DRAFT FINAL REPORT

WA 3-10, Task 8

Sliced Testis Assay Prevalidation: Multichemical Study

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FINAL REPORT

Sliced Testis Assay Prevalidation: Multichemical Study

ABSTRACT

This study was designed to evaluate the sliced testis assay for the ability to screen chemicals with known mechanisms of action for anti-steroidogenic activity and/or for cytotoxicity. The 9 chemicals tested were aminoglutethimide (AG; positive control), 2,4-dinitrophenol (2,4-DNP; cytotoxicity control), flutamide, ketoconazole, vinclozolin, atrazine, dimethoate, finasteride, spironolactone, verapamil, and prochloraz. These chemicals, evaluated for anti-androgenic and/or cytotoxic effects in the *in vitro* sliced testis assay, produced the following results:

Finasteride, a 5α-reductase inhibitor, prevents 5α-reductase from converting testosterone to dihydrotestosterone (DHT) in the testis and locally. DHT plays a major role in the perinatal determination of external male genitalia and structures of the lower male reproductive tract. In this assay, finasteride caused concentration-related decreases in testosterone levels; an opposite response of what was expected. Lactate dehydrogenase (LDH) levels were increased at 1000 μM finasteride, with no effects at 10 or 100 μM.

Flutamide acts to inhibit the P450c17 enzyme, which converts 17α-hydroxypregnenolone to dehydroepiandrosterone (the precursor of androstenedione). Flutamide also produced significant decreases in testosterone concentrations at higher concentrations but was stimulatory at the lowest concentration of 10 μM. A concentration-related increase in LDH concentration was observed with flutamide, which may indicate it is cytotoxic to testicular cells.

Spironolactone acts by 17-α hydroxylase inhibition. Testosterone levels were decreased in a dose-related manner. LDH values increased in a concentration-related manner, although the values were within the control range. This may imply a possible cytotoxic action on the testicular cells.

Vinclozolin is a known antiandrogen and acts by binding to the androgen receptor (AR). The results showed dose-related decreases in the testosterone concentrations at 10 and 100 μM, with variable effects at 1 μM. LDH levels were unaffected in the presence of vinclozolin.

Ketoconazole is a P450scc inhibitor. This enzyme converts cholesterol to pregnenolone at the beginning of the steroidogenesis pathway, so that testosterone levels should be reduced with increasing concentrations of ketoconazole. In fact, it decreased testosterone in a concentration-related manner, with the highest concentration giving the greatest decrease. LDH levels were significantly higher than the control values.

Verapamil is a calcium channel blocker and, as such, was not expected to significantly affect testosterone concentration. There was no measurable effect on testosterone concentrations at lower concentrations of verapamil, as would be expected. However, there was a 32% decrease in testosterone at the highest concentration of verapamil which was not significantly different from the control. LDH levels were unaffected.

Atrazine inhibits the steroidogenesis pathway prior to cholesterol synthesis and also acts at the level of the hypothalamus-pituitary to decrease or abolish the luteinizing hormone (LH) surge at ovulation in female rats. Atrazine decreased testosterone concentration at all 3 of its concentrations, but there was no concentration-dependent relationship (i.e., inhibition was greatest at the lowest concentration); it is considered negative in this assay. Atrazine exposure resulted in slightly higher LDH concentrations than in the controls.

Dimethoate is a StAR (steroid acute regulatory) protein inhibitor. Decreased testosterone concentration was observed at all 3 dimethoate concentrations in a clear, dose-response pattern and there were no effects on LDH levels in Replicate 1. The results for Replicate 2 were not as clear.

Prochloraz (an aromatase inhibitor) produced concentration-related decreases in testosterone levels, and caused a significant linear component of trend, and, at the high dose significantly increased LDH levels.

Therefore, the assay identified all anti-androgenic chemicals regardless of sites or mechanisms of action. For these chemicals, effects on testosterone levels are clearly different in mechanism and potency from induction of increased LDH, which was used as a biomarker of cytotoxicity. Atrazine was interpreted as negative in this assay, which is consistent with its role in the central control of LH surges at ovulation in female rodents. The two positive control chemicals, AG for steroidogenesis and 2,4-DNP for cytotoxicity, were consistently and appropriately active for all test chemicals evaluated and between replicates per chemical.

1.0 INTRODUCTION

1.1 Background

In 1996, the Food Quality Protection Act (FQPA) amendments were enacted by Congress to authorize the EPA to implement an Endocrine Disruptor Screening Program (EDSP) on pesticides and other substances found in food or water sources for endocrine effects in humans (FQPA, 1996). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial substances, and pesticides. A two-tiered approach was

utilized. Tier 1 employed a combination of *in vivo* and *in vitro* screens, and Tier 2 involved *in vivo* testing methods using two-generation reproductive studies. A steroidogenesis assay was proposed as one of the Tier 1 screening battery assays.

A detailed review paper (DRP) about steroidogenesis was prepared. The DRP: (1) summarized the state of the science of the *in vivo, ex vivo,* and *in vitro* methodologies available for measuring gonadal steroidogenesis; (2) presented a review of the individual assays and representative data generated by investigators who used the assay to evaluate a substance for steroidogenic-altering activity for each methodology; (3) provided an evaluation of the various methodologies and assays as tools for screening substances with suspected steroidogenic activity; (4) recommended a particular screening method and assay as a screening tool; and (5) described the strengths, weaknesses, and implications for further research associated with the recommended screening assay.

The *in vitro* sliced testis steroidogenesis assay was selected as the most promising screening tool for identifying substances with steroidogenic-altering activity. The sliced testis assay was recommended because it can be conducted at a minimal cost, quickly, and simply with standard laboratory equipment and basic laboratory training; the preparation is stable and the parenchyma remains viable over a sufficient time period to measure changes in end-product hormone production; the assay is relatively sensitive and specific; the assay uses parenchyma that maintains the cytoarchitecture of the organ; the assay uses a reduced number of animals; the assay will be relatively easy to standardize; and the assay has a well-defined endpoint in testosterone and, if desired, can be modified to include additional intermediate hormonal endpoints. The experiment in this report supports the prevalidation phase. The objectives of the prevalidation studies are to assess the relevance of the sliced testes assay for detecting compounds that inhibit steroidogenesis. Relevance will be assessed by demonstrating that the assay can detect inhibition of steroid hormone synthesis by determining the change in the production of testosterone.

1.2 Objective

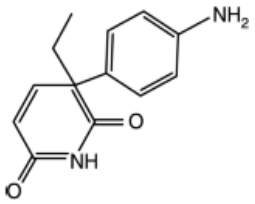
The objective was to evaluate several chemicals to show the relevance of the sliced testis assay for the screening of potential endocrine disruptors for effects on steroidogenesis *in vitro*. Each chemical was used at 3 different concentrations in the assay system.

2.0 MATERIALS AND METHODS

2.1 Chemicals

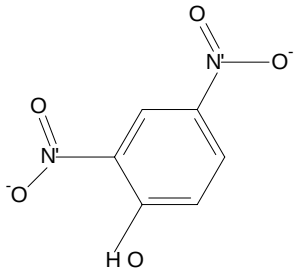
2.1.1 Test Substances

2.1.1.1 Aminoglutethimide (Positive Control)



CAS Number: 125-84-8
Synonyms: AG
Lot Number: 06016JS
Purity: >99%
Appearance: Solid
Molecular Formula: $C_{13}H_{16}N_2O_2$
Molecular Weight: 232.3
Storage, Bulk Chemical: Room temperature
Storage, Test Solution: Refrigerated (1-9°C)

2.1.1.2 2,4-Dinitrophenol (Cytotoxic Control)

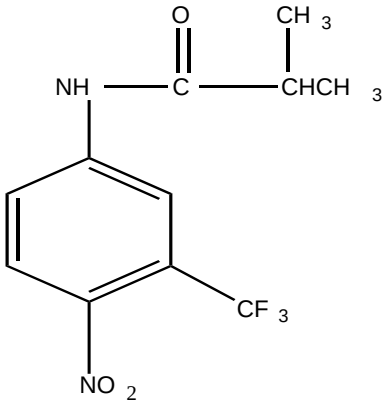


2,4-Dinitrophenol

CAS Number: 51-28-5
Synonyms: DNP
Lot Number: 03713MB
Purity: >98%
Appearance: Solid
Molecular Formula: $C_6H_4N_2O_5$
Molecular Weight: 184.108

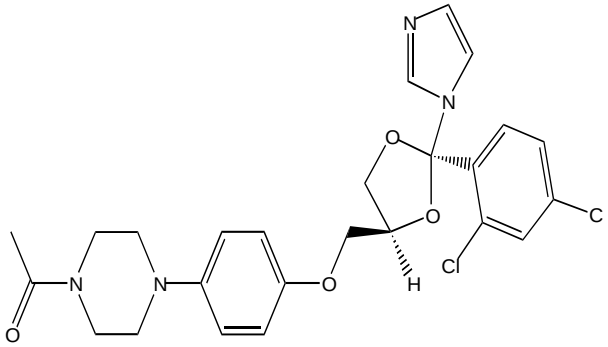
Storage, Bulk Chemical: Room temperature
Storage, Test Solution: Refrigerated (1-9°C)

2.1.1.3 Flutamide



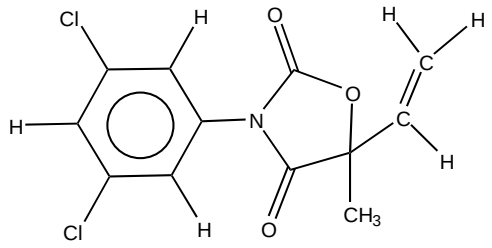
CAS Number: 13311-84-7
Synonyms: 2-Methyl-N-[4'-nitro-3'(trifluoromethyl) phenyl] propanamide
Lot Number: 121K1083
Purity: ≥98%
Appearance: Solid, off-white to yellow, crystals, crystalline powder
Molecular Formula: $C_{11}H_{11}F_3N_2O_3$
Molecular Weight: 276.2147
Storage, Test Solution: 1-8°C

