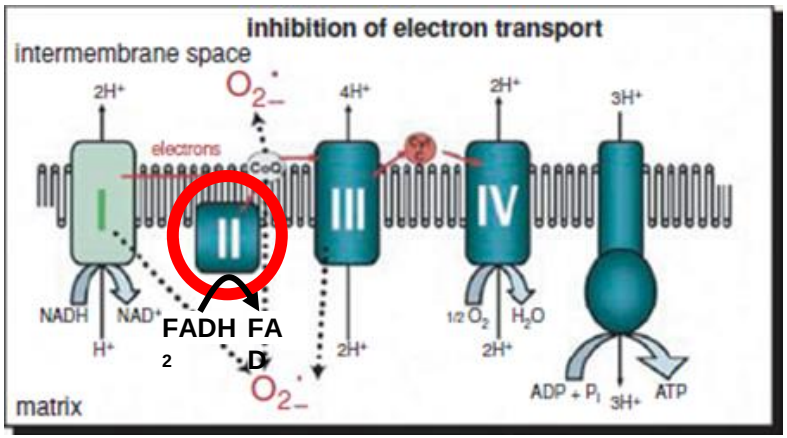
Promethazine



Eakins 2016

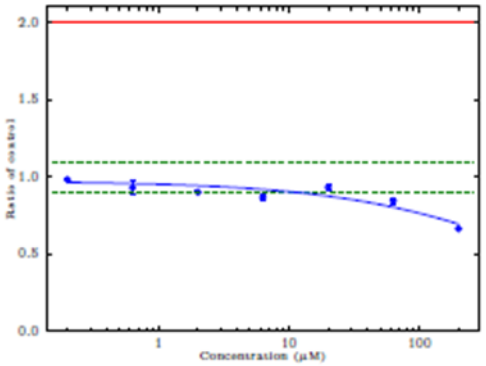
Inhibition of Mitochondrial ETC Complex II May Explain Observed OCR Response

- Complex II accepts electrons from $FADH_2$
  - $FADH_2$ generated in Krebs cycle and via beta oxidation
- $FADH_2$ is responsible for minority of all electrons donated to ETC
  - Full inhibition would not elicit complete full reduction of OCR
  - NADH donates majority of electron flux through ETC
- A plausible alternative hypothesis is that there is an incomplete inhibition of complex I, III, or IV



Nadanaciva 2009

Promethazine



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Preclinical Data

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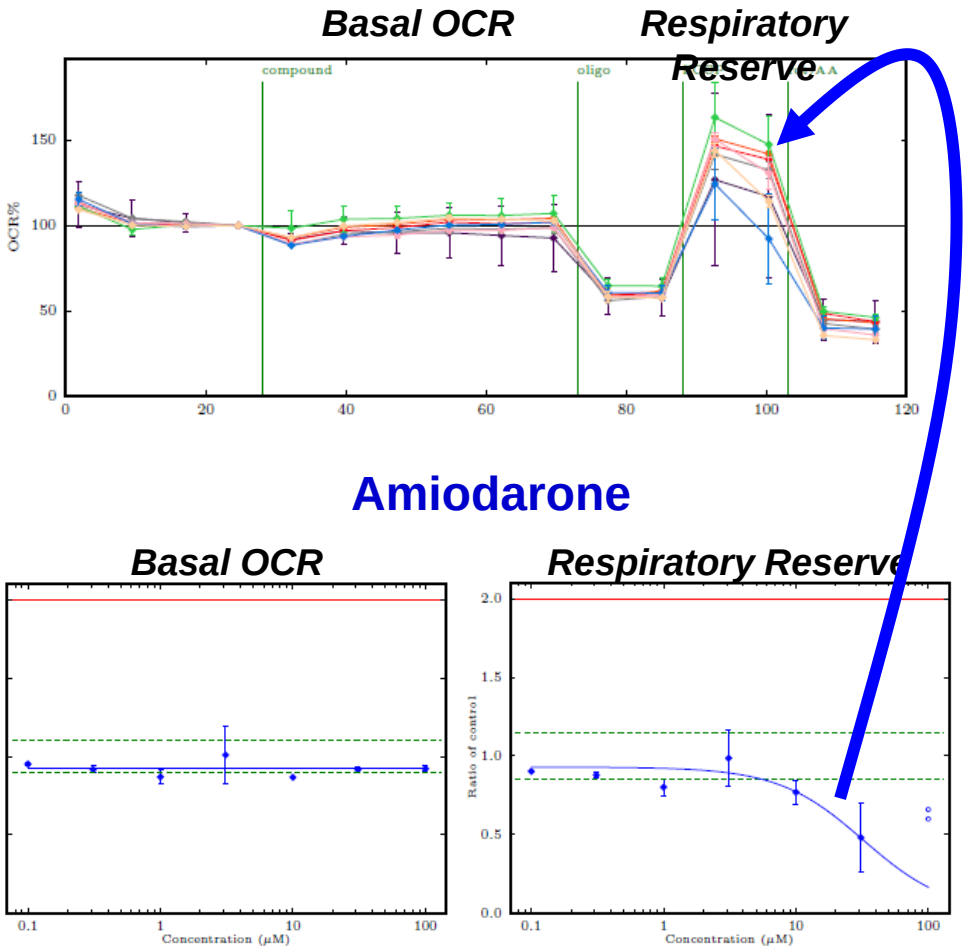
Use of Seahorse Mitochondria Stress Test Assay Provides Ability to Detect Obvious and Subtle ETCi

