DILIsym®

DILIsym® User Training – ROS Data Collection

September 2016

DILIsym® Development Team

CONFIDENTIAL

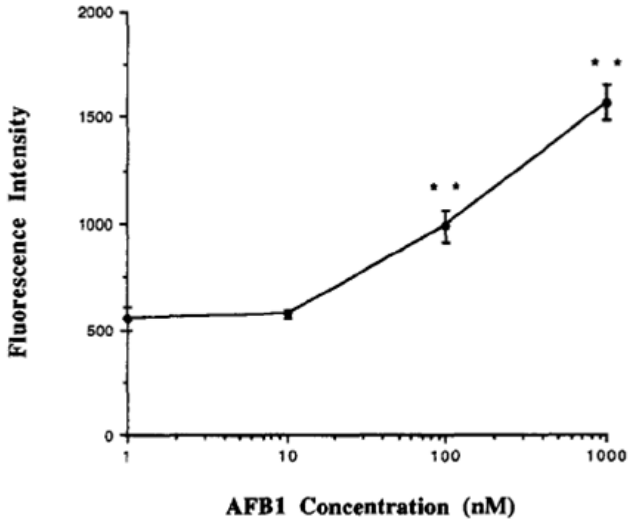
Goal for This Training Session

Participants should understand the following general concepts:

- Methods and tips related to gathering data in the area of oxidative stress for use within DILIsym®

Data Gathering for Modeling Compounds that Disturb the Reactive Oxygen Species Balance

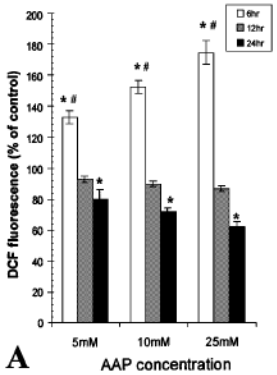
- Data gathering
  - *In vitro* assessments of reactive oxygen or nitrogen species (ROS/RNS)
    - Endpoints recommended by the DILIsym® team
    - Exposure range recommended by the DILIsym® team
    - Intracellular concentration assessments or estimates recommended by the DILIsym® team
  - *In vivo* assessments of reactive oxygen or nitrogen species (ROS/RNS)



Shen 1996

In vitro Indicators of ROS/RNS Recommended by the DILIsym® team

- Several *in vitro* indicators of ROS/RNS are available (examples listed here)
  - **Thiobarbituric acid reactive substances (TBARS), use malondialdehyde (MDA)**
  - **Peroxynitrite**
  - **Lipid hydroperoxide**
  - **Fluorescent probes (DCFDA, DHR123, DHE)**
  - **Mitochondrial superoxide (MitoSOX™ Red)**
- Each potential cell type carries positives and negatives (sample shown in table)
  - Ideally, one molecule can be directly connected to one effect in the experiment (mixtures complicate the parameterization)
  - Active transport is also a consideration
- The more time points measured, the more information garnered on the relationship between exposure and ROS/RNS
  - Depending on stage of development and decision being supported, optimization of *in vitro* exposure may be warranted
  - If a more concise design is required, we recommend the following time points in order of decreasing importance:
    - **24 hour > 12 hour > 6 hour > 1 hour**



Hep3B

Manov 2002

Cell Type	Pros	Cons
HepG2	Availability and cost; lack of drug metabolism if pure metabolites are available for testing individually	Lack of drug metabolism if pure metabolites are not available for testing individually
Primary hepatocytes	Drug metabolism if pure metabolites are not available for testing individually	Availability and cost; drug metabolism if pure metabolites are available for testing individually
HepaRG	Availability and cost; drug metabolism if pure metabolites are not available for testing individually	Drug metabolism if pure metabolites are available for testing individually

Preclinical Data

The Recommended Extracellular Exposure Range is Based on Relevant Plasma Concentrations *in vivo*

