Review of the *In Vivo* Mammalian Assays:

Challenges and Considerations for Conducting and Interpreting these Screening Assays

Leah Zorrilla, PhD
Investigative Toxicology Division
Integrated Laboratory Systems (ILS), Inc.
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ILS Experience with Tier 1 Assays

- 18 Uterotrophic Assays
- 21 Hershberger Bioassays
- 11 Female Pubertal Assays
- 10 Male Pubertal Assays
- 30 Range Finding Assays
EDSP Tier 1 In Vivo Mammalian Assays

- Uterotrophic Assay (OPPTS/OCSTEP 890.1600)
- Hershberger Bioassay (OPPTS/OCSTEP 890.1400)
- Pubertal Female Assay (OPPTS/OCSTEP 890.1450)
- Pubertal Male Assay (OPPTS/OCSTEP 890.1500)

- Study Design and Endpoints
- Dose Range Finding Studies
- Performance Criteria
- Study Interpretation
- Challenges/Solutions
- Future of these Screening Assays
Uterotrophic Assay

**Purpose**
To detect potential estrogenic chemicals through a rapid *in vivo* screening assay

**Study Design**
- 2 Test Substance dose levels, 17α-Ethinyl Estradiol (positive control)
- **Maximum Tolerated Dose (MTD)**- defined as dose that ensures animal survival without significant toxicity or distress up to the limit dose (1000 mg/kg/day)

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**Immature Model**

<table>
<thead>
<tr>
<th>PND</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wean, Assign Treatment Groups</td>
<td>Daily Body Weight and Dose Administration</td>
<td></td>
<td></td>
<td>Necropsy</td>
</tr>
</tbody>
</table>

**Ovariectomized (OVX) Model**

<table>
<thead>
<tr>
<th>PND</th>
<th>42</th>
<th>51</th>
<th>55</th>
<th>56</th>
<th>57</th>
<th>58</th>
<th>59</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OVX</td>
<td>Daily Vaginal Smears for Estrous</td>
<td>Daily Body Weight and Dose Administration</td>
<td></td>
<td></td>
<td>Assign Treatment Groups</td>
<td>Necropsy</td>
</tr>
</tbody>
</table>
Uterotrophic Assay

Study Interpretation

- A statistically significant **increase** in uterine weight (wet and/or blotted) compared to vehicle control is a **positive** result.

Performance Criteria (Blotted Uterine Weight)

- Immature Model: <0.09% of BW
- OVX Model: <0.04% of BW

*Immature Model
Vehicle Selection
- Solubility/stability
- Route of administration
  - Oral vs. subcutaneous

Dose Selection
- Range Finding Studies necessary; dose age matched intact animals for 3 days; assess body weight changes/clinical observations
  - Oral dose level of EE in the OVX model

Experienced Prosectors
- Performance Criteria

Other Considerations
- Positive control in each study
Hershbergerer Bioassay

Purpose
A short term in vivo screening assay for potential androgen agonists and antagonists /5α-reductase inhibitors

Study Design
Agonist assay- Vehicle Control (VC), 2 test substance dose groups, positive control-Testosterone Propionate (TP)
Antagonist assay- Control (VC+ TP), 3 test substance dose groups + TP, and positive control-Flutamide (FT) + TP

MTD
• Dose that avoids death and suffering or distress, and does not cause a final BW loss of >10%, up to the limit dose of 1000 mg/kg/day
**Necropsy**

- Weights of Androgen-dependent tissues are obtained: Ventral Prostate, Seminal vesicle with fluid and coagulating gland, Levator ani and bulbocavernous muscles (LABC), Glans penis, Cowper’s Glands
**Hershberger Bioassay**

**Study Interpretation**
A positive result is a significant change in the weight of two tissues compared to respective controls
- Agonist - increase in weights
- Antagonist/5α-reductase inhibitors - decrease in weights
(note: evaluate body weight changes and coefficients of variation)

**Performance Criteria**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Maximum Allowable CV Androgen Agonist</th>
<th>Maximum Allowable CV Androgen Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glans Penis</td>
<td>22%</td>
<td>17%</td>
</tr>
<tr>
<td>Cowper’s Gland</td>
<td>55%</td>
<td>35%</td>
</tr>
<tr>
<td>LABC</td>
<td>30%</td>
<td>20%</td>
</tr>
<tr>
<td>Ventral Prostate</td>
<td>45%</td>
<td>40%</td>
</tr>
<tr>
<td>Seminal Vesicle</td>
<td>40%</td>
<td>40%</td>
</tr>
</tbody>
</table>
**Hershberger Bioassay Challenges/Lessons Learned**

**Dose Selection/MTD**
- Range Finding Studies - dose intact age matched animals for 10 days; assess body weight changes/clinical observations

**Age at Castration**
- Sexually Mature

**Stagger start dose administration**
- Necropsy over 2 days

**Experienced Prosectors**
- Performance Criteria

**Optional measurements**
- **Liver**, Adrenal, and Kidney weights; T, LH, T\(_4\), T\(_3\) serum hormone analyses; food consumption
Female/Male Pubertal Assays

**Purpose- Female Assay**
To detect test substances that have estrogenic/antiestrogenic or antithyroid activity, or which alter pubertal development via changes in steroidogenesis, gonadotropin secretions, prolactin, or hypothalamic function.

