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Investigative and Predictive Approaches to Drug-Induced Steroid Hormone Perturbation

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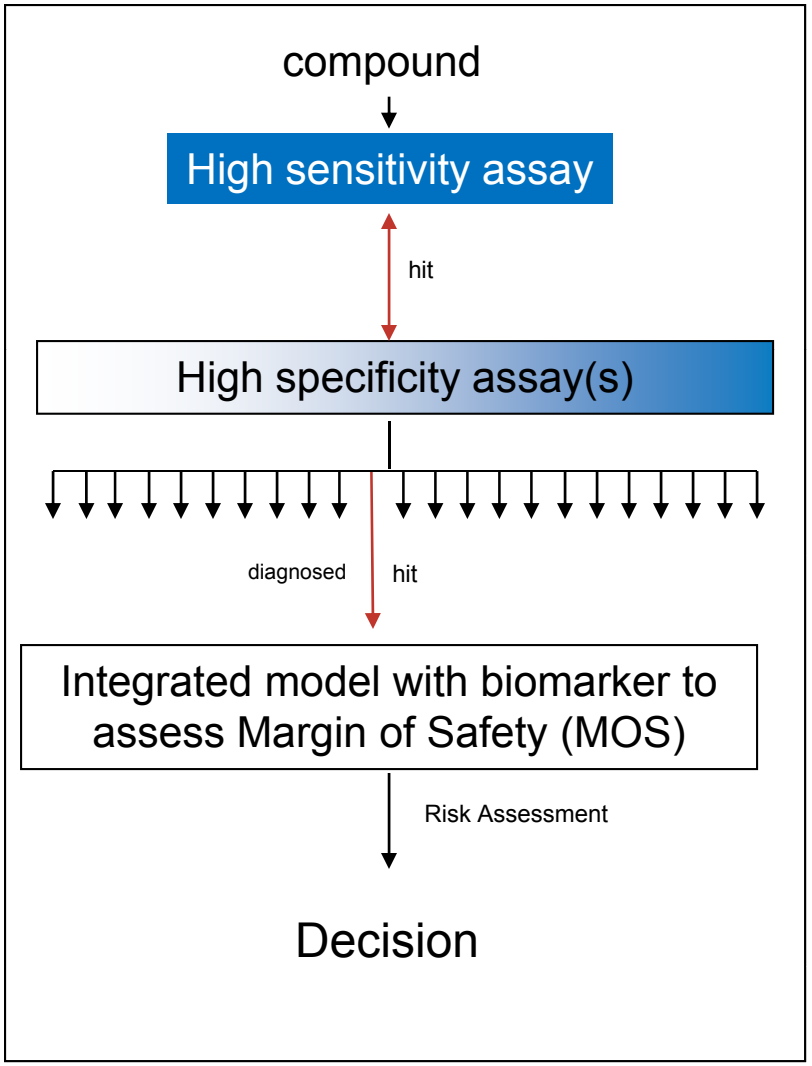
Understanding Mechanisms Helps Provide a Path Forward

- 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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Outline of Presentation

- Program endocrine findings
- In vitro mechanistic studies
- In vivo metabolomics study
- Search for a biomarker
- Conclusions

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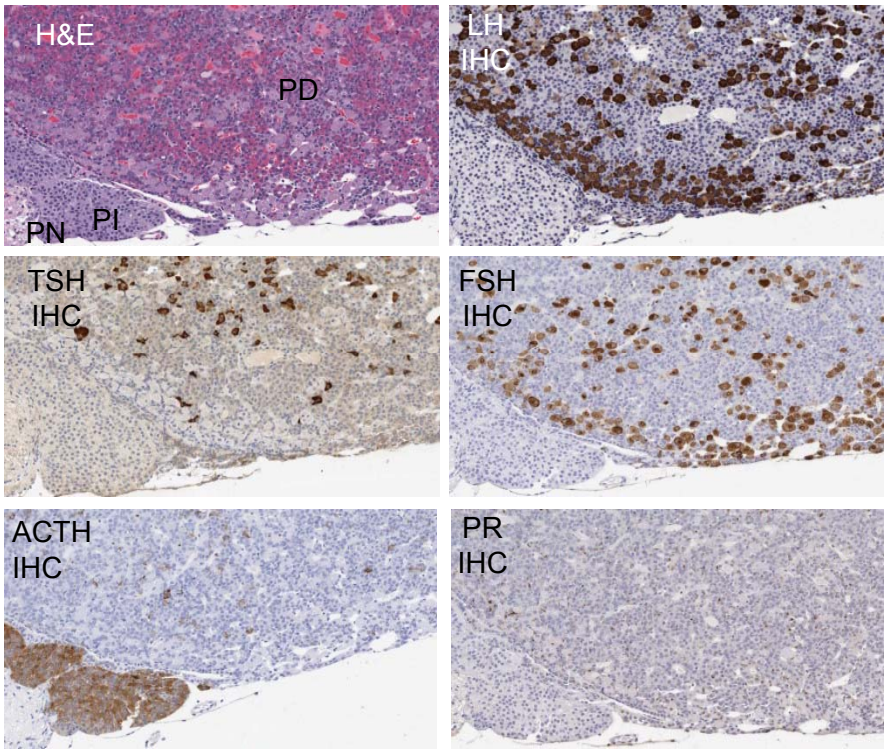
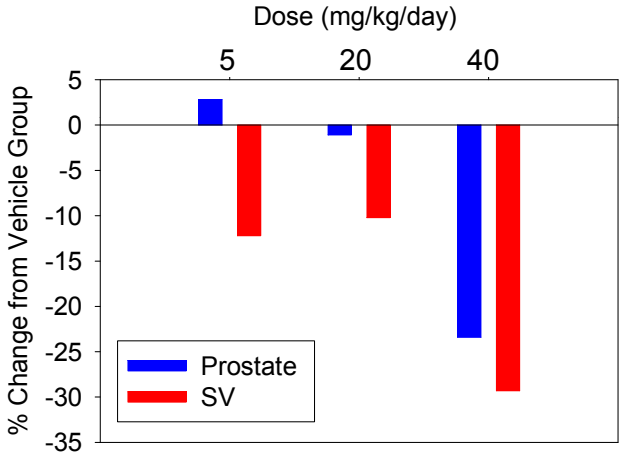
28-Day Sprague-Dawley Rat Study with Cmpd 1