- DILIsym ETC inhibition parameters frequently determined by using basal OCR
  - Able to detect obvious ETCi
- Modest ETC inhibition can be revealed by inspecting respiratory reserve in addition to basal OCR
  - Exposes inhibition only apparent when system is operating a maximum flux
  - Amiodarone is example
- Utilization of mitochondria stress test allows detection of possible compound liabilities
- Can determine ETCi parameter values by simulating mitochondria stress test in MITOsym



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Preclinical Data

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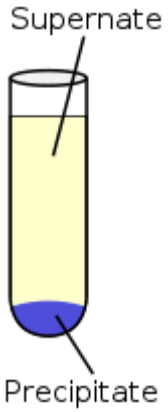
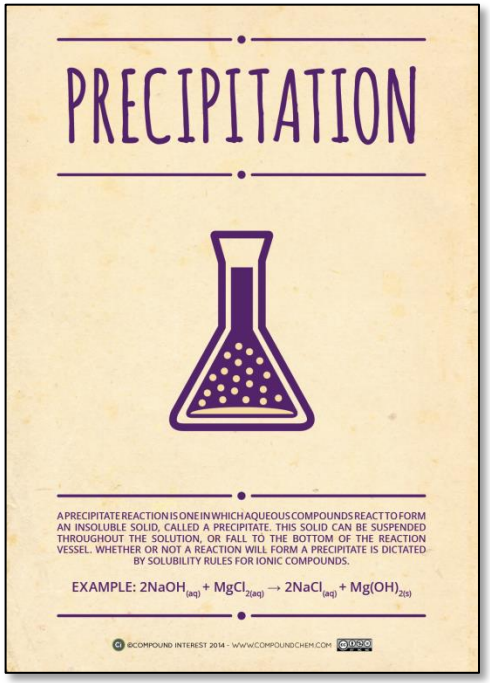
Compound Solubility Testing in Assay Media and/or Buffer Recommended

- Hydrophobic compounds can fall out of solution at high concentrations
  - Inclusion of bovine serum albumin (BSA) minimizes potential for this occurring
- Seahorse media
  - Excludes BSA, as it interferes with assay
  - Upper limit to use of DMSO, as it can have an effect on OCR
- Performing compound solubility testing in assay media and/or buffer can help determine upper limit of exposure-response curve
  - Prevents formation of precipitate during assay
- DILIsym Services routinely performs compound solubility testing in collaboration with Cyprotex and Solvo





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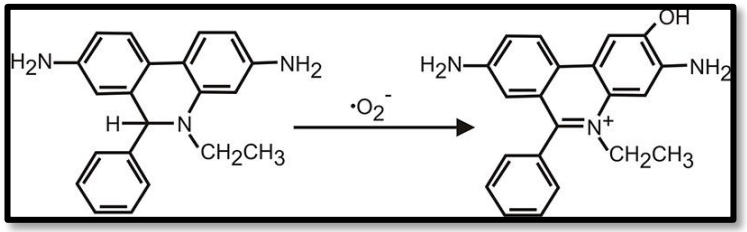
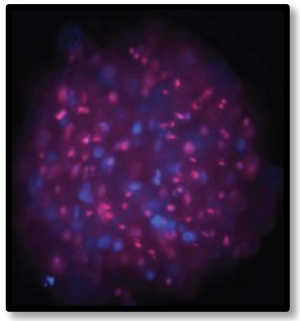
DHE Assay Measures Reactive Oxygen Species (ROS)