2.1.1.4 Ketoconazole



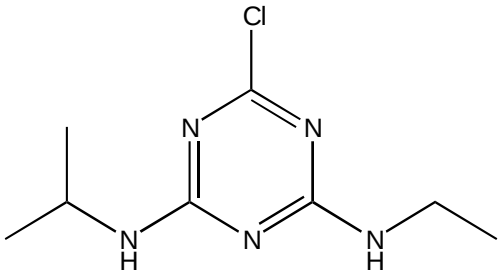
CAS Number: 65277-42-1
Synonyms: Fungarest, Fungarol, Nizoral, Panfungol
Lot Number: QL0352
Purity: ≥98%
Appearance: Solid
Molecular Formula: $C_{26}H_{28}Cl_2N_4O_4$
Molecular Weight: 531.4376
Storage, Test Solution: 1-8°C

2.1.1.5 Vinclozolin



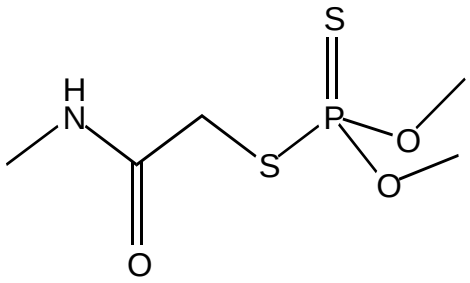
CAS Number: 50471-44-8
Synonyms: Ronilan, Vorlan
Lot Number: 281-94A
Purity: ≥ 98%
Appearance: Solid
Molecular Formula: $C_{12}H_9Cl_2NO_3$
Molecular Weight: 286.114
Storage, Test Solution: 1-8°C

2.1.1.6 Atrazine



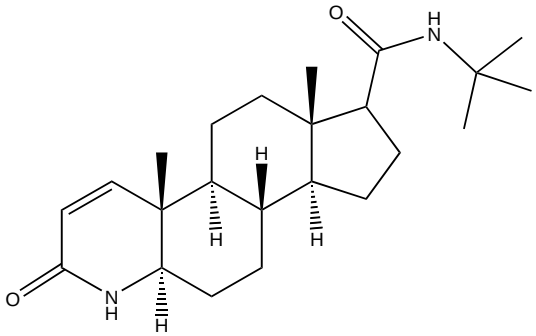
CAS Number: 1912-24-9
Synonyms: Vectal, Weedex, zeazin
Lot Number: 277-93B
Purity: ≥ 98%
Appearance: Solid
Molecular Formula: $C_8H_{14}ClN_5$
Molecular Weight: 215.6851
Storage, Test Solution: 1-8°C

2.1.1.7 Dimethoate



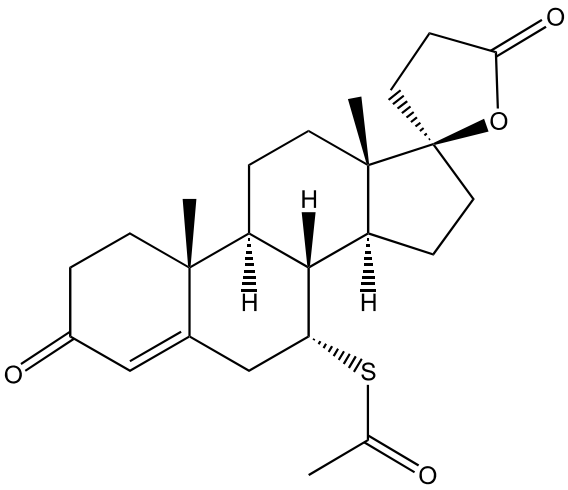
CAS Number: 60-51-5
Lot Number: 021104MS-AC
Purity: ≥98%
Appearance: White powder
Molecular Formula: $C_5H_{12}NO_3PS_2$
Molecular Weight: 229.3
Storage, Test Solution: 1-8°C

2.1.1.8 Finasteride



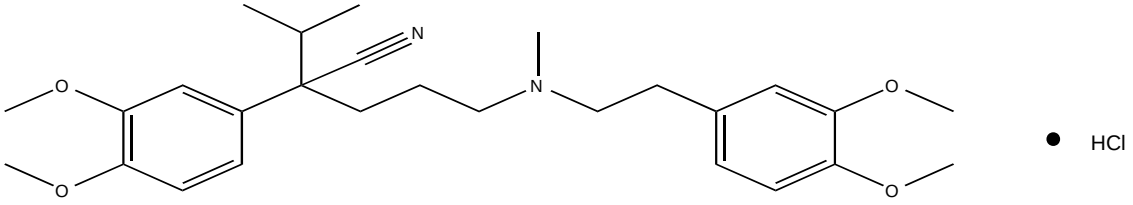
CAS Number: 98319-26-7
Synonyms: Proscar
Lot Number: 23920302
Purity: ≥ 98%
Appearance: White/off-white crystal powder
Molecular Formula: $C_{23}H_{36}N_2O_2$
Molecular Weight: 372.5496
Storage, Test Solution: 1-8 °C

2.1.1.9 Spironolactone



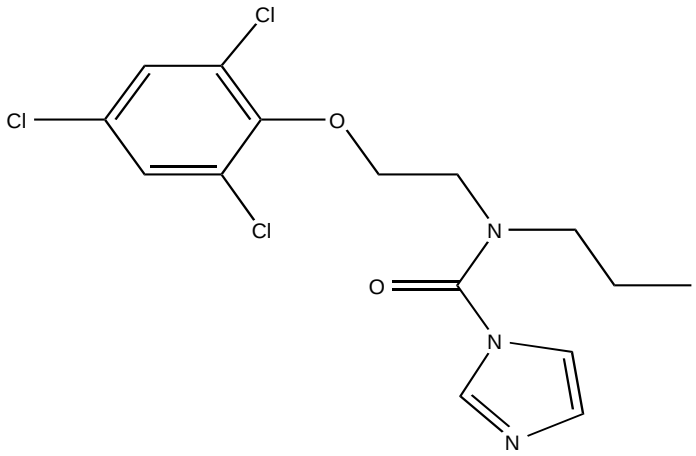
CAS Number: 52-01-7
Synonyms: Spiractin
Lot Number: 10911PB
Purity: ≥ 98%
Appearance: Powder
Molecular Formula: $C_{24}H_{32}O_4S$
Molecular Weight: 416.5744
Storage, Test Solution: 1-8°C

2.1.1.10 Verapamil



CAS Number: 23313-68-0
Lot Number: 062KO325
Appearance: White powder
Molecular Formula: $C_{27}H_{38}N_2O_4$-HCl
Molecular Weight: 491.1
Storage, Test Solution: 1-8°C

2.1.1.11 Prochloraz



CAS Number: 67747-09-5
Synonyms: Octave, Sportak
Lot Number: 2226 X
Purity: 99.4%
Appearance: Colorless, crystals
Molecular Formula: $C_{15}H_{16}Cl_3N_3O_2$
Molecular Weight: 376.6693
Storage, Test Solution: 1-8°C

2.1.2 Standards

Human Chorionic Gonadotropin (hCG) was used as a stimulant of the sliced testis bioassay. This substance was considered a standard and was handled and documented according to the laboratory's Standard Operating Procedures (SOPs) for the Optimized Sliced Testis Assay (SOP LORET 150) section on preparation of hCG.

2.1.2.1 Human Chorionic Gonadotropin (hCG)

Chemical Name: hCG
CAS No.: 9002-61-3
Lot No.: B55956
Molecular Formula/Weight: 36,700
Solubility: H_2O
Supplier: Calbiochem
30% hCG by weight, 3050 IU/mg
Storage Conditions: Freezer (-20°C). Following reconstitution, aliquot and freeze (-20°C); stable for 2 years as supplied

2.2 Animals and Husbandry

2.2.1 Animals

The Sprague Dawley Derived Outbred Albino Rat [Crl:CD® (SD) IGSBR], known as the Charles River CD® Rat (Charles River Laboratories, Inc., Raleigh, NC), was used for these studies. The body weight range for the males used was 337.83-464.50 g. The male rats were individually identified by eartag. A total of 3 males were assigned to each replicate in this study. A total of 30 males were used to test all 9 selected chemicals (including prochloraz which was evaluated later due to procurement problems).

2.2.2 Sentinels

Sentinel animals were not necessary since the animals were not in-house for more than 1 month. The animals were ordered as close as possible to the time of use.

2.2.3 Husbandry

2.2.3.1 Conditions. The animal portions of this study were carried out under standard laboratory conditions. The animals were housed 1 per cage upon arrival, during the acclimation period, and until they were used for testing in solid-bottom polycarbonate cages with stainless steel wire lids (Laboratory Products, Rochelle Park, NJ), with Sani-Chip® cage litter (P.J. Murphy Forest Products Corp., Montville, NJ). The cage dimensions were 8"x19"x10.5" (height) for all phases of this study.

All animals were housed in the RTI International Animal Research Facility following arrival at RTI International and for the duration of the study. RTI International animal rooms are air-conditioned, and temperature and relative humidity are continuously monitored, controlled, and recorded using an automatic system (Siebe/Barber-Colman Network 8000 System with Revision 4.4.1 for Signal® software, Siebe Environmental Controls [SEC]/Barber-Colman Company, Loves Park, IL). The target environmental ranges were 66 to 77˚F (22˚C $\pm$ 3˚C) for temperature and 30 to 70% relative humidity, with a 12 hour light:12 hour dark cycle per day (NRC Guide, 1996). At all times, the animals were handled, cared for, and used in compliance with the NRC *Guide for the Care and Use of Laboratory Animals* (NRC, 1996).

2.2.3.2 Diet. Purina Certified Pelleted Rodent Diet® (No. 5002, PMI Feeds, Inc., St. Louis, MO) was available *ad libitum*. The analysis of each feed batch for nutrient levels and possible contaminants was performed by the supplier, examined by the Study Director, and maintained in the study records. The feed was stored at approximately 60-70˚F, and the period of use did not exceed six months from the milling date.

2.2.3.3 Water. Animals received tap water (source: City of Durham, Department of Water Resources, Durham, NC). Water was available *ad libitum* by plastic water bottles with butyl rubber stoppers and stainless steel sipper tubes. Contaminant levels of the Durham City water were measured at regular intervals by the supplier per EPA specifications and by Balazs Laboratories Inc. (Sunnyvale, CA) and U.S. Biosystems Inc. (Boca Raton, FL). Documentation of these analyses was inspected by the Study Director and maintained in the study records.