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





Incidence of Microscopic Findings and Estimated Margin			
Dose	5	20	40
Pituitary	1/10	9/10	5/10
Prostate atrophy	0/10	2/10	4/10
Seminal vesicle atrophy	1/10	2/10	4/10
Estimated Margin	4	16	25

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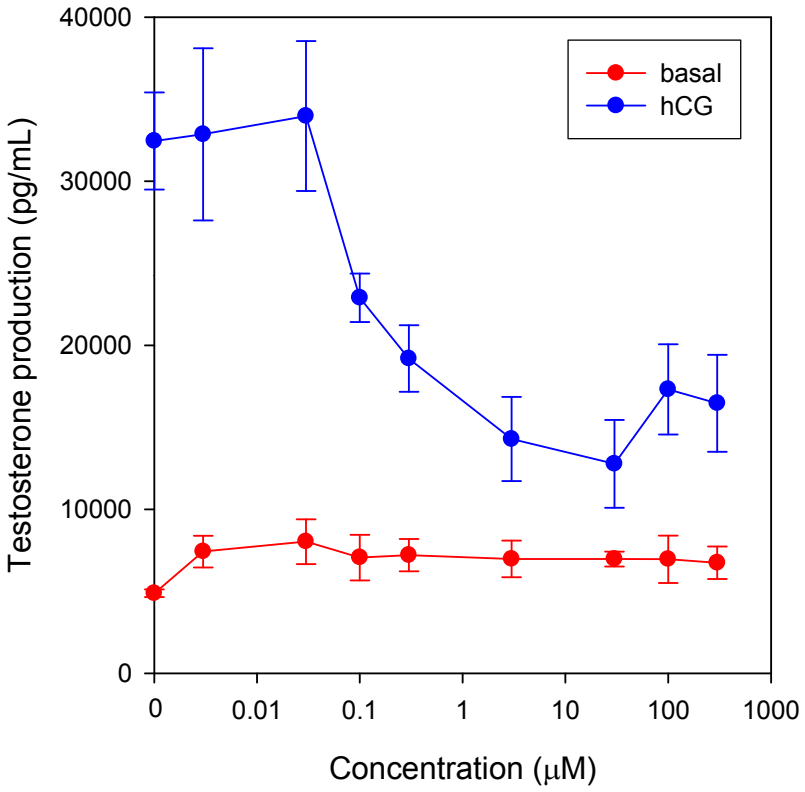
Cmpd 1 Inhibits Only hCG-Induced Testosterone Production