- Dihydroethidium (DHE) interacts with ROS to produce 2-hydroxyethidium
  - DHE fluoresces after accepting electrons
- Magnitude of fluorescence indicates amount of ROS produced
  - Can be measured with high-throughput instruments
- Drug-induced alterations in ROS levels can be measured
  - Expose cells to varying concentrations of parent and/or metabolite
  - Utilize HepG2 and/or HepaRG spheroids





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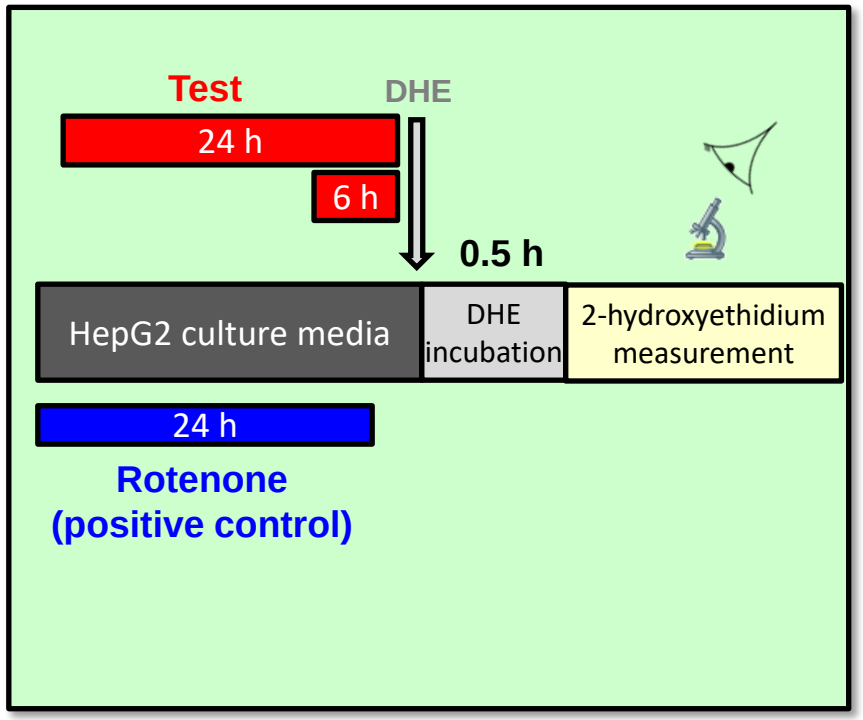
Example Protocol for DILIsym Input Panel ROS Measurements: HepG2

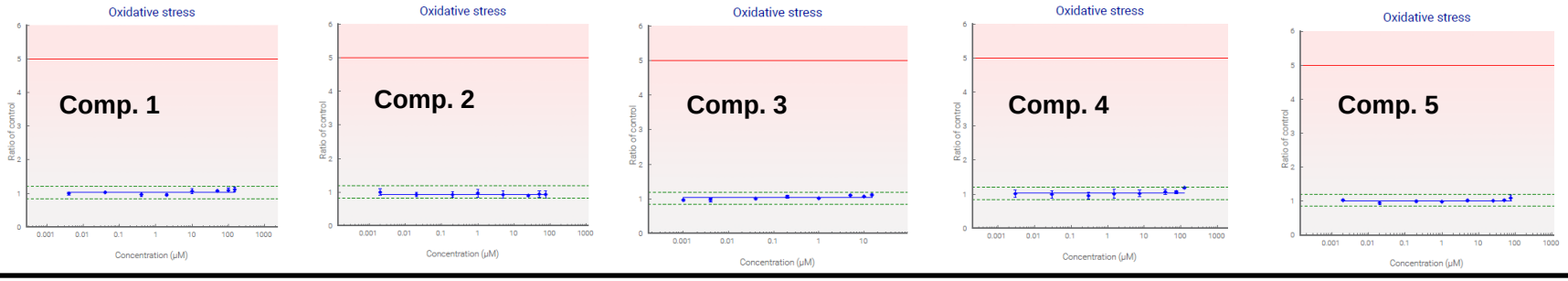
- HepG2 cells lack drug metabolism capacity
  - Exposure with parent, metabolites
  - Cells seeded into plates and cultured for 24-48 h
- Cells incubated with multiple doses of Test compound and a single dose of Rotenone prior to measuring ROS.
  - Cell count for viability and data correction (if necessary)
- Dihydroethidium (DHE) interacts with ROS to produce 2-hydroxyethidium
  - 2-hydroxyethidum fluoresces after accepting electrons
- Measure intracellular compound concentrations coincident with DHE measures
  - Can estimate intracellular concentrations via *in silico* methods