2.3 Sliced Testis Assay Procedure

Male Sprague-Dawley rats, 11-13 weeks old, were euthanized. The testes were surgically isolated and the tunica albicans removed. Whole testis weights were recorded (to the nearest 0.1 mg). Only testes with weights greater than 1000 mg were used. The time from testis removal to the time of slicing was less than 1 hour. The testes were sliced to yield fragments weighing 50-100 mg. Fragment weights were recorded (to the nearest 0.0001 g). Each fragment was placed in individual, tightly capped, 9 mL test tubes containing 2.5 mL of 95% O_2 / 5% CO_2 gassed media (modified medium-199 without phenol red; pH 7.4).

The test tubes containing the testicular fragments and media were incubated at 36°C $\pm$ 0.5°C and placed on a shaker at a speed of 175 rpm. After 30 min (Equilibration Phase), the test tubes were removed from the shaker and centrifuged (800 x g for 5 min). The supernatant was poured off (equilibration wash). Fresh media (2.5 mL) was added to the test tubes and then centrifuged (800 x g for 5 min). The supernatant was poured off, collected, and saved for analysis (Baseline Collection Phase, Time 0 hr).

Fresh media (2.5 mL) without hCG (unstimulated) or with hCG (stimulated; 0.1 IU/mL final concentration) was added to the appropriate test tubes. The Incubation Response Sample Collection Phase began when the test tubes were incubated (36°C) and shaken (175 rpm). After 1 hour, the samples were removed from incubation and the shaker centrifuged, as above, and the media poured off and collected (time 1 hr). This procedure was repeated for collection of media samples at 2, 3, and 4 hours (time 2 hr, 3 hr, and 4 hr). Fresh media, with or without hCG, was added, depending on the test tube study group assignment. [Note: replacement of test chemicals was also made when appropriate.]

After collection of the baseline and all hourly samples for a given fragment, a composite sample was prepared. The composite sample was prepared by taking a 0.5 mL aliquot from the baseline and each hourly sample from a given fragment and combining the aliquots into a single container (final volume 2.5 mL). The original baseline and composite samples from each fragment were analyzed for testosterone and LDH.

For testosterone determinations, media samples collected at the baseline and at hourly time points, as well as the composite sample, were stored frozen at -70 to -80°C. The baseline and composite samples were analyzed within 1 month after collection for testosterone, in duplicate, using a radioimmunoassay (RIA) method. All samples for a given day's set of runs were analyzed in the same testosterone RIA, when possible. For LDH determinations, analysis of the baseline and composite samples were performed within the same day as the sliced testis assay was conducted. LDH samples were stored at room temperature, protected from the light, and transported to LabCorp, Inc. (Alexander Drive, Durham, NC) with a transfer of custody sheet as soon as possible after the conclusion of the assay.

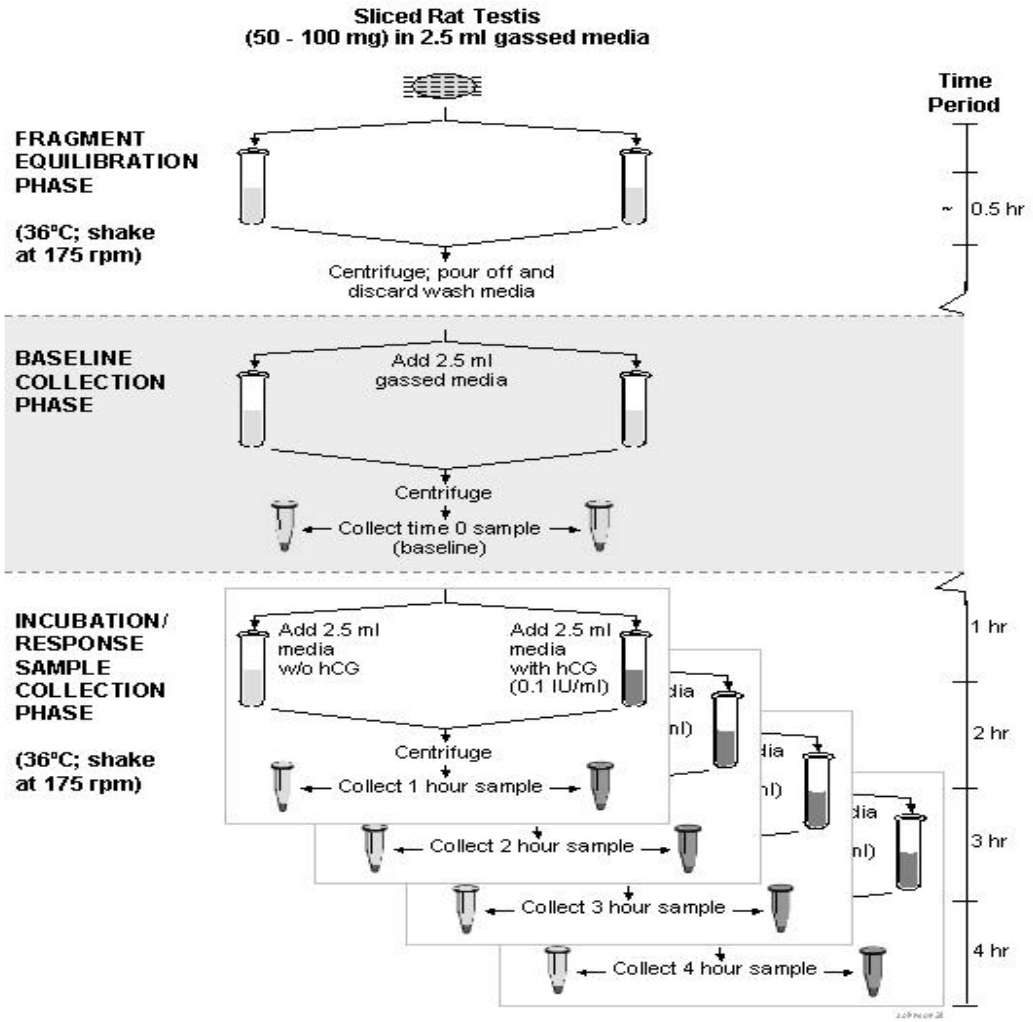


Figure 1. Technical Flow Illustration of the Sliced Testis Assay

2.4 Study Design

The experimental design for each replication of the Multichemical Study for prevalidation is summarized in Table 1; the fragment numbering is for Replicate 1. Consecutive numbers were used for the other replicate, so that each fragment used in these studies had its own unique number for identification purposes.

Table 1. Replicate Organization

Sample Type	hCG	Number of Incubations (Runs)	Testis Fragment(s) Number Assignment
Media-Vehicle control	no	3	1-3
Media-Vehicle control	yes	3	4-6
Media control	yes	3	7-9
Positive control (AG-50 µM)	yes	3	10-12
Cytotoxic control (2,4-DNP-1000 µM)	yes	3	13-15
Media + Chemical 1 (low)	yes	3	16-18
Media + Chemical 1 (mid)	yes	3	19-21
Media + Chemical 1 (high)	yes	3	22-24
Media + Chemical 2 (low)	yes	3	25-27
Media + Chemical 2 (mid)	yes	3	28-30
Media + Chemical 2 (high)	yes	3	31-33

The information presented in Table 1 represents 1 replicate of the experiment. Two replicate experiments were conducted. The overall study used 30 rats for the 9 chemicals studied (3 rats/replicate study, using 1 right testis/rat, 3 testes total/replicate study). The initial eight chemicals tested used 11 fragments/testis. A block design was used for distribution of testis fragments to control bias. The fragments obtained from each testis were divided among the test conditions. For the last chemical tested (prochloraz), there were no fragments exposed to 2,4-DNP. Therefore, the total number of fragments used for those replicates was seven per replicate study. The overall total number of individual fragments and incubations used to conduct this experiment was 306. Due to procurement problems, prochloraz was evaluated later, using AG as the positive control.

The sampling time points (5) from the media are 0 (after a 30 min equilibration) and 1-, 2-, 3-, and 4-hours post-equilibration. A 0.5 ml aliquot was taken from samples taken at 0, 1, 2, 3, and 4 hours and combined to prepare a single composite sample for each fragment. The 0 hour and composite samples were then analyzed for testosterone in duplicate and LDH in

singlet. Thus, the overall total number of testosterone samples for analysis was 1224 [306 runs x 2 sampling time points x 2 (duplicate) analyses]. The samples were also analyzed for LDH at each of the 2 time points for a total of 612 samples.

3.0 CHEMISTRY

3.1 Test Substance Procurement and Purity Determination

The Chemical Repository at Battelle procured all test substances. The cytotoxicant (2,4-DNP), the AG (positive control), atrazine, dimethoate, finasteride, flutamide, ketoconazole, prochloraz, spironolactone , verapamil, and vinclozolin were procured, verified for purity, formulated into a chemical-specific stock solution, and the stock solution formulation was analyzed prior to shipment (to RTI International) by the EDSP Chemical Repository at Battelle. The Chemical Repository only shipped the stock solution of each formulation, and RTI prepared the formulation concentrations to be tested by making the appropriate dilutions of the stock solution. A single lot of each test substance was procured so that all prevalidation experiments were performed using the same lot of test substance. Test substances had a purity >95%. Upon receipt, the test substance was stored in its original container and in storage conditions recommended by the supplier. If the amount of test substance needed was not provided by the supplier in a single container, then the Chemical Repository combined the contents of the individual containers into a single container for a test substance with the same purity and lot number. All test substances were then taken from the container with the combined contents. Additional information is contained in the Analytical Chemistry Reports (Appendix I).

The Chemical Repository at Battelle verified the purity given by the supplier. One chromatographic method was used to determine the purity.

The chemistry results are summarized in Table 2 below. Further information, as reported by the Chemical Repository, is in Appendix I, Analytical Chemistry Reports for the individual chemicals.

