Assessing the Biological Relevance of *In Vitro* Data: A Case Study Using Estrogen Pathway Signaling

Warren Casey, PhD, DABT  
Director, NICEATM

Division of the National Toxicology Program  
National Institute of Environmental Health Sciences

NTP Board of Scientific Counselors (BSC) Meeting  
Dec 9-10, 2014
Interagency Collaboration

- NIEHS / NTP
- EPA OSCP
- EPA NCCT

- Presented at EPA’s FIFRA SAP: Integrated Bioactivity Exposure Ranking, 2-5 Dec 2014.

- White paper available on SAP website:
  
  http://www.epa.gov/scipoly/sap/meetings/2014/120214meeting.html

Positions or views expressed here do not represent official EPA policy or guidance
• For the purposes of prioritization and screening, can *in vitro* assays identify chemicals that *have the potential* to interact with the human estrogen receptor? If so, does *in vitro* potency correlate with *in vivo* bioactivity (uterotrophic)?

• If a chemical is not ER-active *in vitro*, what level of confidence do we have that it will not be ER-active *in vivo* (uterotrophic)?
OECD GD 34, Validation and International Acceptance of New or Updated Test Methods

Validation is a process by which the reliability and relevance of a test method are established for a specific purpose.

Relevance and reliability should be characterized against data generated with a list of reference chemicals tested in the original method accepted by regulatory agencies (uterotrophic bioassay).
20 K ft. View

In vitro activity

Bioactivity

Toxicity

Human Relevance
• 1800 Chemicals run 16 Tox21/ToxCast ER Agonist assays (1800 x 16 dose-response curves)

• Mathematical model developed to summarize results of all 16 assays for each chemical (1800 “AUC” values)

• Database of uterotrophic outcomes, developed from the literature, was used to identify in vivo (uterotrophic) reference chemicals for validation

• Model results assessed using “real world” data from EPA EDSP List 1 chemicals
In Vitro Assays: ER Agonist Pathway

ER Binding
- ER α
- mER α
- bER α

ER Dimerization
- α/α
- α/β
- β/β

DNA Binding
- GFP expression
- HEK293T (Kidney)
- HeLa (Cervix)

RNA Transcription
- ER α Trans
- ERE Cis
- HepG2 (Liver)

Protein Production
- ER α/β
- ER α LBD

Proliferation
- ER α Proliferation
- T47D (Breast)

In Vitro Assays: ER Agonist Pathway
- NovaScreen
- Odyssey Thera
- Odyssey Thera
- Attagene
- NIH-NCGC
- ACEA
Mathematical Model of the ER Agonist Pathway

- **ER Binding**
  - Built using a strictly mathematical approach (i.e., no data used for training/learning)

- **ER Dimerization**
  - Assesses consistency of response across all assays, discounting assay / technology specific results

- **DNA Binding**
  - For each chemical, the model summarizes results from all assays with a composite dose-response curve, which is used to calculate an AUC relative to 17β-estradiol (e.g. AUC for E2 = 1.0)

- **RNA Transcription**
- **Protein Production**
- **Proliferation**
  - AUCs range from 1-0, with ~0.1 representing the approximate limit of detection (~100 µM)
ER AUC Values of 1800 ToxCast Chemicals
ER AUC Values of 1800 ToxCast Chemicals

Most Active

Least Active

ER Agonist AUC

Rank Order (ER Agonist AUC)
A scatter plot showing the rank order of ER agonist AUC for various substances. The x-axis represents the rank order of ER agonist AUC, while the y-axis shows the AUC values. The most active substances are at the top, with the least active at the bottom. The substances plotted include Mestranol, Zearalenone, Bisphenol AF, Genistein, Bisphenol B, Norethindrone, and Bisphenol A.
Uterotrophic Bioassay

Purpose

- Short term *in vivo* screen to evaluate the ability of a chemical to elicit a biological response similar to that of natural estrogens

Principle

- Uterus is under the control of estrogens to stimulate growth
- Production of endogenous estrogens is prevented
  - Ovariectomized (OVX)
  - Immature (Imm)
- Uterus becomes sensitive to external estrogenic substances