- 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

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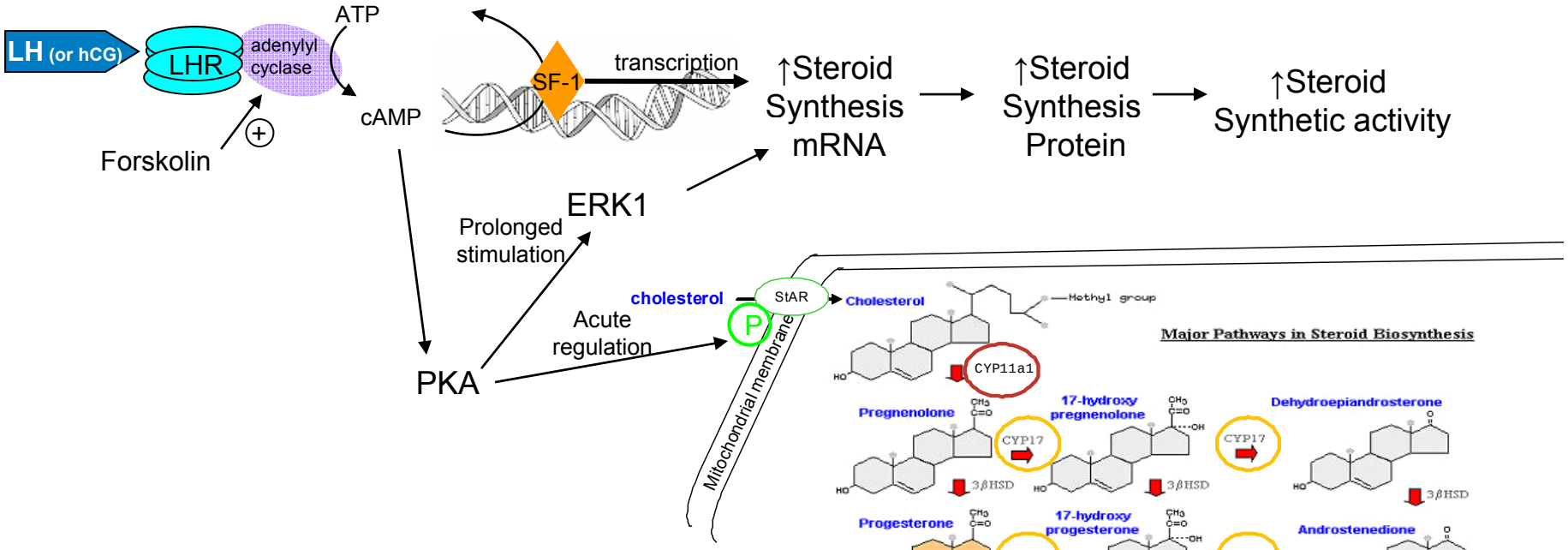
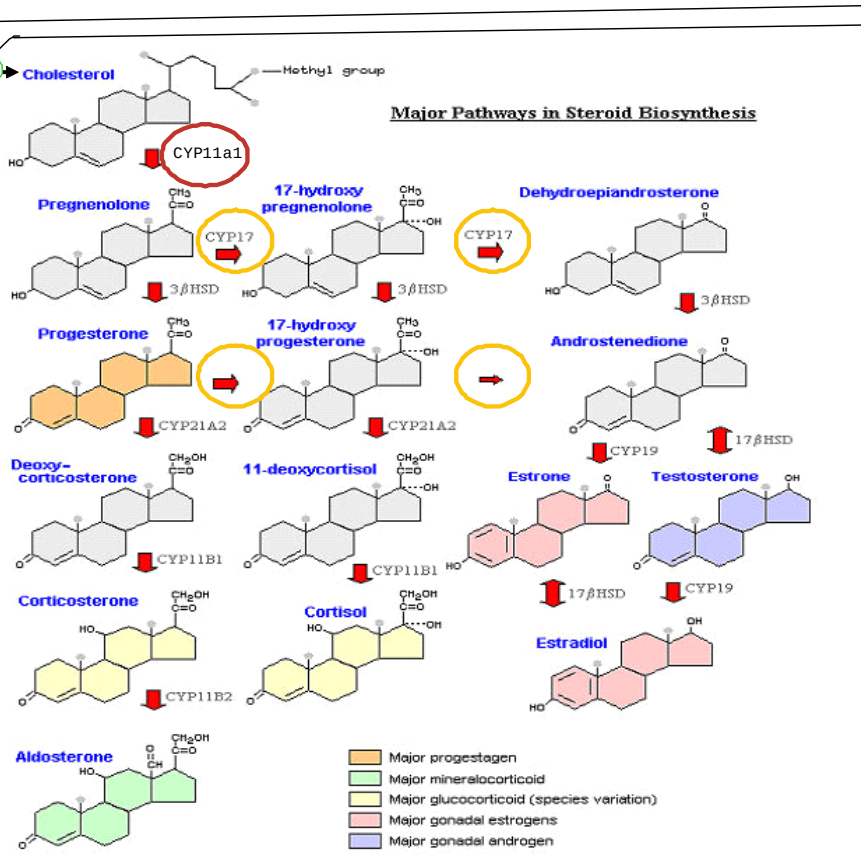
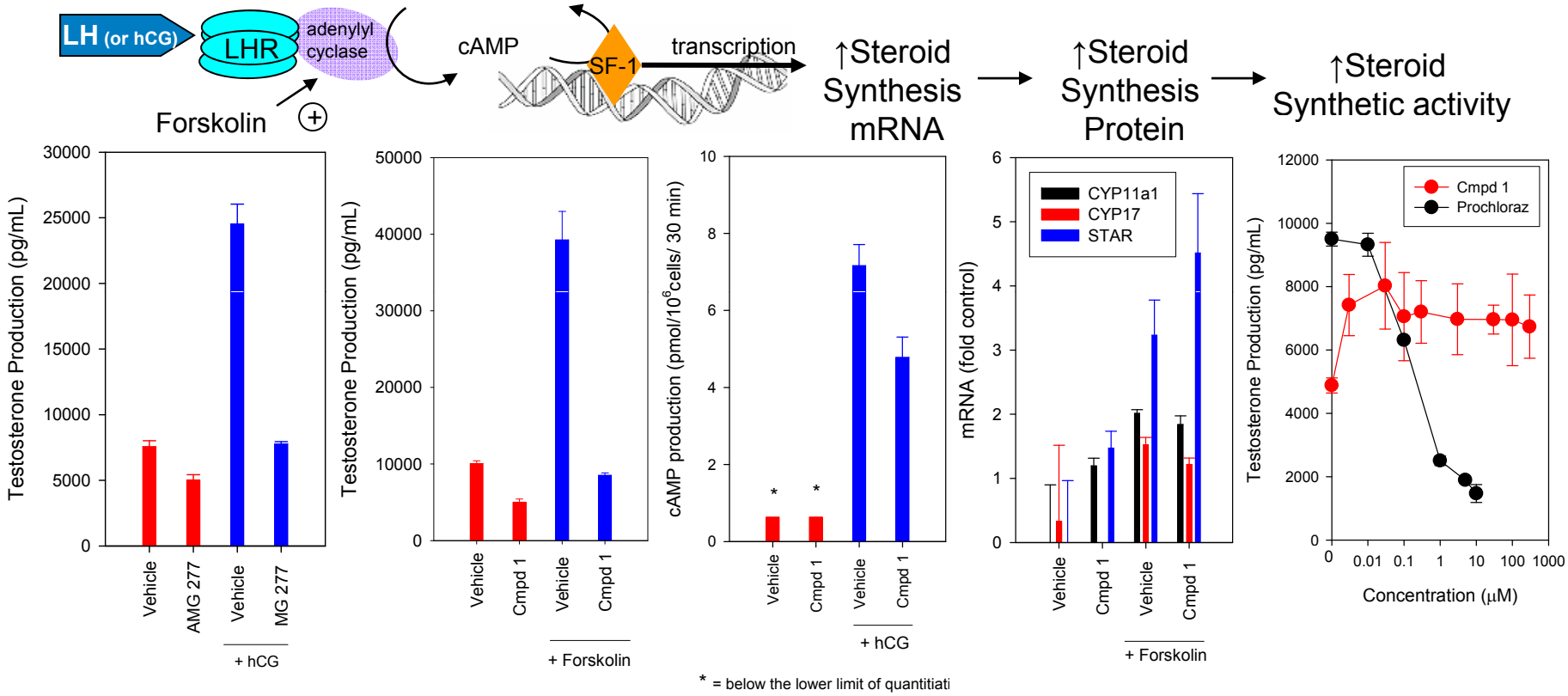
LH Induced Testosterone Production Signaling Pathway in Leydig Cells