Table 2. Chemistry Information

Chemical	Purity	Solubility	Vehicle Used	Concentration		Stability	Analytical Method
				Target	Actual		
AG	99.3%	Acceptable in DMSO	DMSO	23.2 mg/mL	22.85-23.31 mg/mL	39 days at 5° C	Gas chromatography with flame ionization

(continued)

Table 2. Chemistry Information (continued)

Chemical	Purity	Solubility	Vehicle Used	Concentration		Stability	Analytical Method
				Target	Actual		
2,4 DNP	100%	Acceptable in DMSO	DMSO	18.4 mg/mL	21.28-23.02 mg/mL	Unstable but useable for 14 days at 5° C	Liquid chromatography with UV detection
Atrazine	~99.5%	Acceptable in DMSO	DMSO	1.30 mg/mL	1.313-1.324 mg/mL	28 days at 5° C	Gas chromatography with flame ionization
Dimethoate	~ 99.6%	Acceptable in DMSO with a constant impurity present (≤1%)	DMSO	23.0 mg/mL	22.88-23.16 mg/mL	29 days at 5° C	Gas chromatography with flame ionization
Finasteride	99.4%	Acceptable in DMSO	DMSO	37.2 mg/mL	38.26- 38.63 mg/mL	28 days at 5° C	Liquid chromatography with UV detection
Flutamide	~99.8%	Acceptable in DMSO	DMSO	27.6 mg/mL	28.36-28.86 mg/mL	28 days at 5° C	Liquid chromatography with UV detection
Ketoconazole	100%	Acceptable in DMSO	DMSO	0.532 mg/mL	0.5450-0.5485 mg/mL	28 days at 5° C	Liquid chromatography with UV detection
Prochloraz	~99.4%	Acceptable in DMSO	DMSO	1.88 mg/mL	1.868-1.872 mg/mL	28 days at 5° C	Liquid chromatography with UV detection
Spironolactone	~100 %	Acceptable in DMSO	DMSO	41.6 mg/mL	41.66-41.72 mg/mL	28 days at 5° C	Liquid chromatography with UV detection
Verapamil	100%	Acceptable in water	Water	4.92 mg/mL	4.877-4.914 mg/mL	28 days at 5° C	Liquid chromatography with UV detection
Vinclozolin	98%	Acceptable in DMSO	DMSO	2.86 mg/mL	2.884-2.900 mg/mL	29 days at 5° C	Gas chromatography with flame ionizaton

DMSO = Dimethylsulfoxide

3.2 Formulation Preparations

Each test substance formulation was prepared into an individual stock solution. Preparation of the stock solutions was performed by the Chemical Repository at Battelle, whereas the assay testing solutions were prepared by RTI International.

Stock solutions of each test chemical were prepared by weighing an accurate amount of the test chemical and mixing it with the appropriate volume of Milli-Q water or 100% DMSO.

The concentration of the stock solution prepared was the highest of the 3 concentrations to be tested in the assay and was prepared at a "Formulated Stock Solution Concentration." A total volume of 5-10 mL of the stock solution was prepared for shipment to RTI International. The stock solution was shipped in an appropriate glass or plastic container with a taped, screw cap lid, packed in ice (if needed for stability), and other protective shipping materials (e.g. styrofoam peanuts) for delivery by overnight mail.

RTI International, upon receipt of a stock solution, stored it under the appropriate conditions until needed for testing. Prior to testing, the stock solution was used to prepare the mid and low concentrations. The mid and low concentrations were prepared by removing an aliquot of the stock solution and mixing it with the appropriate volume of the vehicle.

Table 3 summarizes the final target concentrations that were tested in the incubation mixture and target stock solution concentrations that were prepared for each cytotoxic test substance.

Table 3. Test Substance Target Concentrations

Test Chemical (relative level)	Target Stock Concentration (mg/mL)[a]	Target Stock Concentration in modified M-199 (mg/mL)[b]	Final Target Concentration in the Incubation Media (µM)
AG (positive control)	23.2	0.0116[c]	50
2, 4- DNP (cytotoxicant control)	18.4	0.184	1000
Atrazine (high)	1.30	0.0130	60
Atrazine (mid)	0.650	0.00650	30
Atrazine (low)	0.217	0.00217	10
Dimethoate (high)	23.0	0.230	1000
Dimethoate (mid)	2.30	0.0230	100
Dimethoate (low)	0.0230	0.000230	1
Finasteride (high)	37.2	0.372	1000
Finasteride (mid)	3.72	0.0372	100
Finasteride (low)	0.372	0.00372	10
Flutamide (high)	27.6	0.276	1000
Flutamide (mid)	2.76	0.0276	100
Flutamide (low)	0.276	0.00276	10
Ketoconazole (high)	0.532	0.00532	10
Ketoconazole (mid)	0.0532	0.000532	1
Ketoconazole (low)	0.00532	0.0000532	0.1

(continued)

Table 3. Test Substance Target Concentrations (continued)

Test Chemical (relative level)	Target Stock Concentration (mg/mL)[a]	Target Stock Concentration in modified M-199 (mg/mL)[b]	Final Target Concentration in the Incubation Media (µM)
Prochloraz (high)	1.88	0.188	50
Prochloraz (mid)	0.188	0.00188	5
Prochloraz (low)	0.0188	0.000188	0.5
Spironolactone (high)	41.6	0.416	1000
Spironolactone (mid)	4.16	0.0416	100
Spironolactone (low)	0.416	0.00416	10
Verapamil (high)	4.92	0.0492	100
Verapamil (mid)	0.492	0.00492	10
Verapamil (low)	0.0492	0.000492	1
Vinclozolin (high)	2.86	0.0286	100
Vinclozolin (mid)	0.286	0.00286	10
Vinclozolin (low)	0.0286	0.000286	1

[a] The high stock solution prepared by the Chemical Repository and shipped to RTI International was used by RTI International to prepare the mid and low stock solutions. The high, mid, and low stock solutions were used to prepare the high, mid, and low stock media formulations, respectively.

[b] The stock media formulations were prepared by adding an appropriate aliquot of the stock solution and diluting it 1:100 in modified media 199.

[c] The AG media formulation was prepared by adding an appropriate aliquot of the stock solution and diluting it 1:2000 in modified media 199.

4.0 ENDPOINT MEASUREMENTS

4.1 Total Testosterone RIA Procedure

The total testosterone RIA used was a no-extraction, solid-phase ^{125}I RIA, which utilized total testosterone specific antibody-coated tubes and ^{125}I-total testosterone, testosterone calibrators as the standard curve and controls with known values of testosterone (DPC, Los Angeles, CA). From the control values, the intra- and interassay coefficient of variation (CV) was determined (see Table 4). The sensitivity of the assay was 0.04 ng/mL. For the RIA procedure, the sample was pipetted into the antibody-coated tube and the ^{125}I-total testosterone was added. The tubes were vortexed and incubated in a 37 ± 1°C water bath for 3 hours. After incubation, the supernatant was decanted and the tubes were counted in a gamma counter. All assays were counted in a Packard Biosciences Cobra II Series Model 5002 gamma counter using RIASMART software, Version 1.0. Results were reported as ng/mL. The intra- and inter-assay CVs are presented in Table 4.

Table 4. CVs for Testosterone RIA

	Low Control CON6 Level 4	Mid Control CON6 Level 5	High Control CON6 Level 6
Intra-assay CV			
Assay 1 (n=4)	7.0%	6.1%	4.5%
Assay 2 (n=4)	3.1%	2.0%	5.6%
Assay 3 (n=4)	5.9%	4.9%	8.0%
Assay 4 (n=4)	4.8%	7.6%	3.7%
Assay 5 (n = 4)	5.2%	5.3%	4.2%
Assay 6 (n=4)	9.0%	3.6%	5.8%
Assay 7 (n=4)	1.0%	4.1%	4.7%
Assay 8 (n=4)	5.9%	5.7%	3.3%
Assay 9 (n=2)	2.8%	8.3%	7.3%
Assay 10 (n=4)	6.7%	2.3%	5.2%
Assay 11 (n=4)	12.7%	9.4%	5.5%
Inter-assay CV			
(n=11)	7.9%	5.8%	6.2%

4.2 LDH Spectrophotometric Procedure

Analyses were performed by LabCorp (Raleigh, NC), on the same day as the sliced testis assay was performed. This laboratory is considered a contributing scientist and issued a report of the results of their assay. The data are reported on the spreadsheets in Appendix II.

The LDH assay measures the rate at which NADH is formed when NAD is reduced when it catalyzes the oxidation of lactate to pyruvate. NADH is measured at 340 nm using a kinetic-spectrophotometric method. The assay and samples are temperature sensitive, and samples were not refrigerated or frozen. The assay has been characterized for assay conditions at 37^{o}C. LDH activity is expressed in U/L. The reportable range for the assay is 5 to 1000 U/L.

All calculations were performed using EP Evaluator, Release 3.0, statistical analysis software from David Rhoads Associates, Inc. (Kennett Square, PA).

5.0 DATA ANALYSIS

The results for each analysis were reported individually, with sufficient identifying information to determine which results correspond to duplicate analyses, to different time points within 1-assay tube, to different assay tubes within the same replicate, and to different replicates.

The laboratory maintains a database to include all data generated during the study. Test conditions, background environmental conditions, and results for each analysis for each sample at each time point are reported.

Data results were processed using a spreadsheet provided by Battelle/Lead Laboratory. Analysis results reported are testosterone concentration (ng T/mg tissue) and LDH concentration (mU/mg tissue). For the testosterone RIA, detection limits and indications of inability to detect were reported, as well as confirmation of the acceptability or nonacceptability of each individual value. The spreadsheet calculated the descriptive data analysis for the testosterone and LDH values. RTI International LORET laboratory personnel are responsible for the accurate transfer of RTI International generated data but Battelle Memorial Institute is responsible for the accuracy of calculations performed by the spreadsheet.

6.0 STATISTICAL ANALYSIS

The statistical analysis was divided into 3 parts.

The statistical analyses for the paired chemicals and prochloraz was performed by Dr. Paul Feder, of the Data Coordination Center, Battelle Memorial Institute. The objectives of the statistical analyses were to:

- Determine whether there was a significant difference, averaged over fragments, between each graded dose of each test chemical and the stimulated media-vehicle control (i.e., analysis of the results of the composite samples, corrected for the results of the 0 hour samples), and determine the slope of the dose trend.
- Compare the four different controls to the stimulated media-vehicle control.
- Estimate the components of variation due to animal (testis), replicate, and fragment within animal.

Analysis was carried out on the results of the composite samples, corrected for the results of the 0 hour (baseline) samples and was based on the logarithms of the baseline-adjusted values.

Mixed effects analysis of variance models were fitted to the data from each analysis part. Random effects were animals (testes), replicates, and fragments within testes. Fixed effects were the study groups (within each part), treated as classification factors. For all comparisons statistical significance was set at the 0.05 level.

In addition, linear, quadratic and cubic dose trends in the graded test chemical dose groups were determined.

The statistical analysis narratives, prepared by Dr. Paul Feder of Battelle Memorial Institute's Data Coordination Center, are located in Appendix III.