Billon-Galés A et al. PNAS 2011
Validation
Organization of Economic Cooperation and Development (OECD)

Guidelines
OECD TG 440 / OCSP 890.1600

Rodent models
- OVX Rat
- Imm Rat
- OVX Mouse

Dosing route
- Oral gavage (Oral)
- Subcutaneous injection (Inj)
Identifying Uterotrophic Reference Chemicals

- Literature Searches: 1800 Chemicals
- Data Review: 700 Papers, 42 Descriptors, x2
- Uterotrophic Database: 98 Chemicals, 442 uterotrophic bioassays
- Reference Chemicals: 31 Active, 13 Inactive
<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Elapsed time between OVX and RX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASRN</td>
<td>Dosing Length</td>
</tr>
<tr>
<td>PMID</td>
<td># of doses per day</td>
</tr>
<tr>
<td>Author</td>
<td># of animals in estrogen control group</td>
</tr>
<tr>
<td>Year</td>
<td># of animals in RX group</td>
</tr>
<tr>
<td>Study Type</td>
<td>Reference Estrogen</td>
</tr>
<tr>
<td>Species</td>
<td>Vehicle/RX control?</td>
</tr>
<tr>
<td>Strain</td>
<td>Diet</td>
</tr>
<tr>
<td>Target</td>
<td>Indicated that Diet is low-PE?</td>
</tr>
<tr>
<td>Route of Administration</td>
<td>necropsy time after last dose</td>
</tr>
<tr>
<td>Age at 1st Dose Administration</td>
<td>Additional Assay Info</td>
</tr>
<tr>
<td>Age at OVX</td>
<td>Source Name SID</td>
</tr>
<tr>
<td>Dose/Response (0 no, 1 yes)</td>
<td>Chemical Tested</td>
</tr>
<tr>
<td># of doses used</td>
<td>Chemical Purity</td>
</tr>
<tr>
<td>Value</td>
<td></td>
</tr>
<tr>
<td>Unit Response</td>
<td></td>
</tr>
<tr>
<td>Value type</td>
<td></td>
</tr>
<tr>
<td>LEL</td>
<td></td>
</tr>
<tr>
<td>Max Conc Tested</td>
<td></td>
</tr>
</tbody>
</table>
Identifying Uterotrophic Reference Chemicals

Minimum Criteria for “Guideline-Like” Studies

1. Animal model
   • Imm rats
   • OVX rats or mice
   • OVX performed between six and eight weeks of age

2. Group size
   • Control group n >=3, test group n >=5

3. Route of administration
   • Oral gavage, subcutaneous or intraperitoneal injection

4. Number of dose groups
   • Minimum of two dose levels plus positive control and vehicle control

5. Dose timing and duration
   • Minimum of three consecutive days
   • Immature rats: dosing should begin between post-natal day (PND) 18 and PND 21, and be completed by PND 25

6. Necropsy timing
   • Animal necropsy should be carried out 18-36 hours after the last dose
Uterotrophic Study Designs

- OVX_Mouse_Oral
- OVX_Mouse_Inj
- OVX_Rat_Oral
- OVX_Rat_Inj
- Imm_Rat_Oral
- Imm_Rat_Inj

# of Bioassays

Uterotrophic
- Active
- Inactive
Uterotrophic Reproducibility

Chemicals with >= 2 Studies

Number of Bioassays

Rank Order (Total # of Bioassays)

Active
Inactive
Same Study Design (Immature Rat): BPA

Uterotrophic Reproducibility

LEL or MDT (mg/kg/day)

Injection

Oral

Uterotrophic

- Active
The relevance of a new test method should be characterized against data generated with a list of reference chemicals in the original test method.

