Investigative and Predictive Approaches to Drug-Induced Steroid Hormone Perturbation

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Comparative Biology and Safety Sciences
Understanding Mechanisms Helps Provide a Path Forward

**Screening:**
- Require higher throughput, high sensitivity assays
- There may be false positives

**Mechanistic Assessment:**
- High specificity assays to distinguish between types of events
- The assay needs to be a different assay
- These tend to be lower throughput

**Risk Assessment:**
- After a hazard is identified and diagnosed, the risk needs to be assessed by determining a safe exposure
  - In animals for Margin of safety determination
  - In the clinical to determine safe exposure and monitor safety

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Decision
Outline of Presentation

• Program endocrine findings
• In vitro mechanistic studies
• In vivo metabolomics study
• Search for a biomarker
• Conclusions
28-Day Sprague-Dawley Rat Study with Cmpd 1
Endocrine Findings in Males

- Prostate and seminal vesicle atrophy with ↓ weights
- Prominent pituitary gonadotrophs
- Adrenal cortical hypertrophy with ↑ weight
- Thyroid follicular hypertrophy ↑ weight
  - Secondary to hepatic enzyme induction

<table>
<thead>
<tr>
<th>Incidence of Microscopic Findings and Estimated Margin</th>
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</thead>
<tbody>
<tr>
<td>Dose</td>
</tr>
<tr>
<td>Pituitary</td>
</tr>
<tr>
<td>Prostate atrophy</td>
</tr>
<tr>
<td>Seminal vesicle atrophy</td>
</tr>
<tr>
<td>Estimated Margin</td>
</tr>
</tbody>
</table>
Cmpd 1 Inhibits Only hCG-Induced Testosterone Production

- **Method**
  - An enriched primary rat Leydig cells were isolated from rat testes by differential centrifugation.
  - Cells were isolated, plated, and incubation with hormones/test articles for 20 hours.
  - Media was assayed for testosterone with an ELISA and cellular ATP measured to assess viability.

- **Cmpd 1 only inhibits the stimulated portion of testosterone production**
  - Doesn’t look like a biosynthetic inhibitor
  - Site of action appears to involve signaling
LH Induced Testosterone Production Signaling Pathway in Leydig Cells

What aspect of signaling is affected?
Understanding The Mode of Action Will Help Identify The Best Screening System

- Cmpd 1 inhibits hCG and forskolin stimulated testosterone production
- Cmpd 1 does not inhibit hCG stimulated cAMP production
- Cmpd 1 does not inhibit forskolin induced StAR or CYP11a1 mRNA
  - CYP17 limited or no induction
- Cmpd 1 does not inhibit basal testosterone production
  - not a biosynthetic pathway inhibitor
LH Induced Testosterone Production Signaling Pathway in Leydig Cells

LH (or hCG) → LHR → adenylyl cyclase → cAMP → SF-1 → transcription → ↑ Steroid Synthesis mRNA → ↑ Steroid Synthesis Protein → ↑ Steroid Synthetic activity

Forskolin + → ERK1 → prolonged stimulation

PKA → cholesterol → acute regulation → StAR

Is the PKA-StAR pathway effected by Cmpd 1?
- 22-OH cholesterol can pass into mitochondria and enter steroid pathway independently of StAR
Effect of Cmpd 1 on 22-OH Cholesterol Stimulated Testosterone Synthesis In Vitro

- No inhibition of 22-OH cholesterol stimulated testosterone by Cmpd 1
- Strong inhibition of basal, 22-OH cholesterol stimulated, and hCG stimulated testosterone by prochloraz
- Data suggests Cmpd 1 is not a biosynthetic inhibitor, but acts at cholesterol transport
In Vitro Chemical Survey
Effects on hCG-Stimulated Testosterone Production in Rat Leydig Cells

- Subtle chemical changes can affect in vitro testosterone production markedly
- Still room for good molecules in this series
  - How do you sift through them?

Only difference is change from F to Me
## Study Design

<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>Number of Animals</th>
<th>Treatment</th>
<th>Dose Level (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 2</td>
<td>Day 5</td>
<td>Day 14</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
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</tr>
</tbody>
</table>

<sup>a</sup> Vehicle 1 (Cmpd1): 20% Captisol, 1% HPMC, 1% Pluronic F68 in RO water, pH 2.1

<sup>b</sup> Vehicle 2 (prochloraz): corn oil

### Terminal Procedures
- Scheduled Necropsy – Approximately 6 hours post-dose on days 2, 5, 14, and 28
- Blood collection in EDTA tubes and plasma collected
- Organ weights, tissue collection for histopathology and freezing

### Analytical Procedures
- Hormone analysis using a multiplex luminex-based analysis platform
- Mass Spectrometry based Metabolomics
  - Broad based biochemical profiling
  - High sensitivity quantitative hormone profiling
- Testicular StAR protein semi-quantification via Western blot
## Metabolomics Study Results