7.0 GOOD LABORATORY PRACTICES

The toxicology laboratories at RTI International are operated in compliance with the U.S. EPA Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Good Laboratory Practices Standards (GLP). The RTI International Animal Research Facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International. Thus, portions of this study conducted at RTI were in compliance with EPA FIFRA regulations for GLPs and in compliance with the AAALAC accreditation standards. The sponsor is responsible for GLP compliance of the initial chemical analyses of the bulk chemicals for identity and purity and the statistical analysis portion of the study and the calculations performed by the spreadsheet that they created. The RTI International Quality Assurance Unit reviewed the protocol, inspected critical phases and audited the data and the final report with the exception of the LDH analysis and statistical analysis portions of the study. The LabCorp QAU was responsible for QA activities relating to the LDH spectrophotometric portion of the study. As of the issue date of this draft of the report, verification of the LabCorp QAU evaluation of the LDH data was still in progress.

8.0 PERSONNEL

This study was conducted at RTI International under contract to Battelle Memorial Institute, Columbus, OH. Dr. David P. Houchens, EDSP Program Manager, Battelle Memorial Institute, was the Sponsor's Representative. Rochelle W. Tyl, Ph.D., DABT, served as RTI International Study Toxicologist and Principal Investigator. Ms. Carol S. Sloan, M.S., RTI International, served as Study Director and Work Assignment Leader, Dr. Jerry D. Johnson, Battelle Memorial Institute, served as Task Leader, and Paul I. Feder, Ph.D., Data Coordination Center, Battelle Memorial Institute, performed the statistical analyses on the data. RTI International personnel included Ms. Amanda B. Goodman, B.S., technical lead for the study and Ms. Susan W. Pearce, B.S., lead for the hormone assays. Bulk chemical analysis and dose formulation analysis were provided by the Sponsor through the Battelle Memorial Institute personnel in Columbus, OH. Mr. M. Michael Veselica, Supervisor, RTI Materials Handling Facility, provided receipt of the initial dose formulations at the RTI International Laboratory. Animal care was provided by Dr. Donald Feldman, DVM, ACLAM, RTI International Veterinarian, and Mr. Frank N. Ali, Manager of the Animal Research Facility at RTI International. Mr. Steven A. Myers, QAU Specialist, audited the final report. LabCorp, Inc. (Alexander Drive, Durham, NC) was responsible for LDH evaluations.

The final report was prepared by Ms. Carol S. Sloan, Dr. Julia D. George, Ms. Amanda B. Goodman, Ms. Susan W. Pearce and Dr. Rochelle W. Tyl. Ms. Denise B. Bynum and Ms. Karen L. Kehagias provided secretarial assistance.

9.0 COMPLIANCE

All specimens and records that remain the responsibility of RTI International will be retained in the RTI International archives for the length of time specified in the FIFRA GLP regulations. These materials will be stored for 2 years at the performing laboratory's expense. The test media samples are retained at -70 ± 10°C. Beyond 2 years, the Sponsor will be notified in writing when the RTI International retention time has expired; continued retention will be at additional cost to the Sponsor. Quality control (QC) and quality assurance (QA) procedures conducted at RTI followed those outlined in the Quality Assurance Project Plan (QAPP) prepared. Verification of LabCorp's compliance is in progress. RTI International's Animal Research Facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International. Animals were housed, handled, and used according to the NRC Guide (NRC, 1996).

10.0 RESULTS

The results of the individual chemicals in this study are presented in Tables 5, 7, 9, 11, and 13 for testosterone concentrations and in Tables 6, 8, 10, 12, and 14 for LDH concentrations (also see Appendix II). A summary of those results is presented here.

The overall control values (mean, sd, n = 10) for testosterone concentration (ng/mg fragment) after 4 hours were 0.77 ± 0.28 for the unstimulated media-vehicle control (-hCG), 6.2 ± 2.1 for the stimulated media control (+hCG), and 5.5 ± 2.2 for the stimulated media-vehicle control. The overall response for the unstimulated media-vehicle control versus the stimulated media control was 8.6 fold; comparison of the unstimulated versus the stimulated media-vehicle controls was 7.5 fold.

The salient results for each test chemical are as follows (percent change is relative to unstimulated media-vehicle control):

Flutamide (P450c17 inhibitor)—at 10 μM, flutamide produced a 44% increase but, at 100 and 1000 μM, it produced an 81 and 88% decrease in testosterone concentration, which was statistically significant. Significant linear, quadratic, and cubic components of trend were also observed. Also, flutamide produced concentration-related increases in LDH levels from 10 to 1000 μM, which were statistically significant at the 2 highest doses. Significant linear and cubic

elements of trend were also noted (Tables 5 and 6). [Note: At 1000 μM, flutamide precipitated out of the media and particulates were visible on the fragments.]

Vinclozolin (antiandrogen; AR-antagonist)—highly variable results at 1 μM. For example, at 1 μM, vinclozolin increased the testosterone concentration by 50% (Replicate 1), but this same concentration of vinclozolin also decreased the testosterone concentration by 37% (Replicate 2). At 10 and 100 μM, vinclozolin produced a 22% and 60% decrease in testosterone concentration. Only the 100 μM group was significantly different from the M-V+hCG group. Significant linear and quadratic components of trend were noted. LDH concentrations for vinclozolin-treated fragments were similar to control values (Tables 5 and 6). LDH had significant cubic element of trend.

Atrazine (inhibitor of pathway prior to cholesterol synthesis and transport)—decreased testosterone concentrations at all 3 test levels (10, 30, and 60 μM), but there was no concentration-dependent pattern. Significant quadratic trend components were noted. The highest concentration caused the least decrease in testosterone concentration. LDH concentrations for atrazine-treated fragments were slightly higher than the control values, but this effect was not dose-related either. A borderline ($p<0.047$) linear trend was observed (Tables 7 and 8).

Ketoconazole (P450scc inhibitor)—at 0.1, 1, and 10 μM ketoconazole, the testosterone concentrations were decreased 53, 88, and 98%, respectively, which was significant at all these dose levels. Significant linear and quadratic components of trend were observed. LDH concentrations for ketoconzaole-treated fragments were all significantly higher than the control values and had significant quadratic element of trend (Tables 7 and 8).

Verapamil (calcium channel blocker)—at 1 μM verapamil, there was no measurable effect on testosterone concentration. At 10 μM verapamil, the effect was mixed (decreased 38% for Replicate 1 and increased 13% for Replicate 2). At 100 μM verapamil, the testosterone concentration was decreased 32%. None of the changes were statistically significant. LDH concentrations for verapamil-treated fragments were similar to control values (Tables 9 and 10).

Spironolactone (17-alpha hydroxylase inhibitor)—at 10, 100, and 1000 μM spironolactone, the testosterone concentrations were decreased 78, 96, and 98%, respectively, which were all significantly different from the M-V+hCG control group. Testosterone had significant linear, quadratic, and cubic components of trend. Also, spironolactone produced a concentration-related increase in LDH from 10 to 1000 μM but still within the values for the control group. Significant linear and quadratic components of trend were noted (Tables 9

and 10). [Note: At 1000 µM, spironolactone precipitated out of the media, and particulates were visible on the fragments.]

Finasteride (5-alpha reductase inhibitor)—at 10, 100, and 1000 µM finasteride, the testosterone concentrations were decreased 50, 94, and 97%, respectively, which was significant at all 3 doses. Significant linear and cubic component of trend were noted. Also, finasteride produced significantly increased LDH levels at 1000 µM, with no concentration-related increases at 10 or 100 µM (Tables 11 and 12). A significant linear trend component was observed.

Dimethoate (StAR protein inhibitor)—at 1, 100, and 1000 µM dimethoate, the testosterone concentrations were decreased 11, 15, and 28%, respectively, which was significant at the high dose. A significant linear component of trend was observed. LDH concentrations for dimethoate-treated fragments were similar to control values (Tables 11 and 12).

Prochloraz (aromatase inhibitor, blocks AR)—at 0.5, 5 and 50 µM prochloraz, the testosterone concentrations were decreased 79, 95, and 98%, respectively (testosterone levels were not determined in the presence of 2,4-DNP). These decreases were statistically significant. Significant linear and quadratic components of trend were observed. LDH levels at 0 and 4 hr (composite) with prochloraz were comparable to the control values at 0.5 and 5 µM. At 50 µM the LDH level was significantly increased. There was a significant linear component of trend (Tables 13 and 14).

Table 5. Flutamide/Vinclozolin–Testosterone

Group	Test Chemical Conc	hCG	Replicate #1			Replicate #2			Overall Response (x-fold) [a] (% I) [b]
			T Conc (ng/mg)		Response (x-fold) [a] (% I) [b]	T Conc (ng/mg)		Response (x-fold) [a] (% I) [b]	
			0 Hr (Baseline)	4 Hr (Comp.)		0 Hr (Baseline)	4 Hr (Comp.)		
Media-Vehicle Control[e]	0	—	0.03	0.55	—	0.05	0.60	—	—
Media Control[e]	0	+	0.04	4.32	8.2x	0.04	5.91	10.7x	9.5x
Media-Vehicle Control	0	+	0.03	2.86	5.4x	0.04	3.87	7.0x	6.2x
Flutamide[c]	10 µM	+	0.04	4.81	68.6%	0.04	4.64	20.1%	44.3%
	100 µM[e]	+	0.05	0.72	- 76.3%	0.04	0.60	-85.4%	-80.9%
	1000 µM[e]	+	0.04	0.46	- 85.2%	0.04	0.42	-90.1%	-87.7%
Vinclozolin[d]	1 µM	+	0.04	4.28	49.8%	0.04	2.44	-37.3%	6.2%
	10 µM	+	0.04	2.60	- 9.5%	0.04	2.55	-34.5%	-22.0%
	100 µM[e]	+	0.04	1.53	-47.3%	0.04	1.12	-71.8%	-60.0%
AG (50 µM)[e]	0	+	0.04	1.02	-65.4%	0.04	1.36	-65.5%	-65.5%
2,4-DNP (1000 µM)[e]	0	+	0.04	0.48	- 84.5%	0.04	0.58	-85.9%	-85.2%

[a]Control Response as an "x- fold" increase = $([T_{4\ Hr}]- [T_{0\ Hr}]_{Control\ with\ hCG})/([T_{4\ Hr}]- [T_{0\ Hr}]_{M\text{-}V\ Control\ without\ hCG})$

[b]Inhibitory Response as a % of Control = $100 - \{([T_{4\ Hr}]- [T_{0\ Hr}]_{Inhibitor\ with\ hCG})/([T_{4\ Hr}]- [T_{0\ Hr}]_{M\text{-}V\ Control\ with\ hCG}) \times 100\}$

[c]Significant linear, quadratic and cubic components of trend ($p<0.05$).