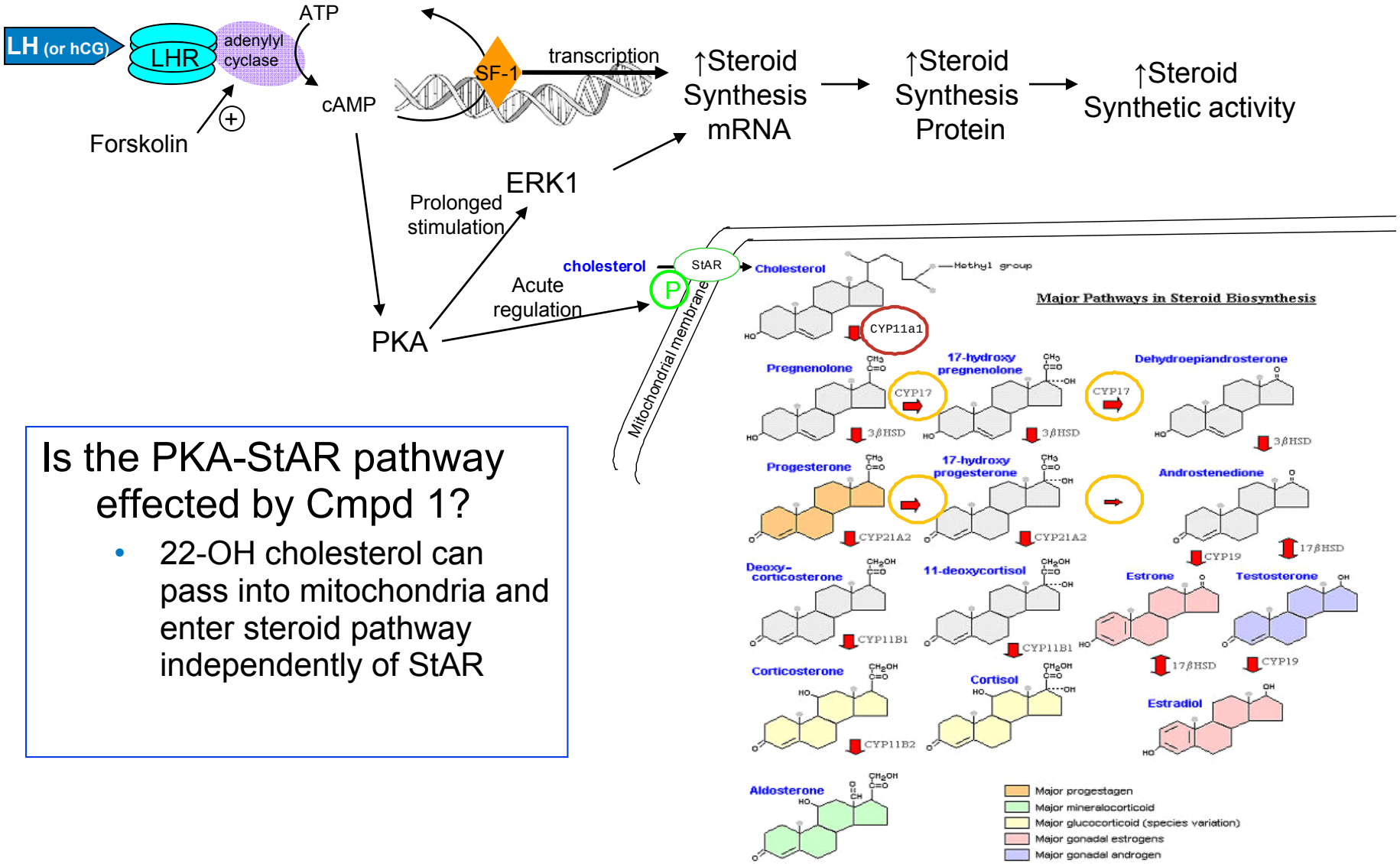
What aspect of signaling is affected?

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

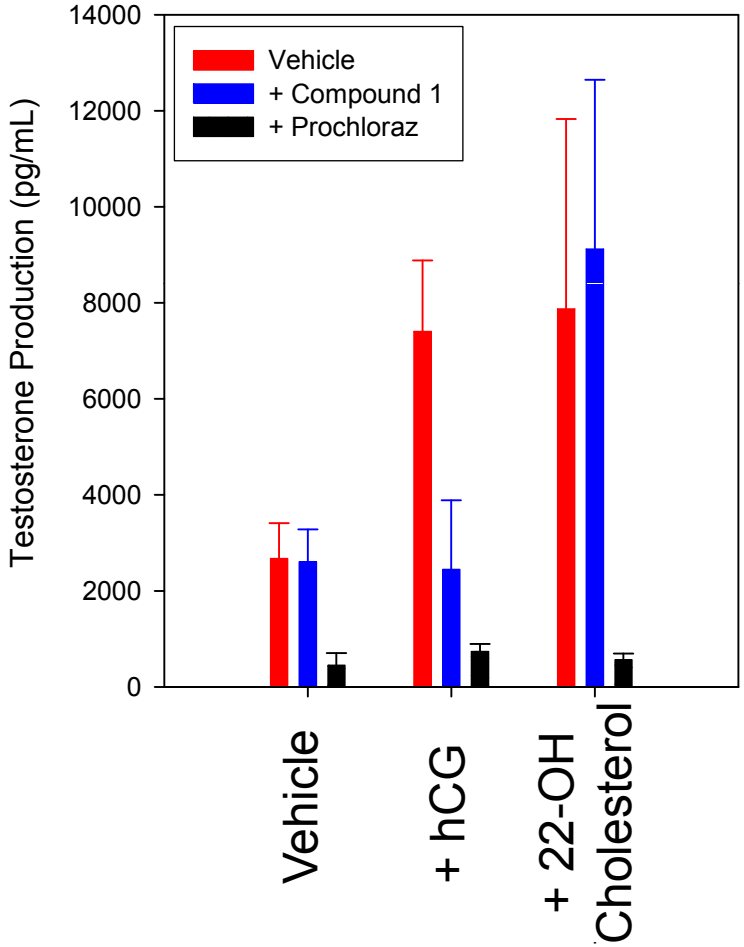
LH Induced Testosterone Production Signaling Pathway in Leydig Cells