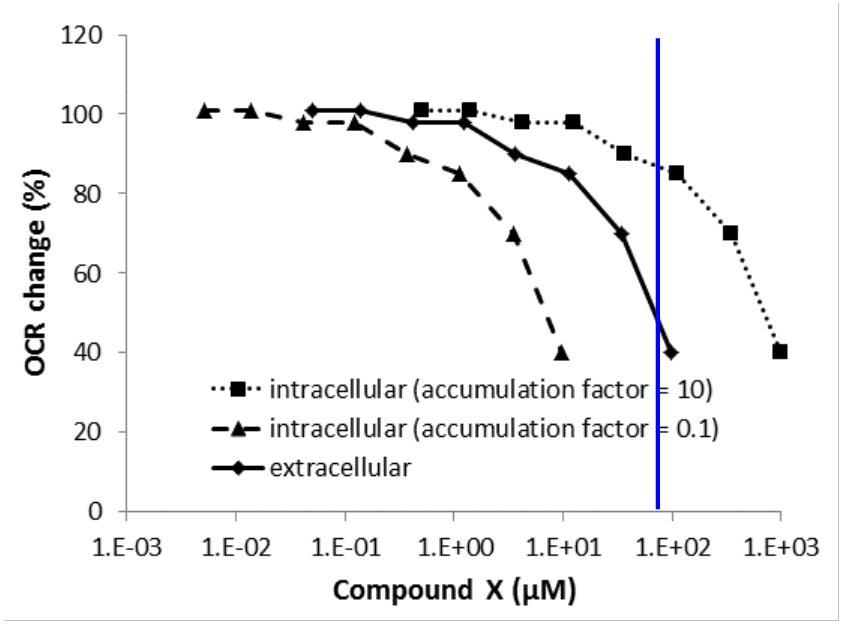
- Extracellular exposure ranges should be relative to the plasma concentrations, or predictions thereof
  - **This is critical to gathering useful data!**
  - *In vitro* studies are often done at extremely high concentrations
  - Contrary to the approach often taken, pushing the cells to a maximal response is not the best approach for this application
- Example table shown that blankets the predicted C_{max}
  - This example is applicable when clinical PK data are unavailable
- Range should be adjusted if more information is available
  - Clinical PK data can help set lower and upper bounds of plasma levels
  - High anticipated variability in exposure may warrant a broader range

Extracellular Exposure Level
100x plasma Cmax
10x plasma Cmax
Plasma Cmax
0.1x plasma Cmax
0.01 plasma Cmax
Vehicle control

Improved DILIsym® Parameter Values When Intracellular Compound Is Measured

- Most Seahorse oxygen consumption rate (OCR) or ROS/RNS data are expressed in an exposure-response relationship
  - OCR change on y-axis
  - Extracellular compound concentration on x-axis
- Numerous compounds have been shown to accumulate in liver
  - Potency relative to intracellular concentrations different than relative to extracellular
  - Intracellular ≠ extracellular
- Basing parameter values on extracellular concentrations introduces inaccuracy for compounds that accumulate in hepatocytes
- Recommend measuring intracellular compound concentration for cell based assays used to provide DILIsym® parameter values
  - OCR, ROS production
  - For compounds that are known to have liver:blood ratio ≠ 1 (or not known)

HepG2



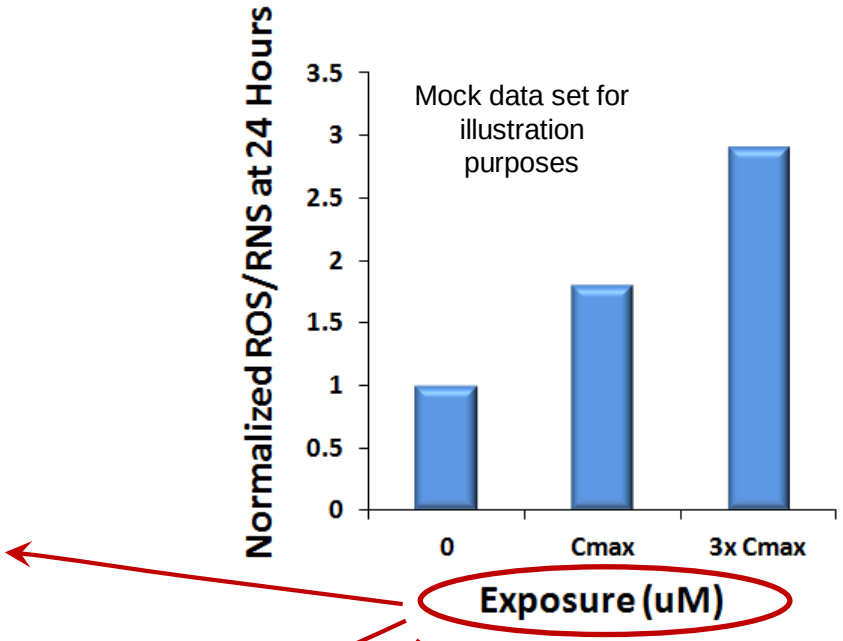
	extracellular	intracellular
10X accumulation	74.4 uM	744 uM
1x accumulation	74.4 uM	74.4 uM
0.1x accumulation	74.4 uM	7.44 uM