[d]Significant linear and quadratic components of trend ($p<0.05$).

[e]Significantly different from the M-V+hCG control ($p<0.05$).

Table 6. Flutamide/Vinclozolin—LDH

Group	Test Chemical Conc	hCG	Replicate #1		Replicate #2	
			LDH Conc (mU/mg), mean ± SD		LDH Conc (mU/mg), mean ± SD	
			0 Hr (Baseline)	4 Hr (Comp.)	0 Hr (Baseline)	4 Hr (Comp.)
Media-Vehicle Control	0	—	0.82 ± 0.23	6.42 ± 1.04	1.09 ± 0.22	6.71 ± 0.72
Media Control	0	+	1.01 ± 0.13	5.24 ± 1.46	1.11 ± 0.09	7.82 ± 0.50
Media-Vehicle Control	0	+	0.88 ± 0.23	5.32 ± 1.93	1.01 ± 0.16	5.72 ± 2.32
Flutamide[a]	10 µM	+	0.91 ± 0.22	5.58 ± 0.93	1.13 ± 0.14	6.80 ± 0.80
	100 µM[b]	+	1.05 ± 0.16	10.64 ± 1.74	1.10 ± 0.09	12.39 ± 1.25
	1000 µM[b]	+	0.91 ± 0.08	17.87 ± 3.26	1.11 ± 0.06	20.02 ± 2.74
Vinclozolin[c]	1 µM	+	1.10 ± 0.37	6.85 ± 0.64	1.27 ± 0.08	6.98 ± 0.94
	10 µM	+	0.87 ± 0.12	5.29 ± 0.14	1.08 ± 0.24	6.19 ± 1.64
	100 µM	+	1.19 ± 0.32	6.35 ± 1.97	1.06 ± 0.17	6.81 ± 1.04
AG (50 µM)	0	+	0.95 ± 0.03	5.64 ± 1.16	1.11 ± 0.20	6.78 ± 0.39
2,4-DNP (1000 µM)	0	+	1.00 ± 0.51	6.37 ± 0.81	1.05 ± 0.09	7.13 ± 0.64

[a]Significant linear and cubic elements of trend ($p<0.05$).
[b]Significantly different from the M-V+hCG control ($p<0.05$).
[c]Significant cubic element of trend ($p<0.05$).

Table 7. Atrazine/Ketoconazole–Testosterone

Group	Test Chemical Conc	hCG	Replicate #1			Replicate #2			Overall Response (x-fold) [a] (% I) [b]
			T Conc (ng/mg)		Response (x-fold) [a] (% I) [b]	T Conc (ng/mg)		Response (x-fold) [a] (% I) [b]	
			0 Hr (Baseline)	4 Hr (Comp.)		0 Hr (Baseline)	4 Hr (Comp.)		
Media-Vehicle Control[e]	0	—	0.05	1.11	—	0.02	0.62	—	—
Media Control	0	+	0.05	6.66	6.2x	0.02	5.97	9.9x	8.1x
Media-Vehicle Control	0	+	0.06	7.68	7.2x	0.03	6.47	10.7x	9.0x
Atrazine[c]	10 µM[e]	+	0.05	5.43	-29.4%	0.02	3.73	-42.4%	-35.9%
	30 µM[e]	+	0.04	4.48	- 41.7%	0.03	5.08	-21.6%	-31.7%
	60 µM	+	0.05	5.85	- 23.9%	0.03	6.83	5.6%	-9.2%
Ketoconazole[d]	0.1 µM[e]	+	0.04	2.64	-65.9%	0.03	3.93	-39.4%	-52.7%
	1 µM[e]	+	0.05	0.68	- 91.7%	0.03	1.10	-83.4%	-87.6%
	10 µM[e]	+	0.05	0.23	-97.6%	0.02	0.18	-97.5%	-97.6%
AG (50 µM)[e]	0	+	0.05	1.81	-76.9%	0.02	1.02	-84.5%	-80.7%
2,4-DNP (1000 µM)[e]	0	+	0.06	0.81	- 90.2%	0.03	0.64	-90.5%	-90.3%

[a]Control Response as an "x- fold" increase = $([T_{4\ Hr}]- [T_{0\ Hr}]_{Control\ with\ hCG})/([T_{4\ Hr}]- [T_{0\ Hr}]_{M\text{-}V\ Control\ without\ hCG})$

[b]Inhibitory Response as a % of Control = $100 - \{([T_{4\ Hr}]- [T_{0\ Hr}]_{Inhibitor\ with\ hCG})/([T_{4\ Hr}]- [T_{0\ Hr}]_{M\text{-}V\ Control\ with\ hCG}) \times 100\}$

[c]Significant quadratic components of trend ($p<0.05$).

[d]Significant linear and quadratic components of trend ($p<0.05$).

[e]Significantly different from the M-V+hCG group ($p<0.05$).

Table 8. Atrazine/Ketoconazole—LDH

Group	Test Chemical Conc	hCG	Replicate #1		Replicate #2	
			LDH Conc (mU/mg), mean ± SD		LDH Conc (mU/mg), mean ± SD	
			0 Hr (Baseline)	4 Hr (Comp.)	0 Hr (Baseline)	4 Hr (Comp.)
Media-Vehicle Control[c]	0	—	0.82 ± 0.08	5.75 ± 0.59	0.76 ± 0.12	4.98 ± 0.78
Media Control[c]	0	+	1.12 ± 0.24	7.03 ± 1.60	0.85 ± 0.18	6.16 ± 1.35
Media-Vehicle Control	0	+	1.20 ± 0.26	5.55 ± 0.47	0.63 ± 0.07	3.21 ± 0.57
Atrazine[a]	10 µM	+	1.15 ± 0.25	7.27 ± 1.75	0.92 ± 0.19	3.83 ± 0.51
	30 µM[c]	+	1.24 ± 0.25	6.97 ± 1.06	0.84 ± 0.12	4.98 ± 1.18
	60 µM	+	1.17 ± 0.19	6.33 ± 1.87	0.73 ± 0.11	4.72 ± 1.48
Ketoconazole[b]	0.1 µM[c]	+	1.10 ± 0.25	6.78 ± 1.18	1.04 ± 0.36	5.35 ± 1.16
	1 µM[c]	+	1.15 ± 0.26	6.17 ± 0.54	0.84 ± 0.07	4.81 ± 1.11
	10 µM[c]	+	1.51 ± 0.80	6.77 ± 1.31	0.95 ± 0.17	4.41 ± 0.62
AG (50 µM)	0	+	1.12 ± 0.13	7.07 ± 1.62	0.75 ± 0.06	3.79 ± 0.57
2,4-DNP (1000 µM)[c]	0	+	1.39 ± 0.04	7.04 ± 1.71	1.07 ± 0.25	7.31 ± 2.05

[a]Significant linear components of trend ($p<0.05$).
[b]Significant quadratic component of trend ($p<0.05$).
[c]Significantly different from the M-V+hCG group ($p<0.05$).

Table 9. Verapamil/Spironolactone–Testosterone

Group	Test Chemical Conc	hCG	Replicate #1			Replicate #2			Overall Response (x-fold) [a] (% I) [b]
			T Conc (ng/mg)		Response (x-fold) [a] (% I) [b]	T Conc (ng/mg)		Response (x-fold) [a] (% I) [b]	
			0 Hr (Baseline)	4 Hr (Comp.)		0 Hr (Baseline)	4 Hr (Comp.)		
Media-Vehicle Control[d]	0	—	0.02	0.65	—	0.06	0.75	—	—
Media Control	0	+	0.03	5.99	9.5x	0.06	5.51	8.0x	8.8x
Media-Vehicle Control	0	+	0.03	5.14	8.1x	0.07	5.17	7.4x	7.8x
Verapamil	1 µM	+	0.02	5.45	6.3%	0.05	4.89	-5.1%	0.6%
	10 µM	+	0.02	3.21	- 37.6%	0.05	5.82	13.1%	-12.3%
	100 µM	+	0.03	4.33	- 15.9%	0.05	2.68	-48.4%	-32.2%
Spironolactone[c]	10 µM[d]	+	0.02	0.97	-81.4%	0.04	1.37	-73.9%	-77.7%
	100 µM[d]	+	0.03	0.25	- 95.7%	0.05	0.25	-96.1%	-95.9%
	1000 µM[d]	+	0.03	0.12	-98.2%	0.05	0.20	-97.1%	-97.7%
AG (50 µM)[d]	0	+	0.03	1.59	-69.5%	0.07	1.67	-68.6%	-69.1%
2,4-DNP (1000 µM)[d]	0	+	0.03	0.63	- 88.3%	0.06	0.52	-91.0%	-89.7%

[a]Control Response as an "x- fold" increase = $([T_{4\ Hr}]- [T_{0\ Hr}]_{Control\ with\ hCG})/([T_{4\ Hr}]- [T_{0\ Hr}]_{M\text{-}V\ Control\ without\ hCG})$

[b]Inhibitory Response as a % of Control = $100 - \{([T_{4\ Hr}]- [T_{0\ Hr}]_{Inhibitor\ with\ hCG})/([T_{4\ Hr}]- [T_{0\ Hr}]_{M\text{-}V\ Control\ with\ hCG}) \times 100\}$

[c]Significant linear, quadratic, and cubic components of trend ($p<0.05$).

[d]Significantly different from the M-V+hCG control group ($p<0.05$).