The Cryopreserved Hepatocyte Model Has Proven Insensitive to ROS/RNS in Many Cases Compared to HepG2 Cells

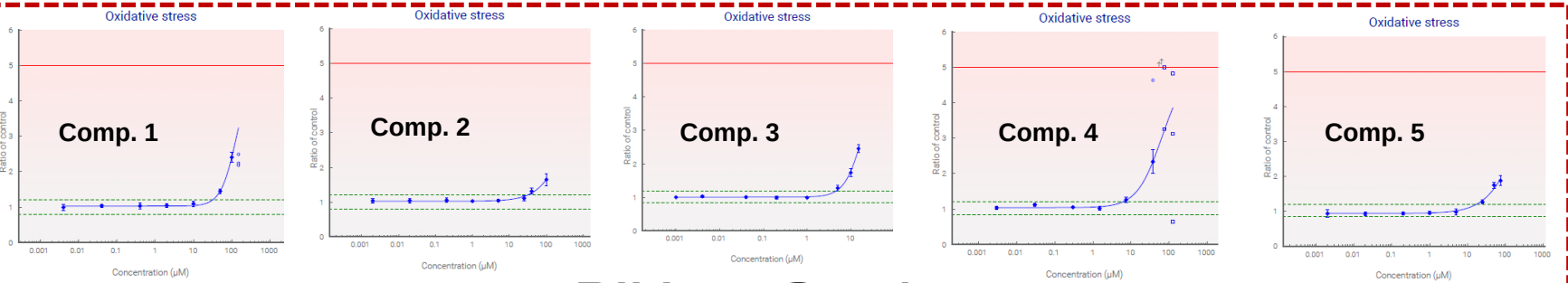
- A large collection of data has suggested to DILIsym Services that the cryopreserved human hepatocyte model is not optimal for purpose
  - 5 example compounds shown below, all blinded
- Cyprotex has agreed with this assessment based on their internal experience
- DILIsym Services would like to test alternative approaches with reasonable cost and reproducibility

Cryo Heps



Cryo Heps

HepG2



HepG2

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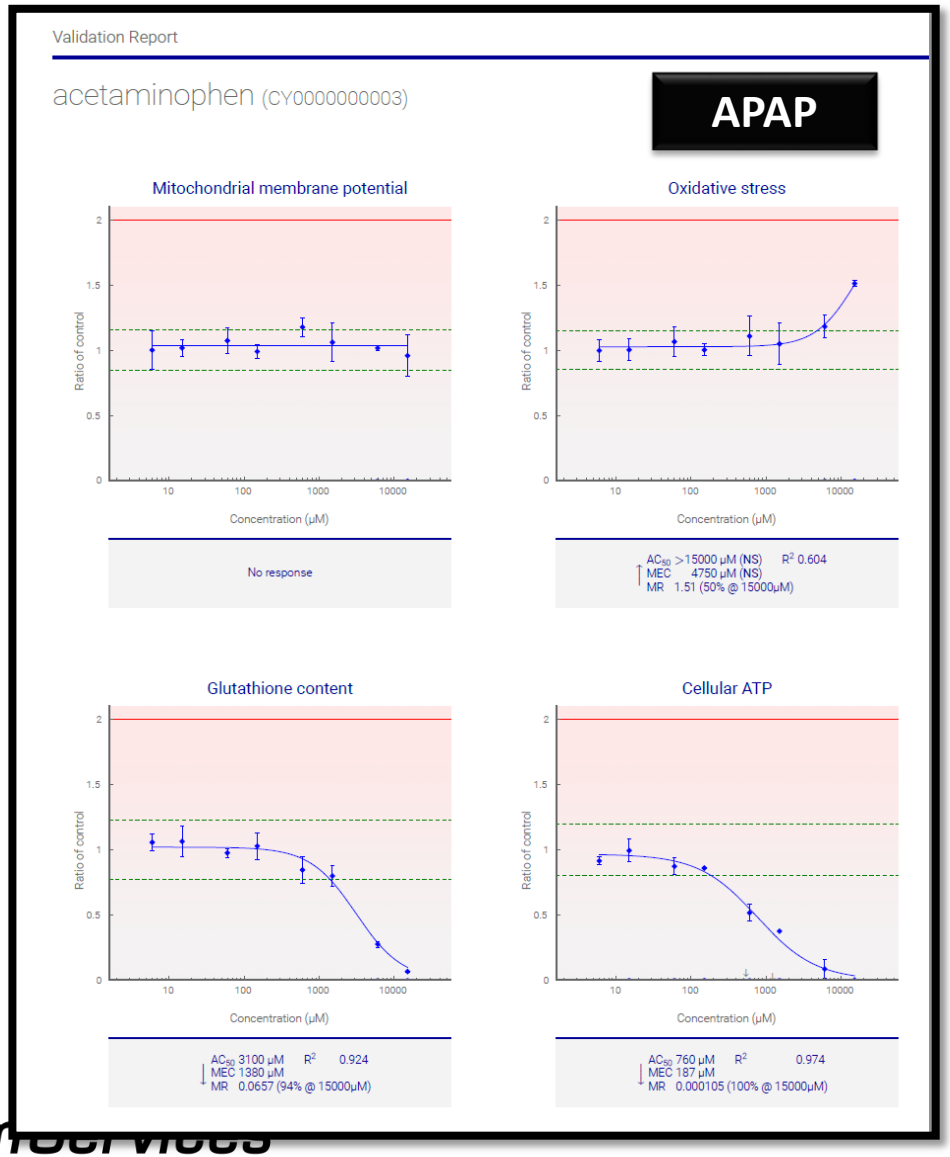
Preclinical Data