**Active** – reported as active in two or more independent GL bioassays, regardless of the number of inactive results

**Inactive** – reported as inactive in two or more independent GL bioassays, with no GL studies reporting bioactivity

**44 Reference Chemicals (31 Active, 13 Inactive)**
Avg LEL of Reference Chemicals (Uterotrophic)

- Rank Order (Avg. LEL)
  - Most Potent
  - Least Potent

Graph showing the average LEL (mg/kg/day) in relation to rank order (avg. LEL), with the x-axis representing rank order and the y-axis representing average LEL (mg/kg/day). The graph illustrates a trend from the most potent to the least potent chemicals.
AUC of Reference Chemicals (In Vitro)

Most Active

ER Agonist AUC

Least Active

Rank Order (ER Agonist AUC)
AUC of Reference Chemicals (In Vitro)

Most Active

Kaempferol

D4

Least Active

ER Agonist AUC

Rank Order (ER Agonist AUC)
### Performance of ER AUC Model

Uterotrophic reference chemicals (31 Active, 13 Inactive)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>True Positive</strong></td>
<td>30</td>
</tr>
<tr>
<td><strong>True Negative</strong></td>
<td>12</td>
</tr>
<tr>
<td><strong>False Positive</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>False Negative</strong></td>
<td>1</td>
</tr>
</tbody>
</table>

**Accuracy**: 0.95

**Sensitivity**: 0.97

**Specificity**: 0.92

ER AUC >= 0.1 as Active
LEL (Uterotrophic) vs. AUC (In Vitro)

Average LEL (mg/kg/day) vs. ER Agonist AUC

Least Potent/Active

Most Potent/Active
LEL (Uterotrophic) vs. AUC (In Vitro)

- **Average LEL (mg/kg/day)**
- **ER Agonist AUC**

- **Least Potent/Active**
  - 17α-estradiol
  - Ethinyl Estradiol
  - Estradiol
  - Estriol
  - Diethylstilbestrol

- **Most Potent/Active**
  - Equilin
  - Estrone
EDSP Tier 1 Battery ER-Specific Endpoints

<table>
<thead>
<tr>
<th>Tier 1 Screening Battery</th>
<th>Endocrine Pathway</th>
<th>E +</th>
<th>E -</th>
<th>A +</th>
<th>A -</th>
<th>HPG$^1$ Axis</th>
<th>HPT$^1$ Axis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER Binding</td>
<td></td>
<td>■</td>
<td>■</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER$\alpha$ Transactivation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterotrophic</td>
<td></td>
<td>■</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pubertal Female</td>
<td></td>
<td>■</td>
<td>■</td>
<td>■</td>
<td>■</td>
<td>■</td>
<td>■</td>
</tr>
<tr>
<td>Fish Short-term Reproduction (male &amp; female)</td>
<td></td>
<td>■</td>
<td>■</td>
<td>■</td>
<td>■</td>
<td>■</td>
<td>■</td>
</tr>
</tbody>
</table>

Note: The full EDSP Tier 1 battery includes five in vitro and six in vivo assays that provide additional information on a chemical’s potential to interact with the androgen and thyroid, as well as alter steroidogenesis.
ER AUC of 42 EPA EDSP List 1 Chemicals

Rank Order (ER Agonist AUC) vs. ER Agonist AUC

- Active
- Inactive

Legend:
- Uterotrophic
- Active
- Inactive
Performance of ER AUC Model

Uterotrophic ref. chemicals + EDSP List 1 (31 Active, 55 Inactive)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>True Positive</td>
<td>30</td>
</tr>
<tr>
<td>True Negative</td>
<td>54</td>
</tr>
<tr>
<td>False Positive</td>
<td>1</td>
</tr>
<tr>
<td>False Negative</td>
<td>1</td>
</tr>
<tr>
<td><strong>Accuracy</strong></td>
<td>0.97</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td>0.97</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>0.98</td>
</tr>
</tbody>
</table>

ER AUC >= 0.1 as Active
• ER Model Reduction / Optimization
• AR Pathway Model (9 In Vitro Assays / Hershberger)
• Evaluate concordance with Repro/Dev endpoints
• *In Vitro* to *In Vivo* Correlation (IVIVE)

• QUESTIONS?
Methoxychlor (AUC=3.8, LEL=128 mg/kg/day)
Non Monotonic Responses

Benzo(a)pyrene

BG1 (Luc) - Antagonist

HEK293 (Bla) - Antagonist

Log Concentration

10 µM