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

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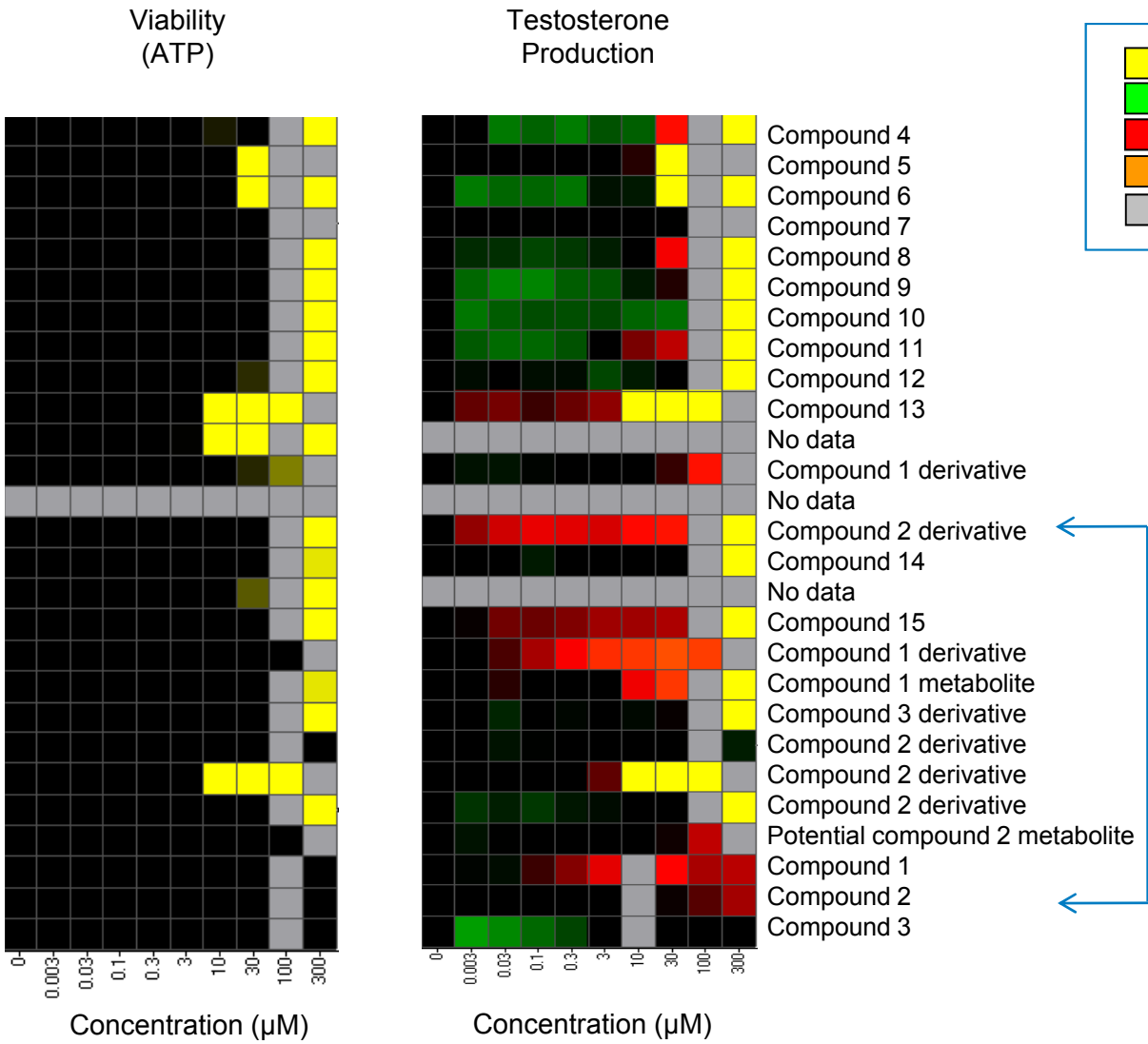
In Vitro Chemical Survey

Effects on hCG-Stimulated Testosterone Production in Rat Leydig Cells



- Cytotoxicity
- Increased T production
- Decreased T production to basal levels
- Decreased T production below basal levels
- No data

- 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

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Metabolomics Study

Demonstrating In Vivo Relevance

Study Design

Group	Number of Animals				Treatment	Dose Level (mg/kg)
	Day 2	Day 5	Day 14	Day 28		
	Subgroup					
	1	2	3	4		
1	6	6	6	6	Vehicle 1[a]	0
2	6	6	6	--	Vehicle 2[b]	0
3	6	6	6	--	Cmpd1	1
4	6	6	6	6	Cmpd1	10
5	6	6	6	6	Cmpd1	40
6	6	6	6	--	Prochloraz	75

[a] Vehicle 1 (Cmpd1): 20% Captisol, 1% HPMC, 1% Pluronic F68 in RO water, pH 2.1

[b] 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 quantititative hormone profiling
  - Testicular StAR protein semi-quantification via Western blot

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Metabolomics Study Results

Histologic Observations

Organ/ change	Day			
	2	5	14	28
Prostate/ Decreased secretion	**No effect**	**No effect**	**No effect**	√ **(40)**
Pituitary/ Increased basophilic pituicytes	**No effect**	**No effect**	**No effect**	√ **(10, 40)**
Adrenal/ Vacuolar hypertrophy	√ **(40)**	√ **(10, 40)**	√ **(1, 10, 40)**	√ **(10, 40)**

- 1 mpk dose of Cmpd 1 was only tested through 14 days
- Prochloraz affected prostate as early as 5 days treatment