DILIsym Services Believes HepaRG Spheroids Show Promise As One of Many Possible Solutions

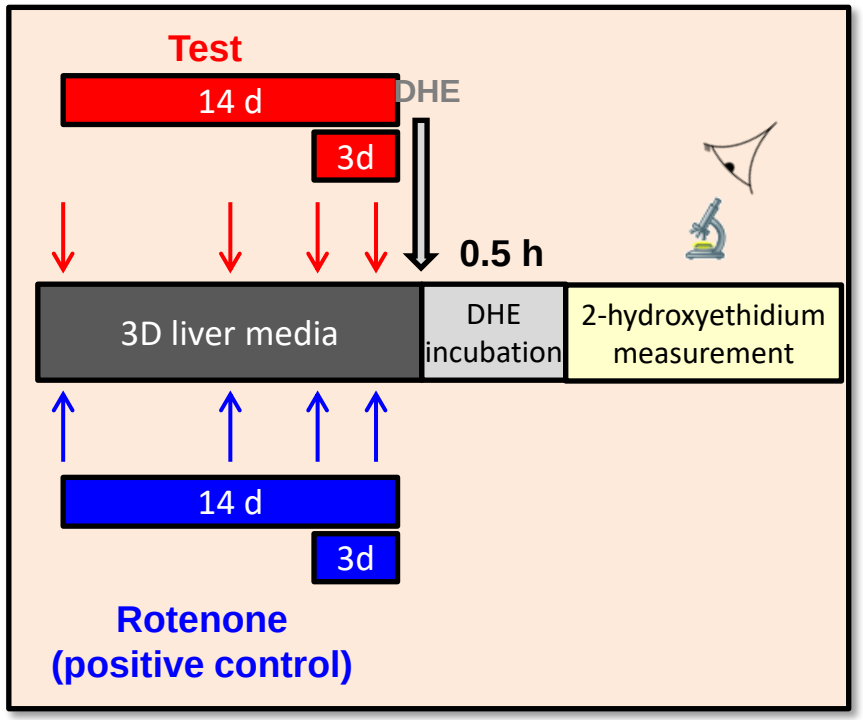
- Cyprotex recommends their HepaRG spheroid model for the intended purpose of ROS production by metabolites
- HepaRG cells are robust, reproducible and metabolically competent
- System offers the added benefit of longer term time points (up to 2 weeks)
- Validation data suggest the system is capable of picking up metabolite-mediated ROS
  - Have tested 30 compounds within validation set



Preclinical Data

Example Protocol for DILIsym Input Panel ROS Measurements: HepaRG Spheroids

- HepaRG spheroids include drug metabolism capacity
  - Responses to exposure with parent includes potential contributions from metabolite(s)
  - Cells seeded into plates and cultured for 3 d or 14 d
  - Conduct this assay in parallel with HepG2 to assess contribution of metabolites (particularly RM)
- Cells incubated with varying doses of Test compound and a single dose of Rotenone prior to measuring ROS.
  - Cell count for viability and data correction (if necessary)
  - Re-dosing done on days 4, 7, 10, 16
- Dihydroethidium (DHE) interacts with ROS to produce 2-hydroxyethidium
  - 2-hydroxyethidum fluoresces after accepting electrons
- Measure intracellular compound concentrations coincident with DHE measures
  - Can estimate intracellular concentrations via *in silico* methods



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