### Histologic Observations

<table>
<thead>
<tr>
<th>Organ/ change</th>
<th>Day</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>No effect</td>
</tr>
<tr>
<td>Prostate/ Decreased secretion</td>
<td></td>
</tr>
<tr>
<td>Pituitary/ Increased basophilic pituicytes</td>
<td>No effect</td>
</tr>
<tr>
<td>Adrenal/ Vacuolar hypertrophy</td>
<td>✓</td>
</tr>
</tbody>
</table>

• 1 mpk dose of Cmpd 1 was only tested through 14 days
• Prochloraz affected prostate as early as 5 days treatment
Metabolomics Study
Plasma Testosterone

Day 2

Day 5

Day 14

Day 28

Note variability in vehicle testosterone levels

- At 40 mg/kg/day (C), Cmpd 1 appears to “tighten up” the testosterone levels
  - Decreased influence of stress?
- Cmpd 1 did not affect LH hormone levels
- Cmpd 1 did not affect ACTH levels
Metabolomics Study  
Supervised Multivariate Overview of Treatment Effect

Cmpd 1

- Weak metabolic effects of Cmpd 1

Prochloraz

- Strong metabolic effects of prochloraz

Score plots of OPLS on log ratios normalized to vehicle control
Metabolomics Study
Supervised Multivariate Overview of Treatment Effect
Hormone Method Analytes Only

OPLS loadings SUS plot of log ratios normalized to vehicle control

Cmpd 1
- Decreases below horizontal line
- Increases above horizontal line

Prochloraz
- Decreases left of vertical line
- Increases right of vertical line
Metabolomics Study
Supervised Multivariate Overview of Treatment Effect of Steroids Only

Cmpd 1
- All gonadal steroids are decreased by Cmpd 1

Prochloraz
Decreased
- Testosterone
- Androstenedione

Increased
- Progesterone
- 21-hydroxyprogesterone
  - 11-Deoxycorticosterone

Consistent with CYP17 inhibition

OPLS loadings SUS plot of log ratios normalized to vehicle control
Metabolomics Study
Summary

• Few biochemical changes observed in metabolomics analysis for Cmpd 1
  • strong effects observed with prochloraz
• Prochloraz decreased male gonadal steroids distal to CYP17 and increased gonadal steroids proximal to CYP17 consistent with its known mechanisms of testosterone synthesis inhibition (CYP17)
• Cmpd 1 decreased all gonadal steroids consistent with in vitro testosterone synthesis results
  • cholesterol transport
Biomarker Assessment
StAR expression

• 22-OH cholesterol expt implicates role of transport in defect
  • Herbicide “Round-Up” affects steroid synthesis by decreasing StAR quantity

• What does Cmpd 1 do to StAR levels?
  • Can StAR quantity be a biomarker useful in a 4-day study?
    • Eliminate need for 28 day studies to detect changes?
Biomarker Assessment
StAR Protein Expression

- Rat were treated with Cmpd 1 at 1, 10, and 40 mpk for 4 days
- At necropsy, testes were harvested and frozen at -70°C
- A portion of the frozen testes was homogenized and the StAR protein semi-quantified by Western blot
- StAR content was increased compared to vehicle treated animals at day 4
- This suggests that cholesterol transport inhibition may lead to increased StAR protein levels through a feedback loop

StAR content may be a useful biomarker in 4 day studies to assess the testosterone disruption effects that manifest in prostate weight changes at 28 days
Mechanistic Summary

• Cmpd 1 can inhibit testosterone production
  • Only hormone stimulated testosterone production inhibited
    • Does not affect intramitochondrial biosynthetic targets
      • Affects all steroid intermediates equally
    • Is not a transcriptional mechanism
    • Is not a cAMP production inhibitor mechanism
    • Is not a hormone receptor inhibitor mechanism
    • Is not a LH hormone deficiency mechanism
    • Is not a androgen nuclear receptor antagonist (data not shown)
    • Does not inhibit PKA (data not shown)
  • Defect involves cholesterol transport
    • Does not decrease StAR (cholesterol transporter) quantity
      • Increase observed
What can we conclude?

- Program compounds affected steroid biosynthesis
  - Cmpd 1 decreased all gonadal steroids in vivo, consistent with in vitro evidence of testosterone synthesis inhibition at cholesterol transport
  - Metabolomics quantitative hormone profiling can distinguish types of testosterone synthesis inhibitors in the in vivo setting
- Value of in vitro Leydig cell assay:
  - Good positive predictive value (3/3)
  - Negative predictive value less reliable (1/2 for molecules showing no effect up to 300 μM)
    - Edge of sensitivity for distinguishing compounds like Cmpd 2 (100-300 μM)
  - Cytotoxicity limitations
- Knowing the mechanism can help identify potential predictive biomarkers
  - Cmpd 1 treatment increased StAR protein levels at early time points (Day 4) compared to organ weight or histopathological changes (Day 28)
  - This can be a useful biomarker to assess testosterone synthesis disruption for this mechanism in screening studies
Acknowledgements

• Michelle Horner
• Jon Werner
• Chris DiPalma
• Rocio Hernandez
• Patrick Cosgrove
• Rong Hu
• Paul Acton
• Ihsan Senal

• Victor Cee
• Ra’Shun Conner
• Yang Xu
• Han Xu
• Min Wong