**Purpose- Male Assay**
To detect chemicals with antithyroid, androgenic, or antiandrogenic [androgen receptor (AR) or steroid-enzyme-mediated] activity or agents which alter pubertal development via changes in gonadotropins, prolactin, or hypothalamic function.

**MTD-** Statistically significant decrease in body weight gain, no more than approximately 10% body weight loss compared to vehicle controls, without toxicity
Study Designs

Female Pubertal

- PND: 3 or 4
- Cull Pups
- Wean, Assign Treatment Groups
- Daily Examination for Vaginal Opening and Estrous Cyclicity
- Daily Body Weight and Dose Administration
- Necropsy

Male Pubertal

- PND: 3 or 4
- Cull Pups
- Wean, Assign Treatment Groups
- Daily Examination for Preputial Separation
- Daily Body Weight and Dose Administration
- Necropsy
## Pubertal Assay-Endpoints

<table>
<thead>
<tr>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight</strong></td>
<td><strong>Body Weight</strong></td>
</tr>
<tr>
<td>- Initial, final, BW at vaginal opening (VO)</td>
<td>- Initial, final, BW at preputial separation (PPS)</td>
</tr>
<tr>
<td><strong>Tissue weights</strong></td>
<td><strong>Tissue weights</strong></td>
</tr>
<tr>
<td>- Adrenal glands, liver, pituitary gland, kidneys, thyroid, ovaries, uterus</td>
<td>- Adrenal glands, liver, pituitary gland, kidneys, thyroid, epididymides, LABC, prostate (ventral and dorsolateral), seminal vesicles (w/ and w/out fluid), testes</td>
</tr>
<tr>
<td><strong>Histopathology</strong></td>
<td><strong>Histopathology</strong></td>
</tr>
<tr>
<td>- Kidney, thyroid, ovary, uterus</td>
<td>- Kidney, thyroid, testis, epididymis</td>
</tr>
<tr>
<td><strong>Serum Endpoints</strong></td>
<td><strong>Serum Endpoints</strong></td>
</tr>
<tr>
<td>- T(_4), TSH, clinical chemistry panel</td>
<td>- T(_4), TSH, Testosterone, clinical chemistry panel</td>
</tr>
<tr>
<td><strong>Age at Pubertal Development</strong></td>
<td><strong>Age at Pubertal Development</strong></td>
</tr>
<tr>
<td>- VO</td>
<td>- PPS</td>
</tr>
<tr>
<td><strong>Estrous cycle evaluations</strong></td>
<td></td>
</tr>
<tr>
<td>- Estrus stage, age at first estrus, cycle length, percent cycling</td>
<td></td>
</tr>
</tbody>
</table>


**Pubertal Assay Performance Criteria**

**Performance Criteria**
Mean, acceptable range, and CV criteria for vehicle control group given for study to be acceptable for most endpoints:
- Males given for SD and Wistar rats, Females SD only
- Weaning BW (males), Final BW; BW at VO/PPS
- Day of VO/PPS
- Tissue Weights
- Hormone Concentrations

**No Criteria given for:**
- Female TSH, weaning BW
- Dorsolateral prostate
- Clinical chemistries
- Histopathology endpoints
Data Interpretation for Pubertal Assays

**Potential for test substance to interact with endocrine system**
Dose levels examined for MTD (limit dose 1000 mg/kg/day)
- Body weight loss does not exceed ~10%, BW gain decrease
- Adverse clinical observations and histopathology of kidney or other target organs
- Blood clinical chemistry values

Negative results
- Was the high dose tested at or near the MTD?
- Evaluate performance criteria

Positive results
- Evaluate body weight loss
- Evaluate performance criteria

Emphasis on the complementary and redundant effects across Tier 1 Assays
# Female Pubertal Assay Potential MOA