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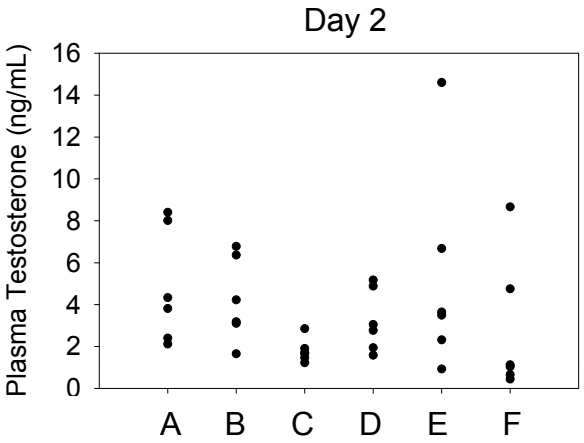
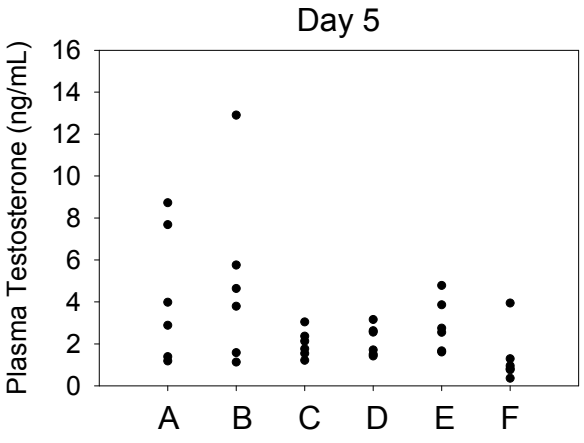
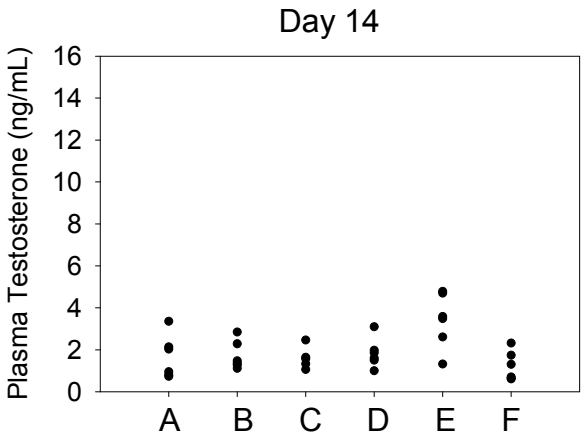
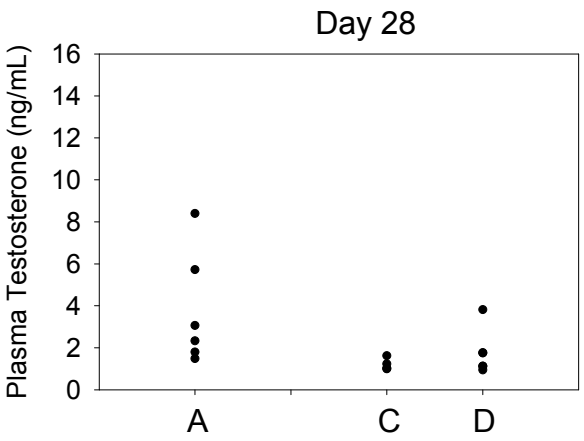
Metabolomics Study
Plasma Testosterone





A- vehicle
B- corn oil
C- 40 mpk Cmpd 1
D- 10 mpk Cmpd 1
E- 1 mpk Cmpd 1
F- 75 mpk Prochloraz





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

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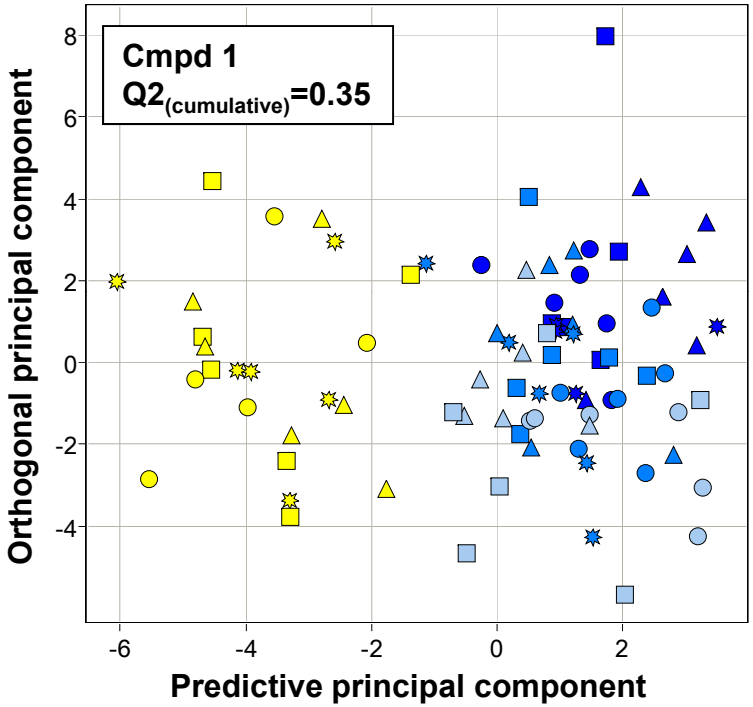
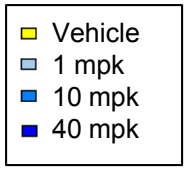
Metabolomics Study