Table 10. Verapamil/Spironolactone—LDH

Group	Test Chemical Conc	hCG	Replicate #1		Replicate #2	
			LDH Conc (mU/mg), mean ± SD		LDH Conc (mU/mg), mean ± SD	
			0 Hr (Baseline)	4 Hr (Comp.)	0 Hr (Baseline)	4 Hr (Comp.)
Media-Vehicle Control	0	—	0.99 ± 0.01	6.67 ± 0.04	0.90 ± 0.08	6.24 ± 1.79
Media Control	0	+	0.86 ± 0.21	6.65 ± 0.74	1.12 ± 0.19	6.01 ± 1.05
Media-Vehicle Control	0	+	1.17 ± 0.09	8.70 ± 4.67	1.15 ± 0.27	6.21 ± 0.87
Verapamil	1 µM	+	1.02 ± 0.10	6.60 ± 0.22	1.01 ± 0.11	5.56 ± 0.21
	10 µM	+	1.12 ± 0.20	5.96 ± 1.46	1.02 ± 0.08	5.79 ± 1.96
	100 µM	+	1.02 ± 0.07	7.88 ± 1.83	0.96 ± 0.19	3.51 ± 0.36
Spironolactone[a]	10 µM	+	1.02 ± 0.05	5.31 ± 0.71	0.92 ± 0.16	5.23 ± 0.58
	100 µM	+	1.02 ± 0.15	6.48 ± 1.81	1.13 ± 0.04	6.50 ± 0.94
	1000 µM	+	1.27 ± 0.32	10.07 ± 1.16	1.11 ± 0.15	8.62 ± 0.97
AG (50 µM)	0	+	0.94 ± 0.18	5.68 ± 1.68	1.18 ± 0.21	6.01 ± 0.39
2,4-DNP (1000 µM)	0	+	1.11 ± 0.22	7.79 ± 0.56	1.11 ± 0.25	6.53 ± 1.30

[a]Significant linear and quadratic components of trend ($p<0.05$).

Table 11. Finasteride/Dimethoate–Testosterone

Group	Test Chemical Conc	hCG	Replicate #1			Replicate #2			Overall Response (x-fold) [a] (% I) [b]
			T Conc (ng/mg)		Response (x-fold) [a] (% I) [b]	T Conc (ng/mg)		Response (x-fold) [a] (% I) [b]	
			0 Hr (Baseline)	4 Hr (Comp.)		0 Hr (Baseline)	4 Hr (Comp.)		
Media-Vehicle Control[e]	0	—	0.06	1.43	—	0.02	0.72	—	—
Media Control	0	+	0.05	11.56	8.4x	0.03	5.25	7.5x	8.0x
Media-Vehicle Control	0	+	0.06	10.24	7.4x	0.02	4.17	5.9x	6.7x
Finasteride[c]	10 µM[e]	+	0.07	4.95	-52.1%	0.03	2.21	-47.5%	-49.8%
	100 µM[e]	+	0.05	0.45	-96.1%	0.02	0.36	-91.8%	-94.0%
	1000 µM[e]	+	0.05	0.31	-97.4%	0.02	0.17	-96.4%	-96.9%
Dimethoate[d]	1 µM	+	0.05	8.18	-20.1%	0.02	4.12	-1.2%	-10.7%
	100 µM	+	0.05	6.91	-32.6%	0.02	4.31	3.4%	-14.6%
	1000 µM[e]	+	0.05	5.47	-46.8%	0.03	3.81	-8.9%	-27.9%
AG (50 µM)[e]	0	+	0.05	2.06	-80.3%	0.02	0.98	-76.9%	-78.6%
2,4-DNP (1000 µM)[e]	0	+	0.05	0.94	-91.3%	0.02	0.44	-89.9%	-90.6%

[a]Control Response as an "x- fold" increase = $([T_{4\ Hr}]- [T_{0\ Hr}]_{Control\ with\ hCG})/([T_{4\ Hr}]- [T_{0\ Hr}]_{M\text{-}V\ Control\ without\ hCG})$

[b]Inhibitory Response as a % of Control = $100 - \{([T_{4\ Hr}]- [T_{0\ Hr}]_{Inhibitor\ with\ hCG})/([T_{4\ Hr}]- [T_{0\ Hr}]_{M\text{-}V\ Control\ with\ hCG}) \times 100\}$

[c]Significant linear and cubic components of trend ($p<0.05$).

[d]Significant linear components of trend ($p<0.05$).

[e]Significantly different from the M-V+hCG control ($p<0.05$).

Table 12. Finasteride/Dimethoate—LDH

Group	Test Chemical Conc	hCG	Replicate #1		Replicate #2	
			LDH Conc (mU/mg), mean ± SD		LDH Conc (mU/mg), mean ± SD	
			0 Hr (Baseline)	4 Hr (Comp.)	0 Hr (Baseline)	4 Hr (Comp.)
Media-Vehicle Control	0	—	1.21 ± 0.40	6.70 ± 1.71	1.09 ± 0.24	5.55 ± 1.09
Media Control	0	+	1.28 ± 0.17	5.91 ± 1.11	1.49 ± 0.43	7.29 ± 1.93
Media-Vehicle Control	0	+	1.10 ± 0.29	5.75 ± 1.51	1.24 ± 0.26	6.83 ± 1.33
Finasteride[a]	10 µM	+	1.32 ± 0.08	7.52 ± 2.46	1.44 ± 0.33	7.06 ± 1.95
	100 µM	+	1.16 ± 0.07	8.88 ± 1.11	1.21 ± 0.18	6.89 ± 1.42
	1000 µM[b]	+	1.08 ± 0.16	8.88 ± 1.07	1.02 ± 0.25	9.07 ± 1.82
Dimethoate	1 µM	+	1.08 ± 0.23	6.66 ± 0.86	1.46 ± 0.58	6.33 ± 1.43
	100 µM	+	1.23 ± 0.06	7.92 ± 2.13	1.02 ± 0.25	5.96 ± 1.03
	1000 µM	+	1.13 ± 0.19	7.44 ± 1.02	1.07 ± 0.10	5.34 ± 1.01
AG (50 µM)[b]	0	+	1.20 ± 0.20	4.13 ± 1.64	1.21 ± 0.48	5.32 ± 0.81
2,4-DNP (1000 µM)	0	+	1.04 ± 0.02	7.58 ± 2.21	1.23 ± 0.16	7.20 ± 0.70

[a]Significant linear component of trend ($p<0.05$).
[b]Significantly different from the M-V+hCG control group ($p<0.05$).

Table 13. Prochloraz–Testosterone

Group	Test Chemical Conc	hCG	Replicate #1			Replicate #2			Overall Response (x-fold) [a] (% I) [b]
			T Conc (ng/mg)		Response (x-fold) [a] (% I) [b]	T Conc (ng/mg)		Response (x-fold) [a] (% I) [b]	
			0 Hr (Baseline)	4 Hr (Comp.)		0 Hr (Baseline)	4 Hr (Comp.)		
Media-Vehicle Control[c]	0	—	0.04	0.54	—	0.03	0.73	—	—
Media Control	0	+	0.04	4.12	8.2x	0.03	6.44	9.2x	8.7x
Media-Vehicle Control	0	+	0.06	4.04	8.0x	0.03	5.74	8.2x	8.1x
Prochloraz[d]	0.5 uM[c]	+	0.05	1.04	-75.1%	0.03	1.04	-82.3%	-78.7%
	5 uM[c]	+	0.04	0.26	-94.5%	0.02	0.27	-95.6%	-95.1%
	50 uM[c]	+	0.04	0.15	-97.2%	0.03	0.16	-97.7%	-97.5%
AG (50 uM)[c]	0	+	0.03	1.03	-74.9%	0.02	0.89	-84.8	-79.9%
2,4-DNP (1000 uM)	0	+	ND	ND	ND	ND	ND	ND	ND

[a]Control Response as an "x- fold" increase = $([T_{4\ Hr}]- [T_{0\ Hr}]_{Control\ with\ hCG})/([T_{4\ Hr}]- [T_{0\ Hr}]_{M\text{-}V\ Control\ without\ hCG})$

[b]Inhibitory Response as a % of Control = $100 - \{([T_{4\ Hr}]- [T_{0\ Hr}]_{Inhibitor\ with\ hCG})/([T_{4\ Hr}]- [T_{0\ Hr}]_{M\text{-}V\ Control\ with\ hCG}) \times 100\}$

[c]Significantly different from the M-V+hCG control group ($p<0.05$).

[d]Significant linear and quadratic components of trend ($p<0.05$).

ND = Not determined.

Table 14. Prochloraz—LDH

Group	Test Chemical Conc	hCG	Replicate #1		Replicate #2	
			LDH Conc (mU/mg), mean ± SD		LDH Conc (mU/mg), mean ± SD	
			0 Hr (Baseline)	4 Hr (Comp.)	0 Hr (Baseline)	4 Hr (Comp.)
Media-Vehicle Control	0	—	0.76 ± 0.12	4.38 ± 0.24	0.95 ± 0.39	5.08 ± 0.97
Media Control	0	+	1.02 ± 0.31	4.64 ± 0.48	0.86 ± 0.08	4.65 ± 1.38
Media-Vehicle Control	0	+	1.06 ± 0.63	3.78 ± 1.27	0.66 ± 0.06	4.73 ± 0.72
Prochloraz[b]	0.5 uM	+	1.15 ± 0.20	4.47 ± 0.32	1.02 ± 0.18	5.90 ± 1.43
	5 uM	+	0.83 ± 0.12	5.12 ± 1.17	0.88 ± 0.05	5.27 ± 1.47
	50 uM[a]	+	1.08 ± 0.39	5.47 ± 0.19	0.74 ± 0.19	5.32 ± 0.99
AG (50 uM)	0	+	0.88 ± 0.35	4.08 ± 0.93	1.24 ± 0.54	6.72 ± 0.57

[a]Significantly different from the M-V+hCG control group ($p<0.05$).
[b]Significant linear component of trend ($p<0.05$).

11.0 DISCUSSION

The 9 chemicals evaluated for anti-androgenic and/or cytotoxic effects on Leydig cells (reduced testosterone levels and/or increased LDH levels) can be categorized into four groups.

1. Inhibitors of Steroidogenesis Enzymes
   - Flutamide (P450c17)
   - Spironolactone (17α-hydroxylase)
   - Ketoconazole (P450scc; P450c17)
   - Dimethoate (StAR protein which transports cholesterol into the mitochondrion to begin steroidogenesis)
   - Vinclozolin (an AR antagonist)

2. Inhibitors of Aromatase (which converts testosterone into 17β-estradiol in males and females, and converts androstenedione into estrone in females)
   - Prochloraz
   - Vinclozolin

3. Inhibitor of 5α-reductase (which converts testosterone to DHT in the testis and locally; especially important in perinatal male lower reproductive tract and external sex structures and characteristics)
   - Finasteride

4. Other
   - Verapamil (a calcium channel blocker)
   - Atrazine (acts on pathways prior to cholesterol synthesis and acts centrally, in the hypothalamus and pituitary in female rodents, to suppress the LH surge at ovulation; Goldman et al., 2000).