<table>
<thead>
<tr>
<th>Estrogen Agonist</th>
<th>Inhibition of Steroidogenesis</th>
<th>Disruption of Hypothalamic-pituitary axis</th>
<th>Thyrotoxicants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early VO, pseudoprecocious puberty</td>
<td>Delayed VO</td>
<td>Alterations in VO</td>
<td>↓ T₄</td>
</tr>
<tr>
<td>↓ BW at VO</td>
<td>Delayed first estrus</td>
<td>Alterations in cyclicity</td>
<td>↑ TSH</td>
</tr>
<tr>
<td>Early first estrus</td>
<td>Persistent diestrus</td>
<td>Altered ovarian uterine or pituitary weights</td>
<td>↑ Follicular cell height ↓ Colloid area</td>
</tr>
<tr>
<td>Altered organ histology</td>
<td>↓ uterine weight</td>
<td>Altered organ histology</td>
<td>↑ Thyroid weight</td>
</tr>
<tr>
<td>Possible persistent estrus</td>
<td>Altered organ histology</td>
<td></td>
<td>↑ Liver weight (for compounds which induce hepatic clearance of thyroxine) or no effect</td>
</tr>
<tr>
<td>↓ ovarian weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ uterine weight</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
# Male Pubertal Assay Potential MOA

<table>
<thead>
<tr>
<th>Androgen Agonist</th>
<th>Steroidogenesis Inhibitor or HPG Suppression</th>
<th>Thyrotoxicants</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ Age of Puberty</td>
<td>↑ Age of Puberty</td>
<td>↓ T₄</td>
</tr>
<tr>
<td>↓ Ventral prostate, seminal vesicles, LABC, epididymis weight</td>
<td>↓ Ventral prostate, seminal vesicles, LABC, epididymis weight</td>
<td>↑ TSH</td>
</tr>
<tr>
<td>↑ Testosterone</td>
<td>↓ Testosterone or no effect</td>
<td>↑ Thyroid weight</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Follicular cell height ↓ Colloid area</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Liver weight (for compounds which induce hepatic clearance of thyroxine) or no effect</td>
</tr>
</tbody>
</table>
Pubertal Assay Challenges/Lessons Learned

**Dose Selection to meet MTD**
- Solubility/Stability
- Range Finding Studies necessary; dose age matched animals for 7-14 days; assess body weight changes/clinical observations, liver, kidney weights, clinical chemistry

**Logistical Considerations**
- Animal Availability
- Diet, Water, Bedding
- Pup DOB
- Utilization of all pups (run male/female concurrently)
- Randomization/Allocation (1 pup/sex/group)
- Experienced staff for in-life endpoints - VO/PPS
- Separate necropsy holding room
Pubertal Assay Challenges/Lessons Learned

Necropsy
• Euthanasia route
• Limit number of prosectors to meet performance criteria
• Necropsy over 2 days

Hormone Levels
• Variability in hormone levels due to stress, method of euthanasia
• Interpretation of thyroid hormones ($T_4$ and/or TSH) without corresponding changes in follicular cell height and colloid area
• Hepatic enzyme induction
• Retain liver
Pubertal Assay Challenges/Lessons Learned

**Estrous Cycle Evaluations**
- Only 1-2 cycles by time of necropsy
- Cycles are often irregular
- Inherent differences in uterine/ovarian weights due to different stages of the cycle

**Statistics**
- ANOVA, ANCOVA, and trend analysis
- Covariate for date of weaning (21) or day of first dose administration (22/23)
- Evaluation of thyroid and ovarian histopathology
Pubertal Assay Challenges/Lessons Learned

**Histopathology- Female Pubertal**
Five sections of left ovary, two sections of uterine horns

**Histopathology- Male Pubertal**
Left testis and left epididymis

**Pathologist recommendations (from Regional STP meeting)**
- Standardize terminology and ovarian sectioning
- Follicular counts not necessary for screening
- Rely on necropsy vaginal smears for estrous staging
- Standardize fixative for testis/epididymis- Modified Davidson’s fluid rather than Bouin’s fixative (Latendresse et al., 2002)
- Save testis and epididymis not used for histology
Pubertal Assay Challenges/Lessons Learned

Histopathology- Thyroid Gland
- Two sections of thyroid gland
- Subjectively assessed for follicular cell height and colloid area using a five point grading scale (1=shortest follicular cell height/least colloid area; 5=tallest follicular cell height/largest colloid area)

Challenges
Hepatic Enzyme Inducers
- Increases in liver weights, T₄/TSH changes
- Retain liver

Histopathology- L. Kidney
- Systemic toxicity
Considerations for Future Testing

Overcoming challenges presented took **time** and **resources**

- Understanding available data on test substance
- Choosing an appropriate MTD (range finding studies)
- Logistical considerations

Some additional clarifications/considerations for future test orders

- Statistical analyses
- Randomization/Allocation
- Usefulness of Clinical Chemistry
- EPA deadlines and scheduling
Strengths of Tier 1 *In Vivo* Mammalian Assays

- *In vivo* assays incorporate ADME

- These assays utilize models that evaluate the developing endocrine system

- Evaluate more than 1 MOA

- Complementary endpoints within the assay (i.e. PPS, tissue weights, hormones, histopathology)
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