Supervized Multivariate Overview of Treatment Effect

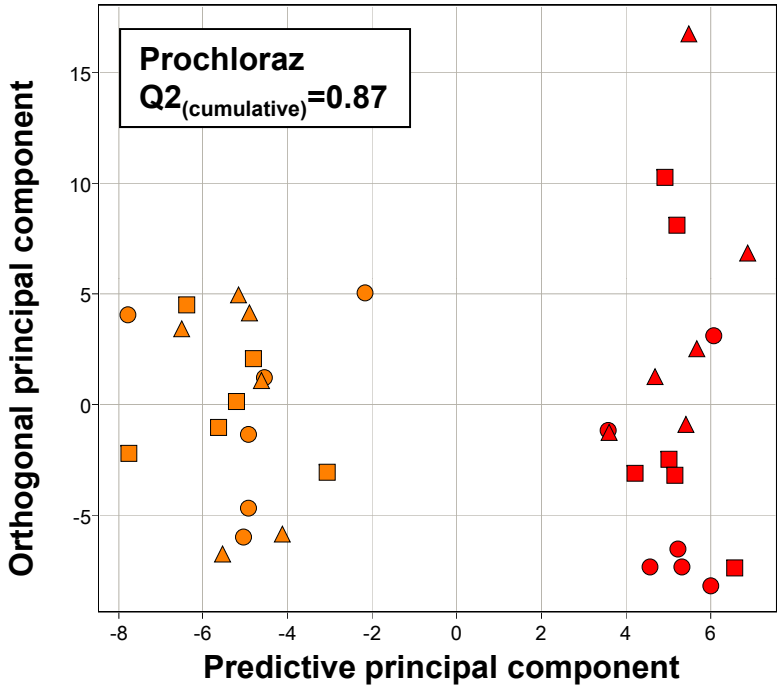
Cmpd 1



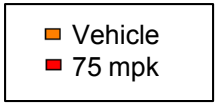
- Weak metabolic effects of Cmpd 1



Prochloraz



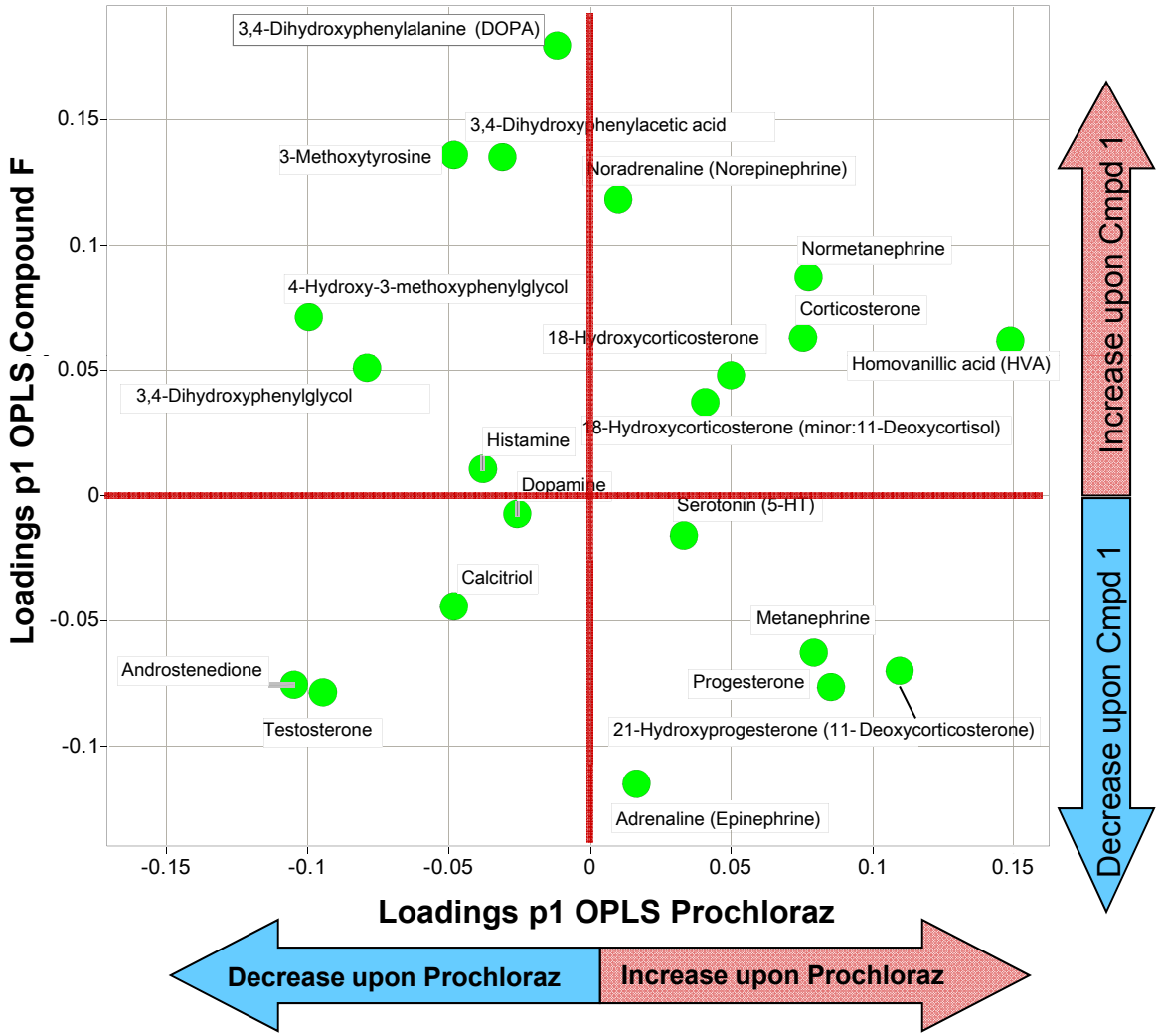
- Strong metabolic effects of prochloraz



Score plots of OPLS on log ratios normalized to vehicle control

Metabolomics Study

Supervized Multivariate Overview of Treatment Effect
Hormone Method Analytes Only