Theoretical Preclinical Data

DILI-sim Initiative

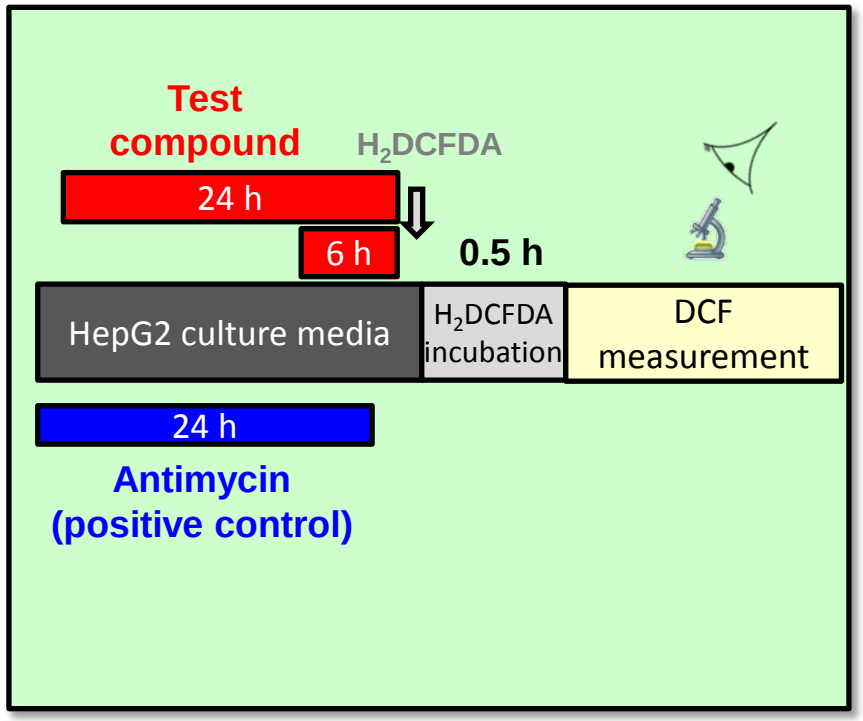
Establishing a Dose Response Requires Choosing an Exposure Estimate and Preferred Time Point(s)

- A relationship between exposure and normalized ROS/RNS response at a single time point is typically used
  - Could also choose to fit multiple time courses
- Intracellular exposure can be estimated in three ways
  - Measured (see previous slide); this is the recommended method, but is not always possible
  - Estimated using media concentration and PBPK parameters such as liver to blood concentration ratio
  - Assumed from media concentration; this is the least optimal approach, in our opinion



Example Protocol for DILIsym® Input Panel ROS Measurements

- 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 Antimycin for 24h prior to measuring ROS.
  - Cell count for viability and data correction (if necessary)
- 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) acetate groups are cleaved by esterases and then oxidated to fluorescent 2',7'-dichlorofluorescein (DCF) during a 30 min incubation
- Measure intracellular compound concentrations coincident with DCF measures
  - Can estimate intracellular concentrations via PBPK methods

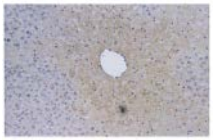


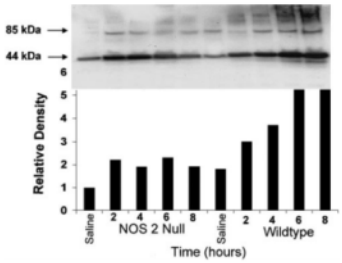
In vivo Assessments of ROS/RNS are Useful but Rarely Available

- *In vivo* assessments of ROS/RNS in the liver after drug exposure are sometimes (but rarely) available
- These assessments are useful for obtaining the necessary parameter within DILIsym® to represent ROS/RNS increases if:
  - A PBPK model has been established for the drug and species from which the data originate
  - The molecular entity (parent or metabolite) suspected of causing the ROS/RNS is represented in the liver within the PBPK model
    - e.g. NAPQI in an APAP PBPK model
- Simulations of ROS/RNS are fitted to the data
- The use of *in vitro* data is much more common
  - *In vivo* endpoints related to ROS/RNS are practically never available for compounds in development with multiple doses, time courses, etc.
  - *In vivo* endpoints related to ROS/RNS can be used for validation and refinement of the *in vitro* approach post-hoc if they become available during the latter stages of development

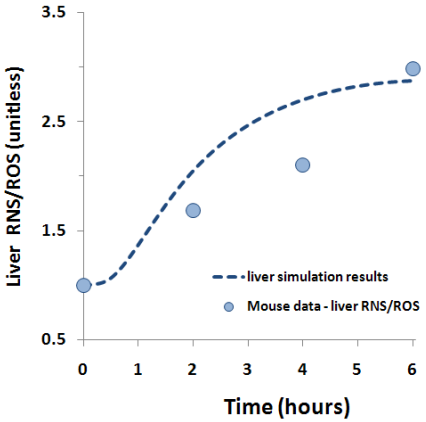
Michael 2001 - 300 mg/kg APAP, liver RNS/ROS

MICE





RNS indicator



Thank You!