Cmpd 1

- Decreases below horizontal line
- Increases above horizontal line

Prochloraz

- Decreases left of vertical line
- Increases right of vertical line

OPLS loadings SUS plot of log ratios
normalized to vehicle control

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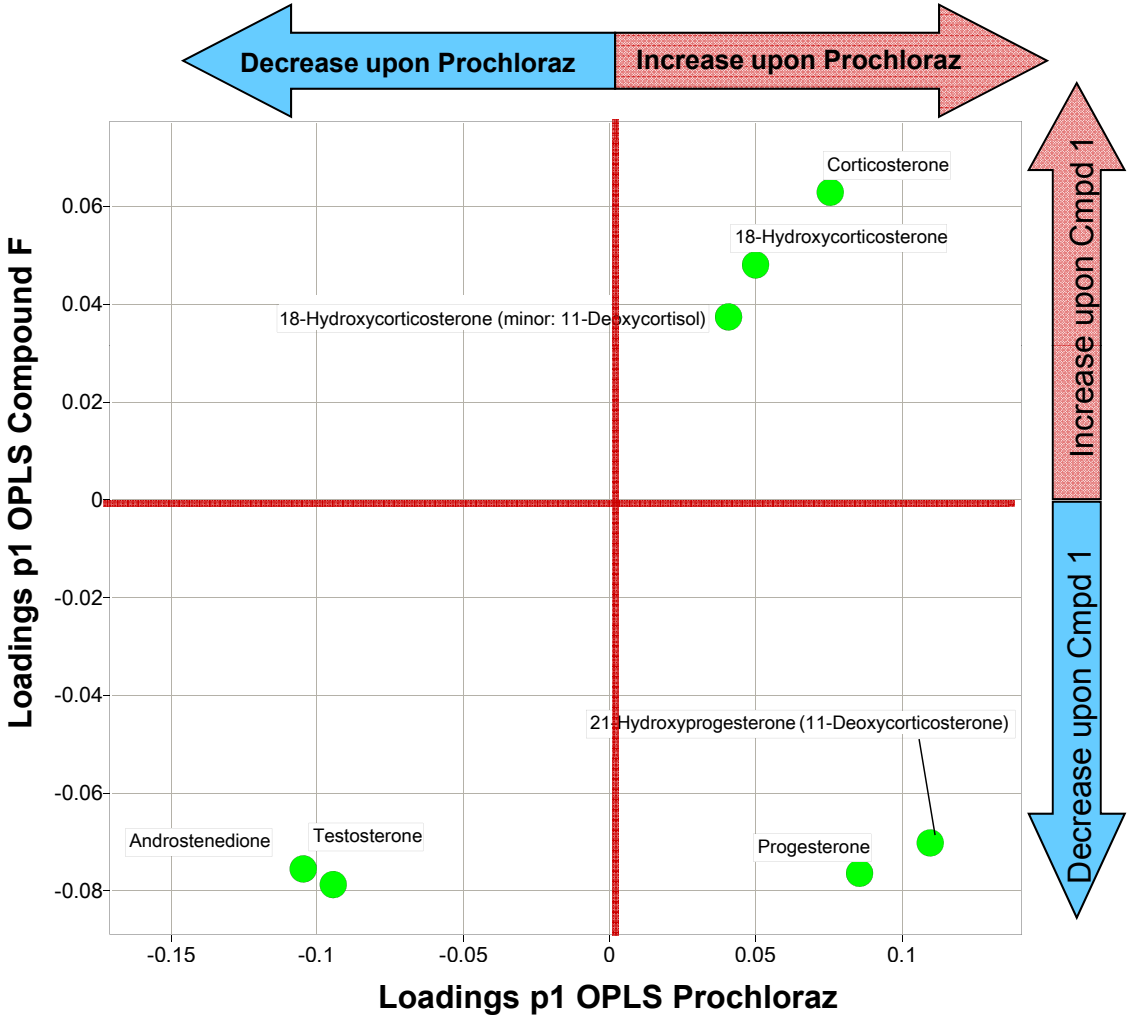
Metabolomics Study

Supervized 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-Deoxycorticosterstone

Consistent with CYP17 inhibition

OPLS loadings SUS plot of log ratios normalized to vehicle control

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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 know mechanisms of testosterone synthesis inhibition (CYP17)
- Cmpd 1 decreased all gonadal steroids consistent with in vitro testosterone synthesis results
  - cholesterol transport

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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?

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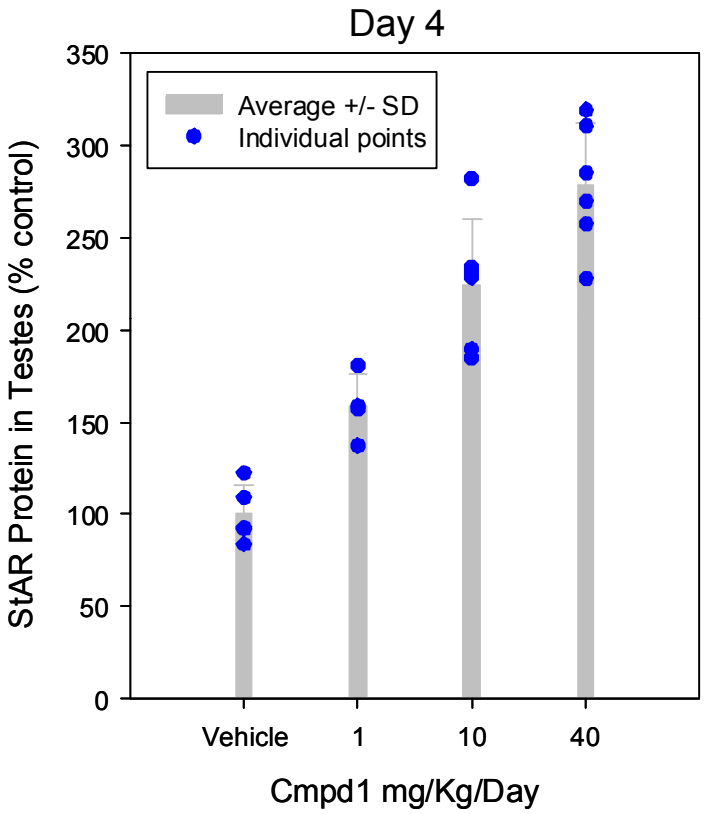
Biomarker Assessment
StAR Protein Expression



- 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

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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

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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

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