A priori, one would expect that those chemicals with direct effects on steroidogenesis would be the most potent in reducing testicular testosterone levels (see Figure 2 for the steroidogenesis pathway). By and large this was true. Flutamide was a potent inhibitor at 100 and 1000 μM, down to 88% inhibited (but a stimulant at 10 μM); vinclozolin was variable at 1 μM but a relatively potent inhibitor at 10 and 100 μM, down to 60% inhibited; ketoconazole was a potent inhibitor at all 3 concentrations, 0.1, 1, and 10 μM, down to 98% inhibited, and spironolactone was also a potent inhibitor at all 3 concentrations, 10, 100 and 1000 μM, down to 98% inhibited. Dimethoate was a less potent inhibitor but did reduce testosterone levels at all 3 concentrations, down to 28% inhibited.

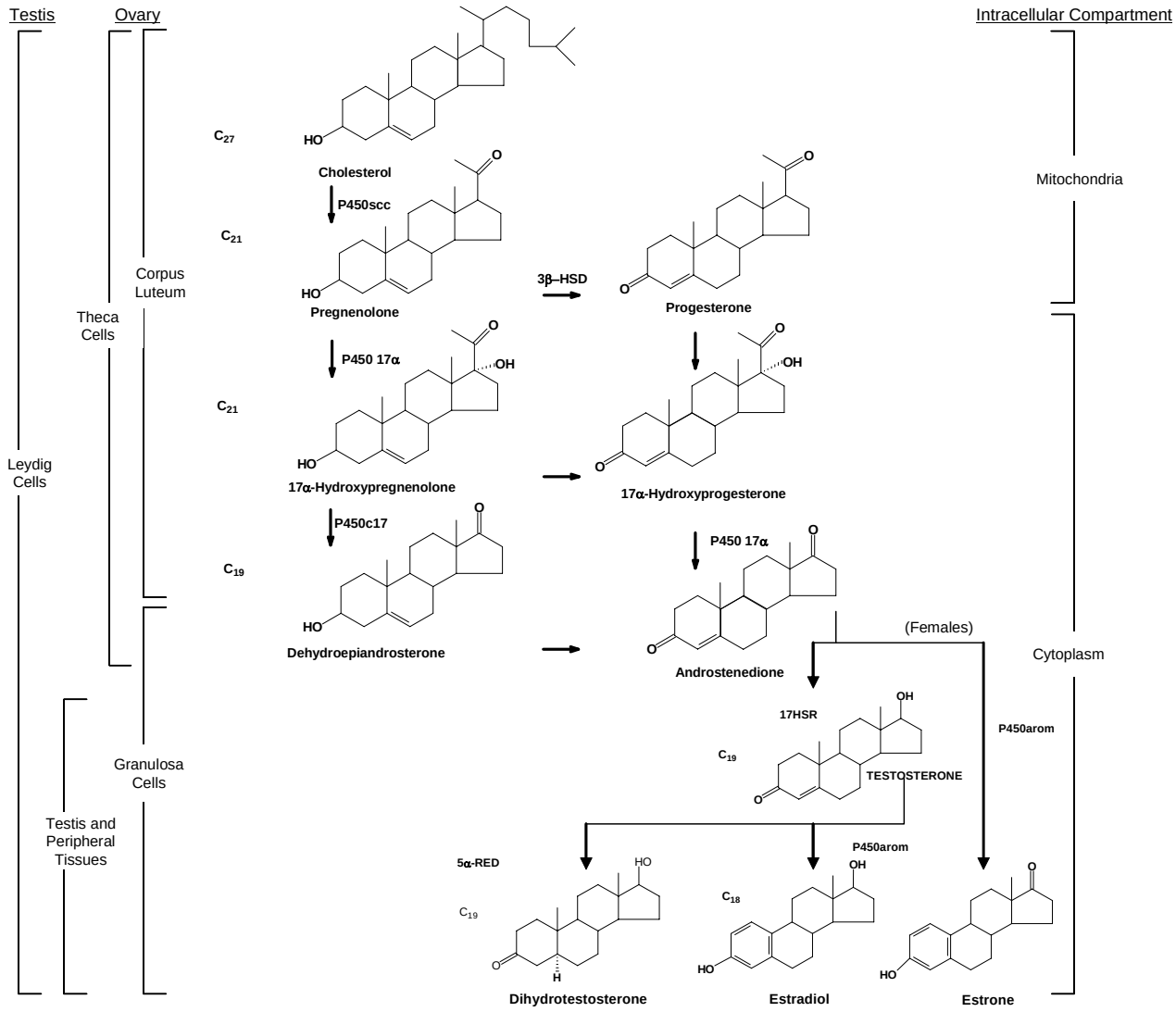


Figure 2. Steroidogenesis Pathway

Also *a priori*, one would not expect aromatase inhibitors to affect testosterone levels. Vinclozolin which inhibits aromatase but also acts on steroidogenesis was variable at 1 μM but inhibited testosterone levels at 10 and 100 μM down to 60% inhibition–the inhibition may be due, in large part, or entirely to its effects on steroidogenesis. Prochloraz, which is reported to be an aromatase inhibitor, was a potent inhibitor of testosterone levels at 0.5, 5 and 50 μM, down to 98%. There are 2 explanatory possibilities: either the mechanistic designation of these 2 chemicals is in error and they do not inhibit aromatase (or they inhibit aromatase and testicular steroidogenesis), or the reduction in aromatase in the testis (if it occurs) causes a local feedback loop (it has to be local since this is an *in vitro* testicular preparation) to suppress testosterone production in the reduced presence or absence of 17β-estradiol, i.e., there is a homeostatic ratio of testosterone and estradiol and reduction of one steroid results in a reduction of the second steroid. Powlin et al. (1998) evaluated the *in vitro* testis explant culture; when they added fetal calf serum to the medium, they did detect estradiol in the assay (estradiol is also present in the male rat brain and acts to masculinize male rat behaviors *in situ*), so the second possibility may be possible. Parenthetically, steroidogenic enzymes are also found in the rat brain, liver, duodenum, adrenals, and ovaries (Stoker et al., 2000).

Also *a priori*, one would not expect inhibitors of 5α-reductase (which converts testosterone to dehydrotestosterone in the testis and locally) to affect testicular testosterone levels. However, finasteride, a 5α-reductase inhibitor, was a potent inhibitor of testosterone levels at all 3 concentrations, down to 97% reduction at 1000 μM. Again, either there is a local feedback loop or a hemostatic equilibrium between testosterone and DHT (so that reductions in DHT cause reductions in testosterone), or finasteride affects steroidogenesis in the sequence to produce testosterone. This latter possibility is very unlikely since finasteride has been well researched, its mechanism of action identified and it is used in pharmaceutical preparations to restore hair growth in men (and women) with male pattern baldness, under DHT control. The assay response was opposite of what was expected.

Powlin et al. (1998) evaluated *in vitro* testis and ovarian explants as a screen to identify potential inhibitors of steroid biosynthesis. Chemicals tested in common by Powlin et al. (1998) and the present study, using the *in vitro* testis explant, included ketoconazole, aminoglutethimide, finasteride, and flutamide. In their *in vitro* testis assay, ketoconazole and flutamide (as well as 17β-estradiol and haloperidol [a D2 receptor antagonist]) inhibited steroid biosynthesis. Their testis culture did not detect aromatase or 5α-reductase inhibitors. Their *in vivo* assay also detected ketoconazole and flutamide (as well as other compounds). They concluded that "Because of the difficulties in assessing cytotoxicity and the high false positive/negative rates, the ovary and testis explant assays are not useful as routine screening

procedures for detecting steroid biosynthesis inhibitors; however, they may have utility in confirming *in vivo* findings." (Powlin et al., 1998, p. 61).

Gray et al. (1995) used rat and hamster quarter testes *in vitro* to evaluate effects of ethane dimethanesulfonate (EDS), a Leydig cell toxicant, on testicular steroidogenesis by assaying for testosterone production. The IC50 (EDS concentration at 50% inhibition) in the rat testis was 320 ng EDS/ml while the hamster IC50 was greater than 1800 ng EDS/ml. *In vivo* studies in rats demonstrated that oral 100 mg/kg EDS inhibited the activity of testicular 5-ene-3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) enzyme by 99% of control with severely reduced Leydig cell numbers; while the hamster exhibited only a 35% reduction in 3β-HSD. 3β-HSD converts pregnenolone to progesterone (which is further converted to 17α-hydroxyprogesterone and then to androstenedione, etc.) in the steroidogenesis pathway (Figure 2). Therefore, the testicular *in vitro* culture was able to identify the inhibition of the steroidogenesis (with the site of action identified *in vivo*).

The last category, "other," has 2 chemicals, verapamil (which blocks calcium channels) and atrazine (which supposedly acts on pathways prior to cholesterol synthesis and affects the LH surge in female rodents by acting at the level of the hypothalamus-pituitary; Goldman et al., 2000). Verapamil reduced testosterone levels consistently only at the highest concentration (100 μM), down only to 32% inhibited. Atrazine also reduced testosterone levels at all 3 concentrations, (10, 30, and 60 μM), with no concentration-response pattern; i.e., greatest inhibitions were at 10 and 30 μM (36 and 32%, respectively) and only 9% inhibited at 60 μM. Atrazine is therefore interpreted as "negative" in this assay.

In fact, this assay did identify all anti-androgenic chemicals, regardless of sites or mechanisms of action. It is also notable that an anti-androgenic chemical, even a potent one, need not be cytotoxic, and conversely, a cytotoxic chemical may not affect testosterone levels until the cytotoxicity becomes significant.

LDH levels were used as a biomarker of testicular cell cytotoxicity. Of the anti-steroidogenic chemicals, flutamide was a potent LDH inducer, ketoconazole produced significantly increased LDH levels, and spironolactone increased values but not significantly; there were no effects on LDH levels with vinclozolin or dimethoate. LDH levels were also unaffected with prochloraz and verapamil. Finasteride exhibited increased LDH levels only at the highest concentration (1000 μM). Atrazine, negative for effects on testosterone levels, induced LDH levels only very slightly higher than the control values.

The results from the positive controls, AG and 2,4-DNP, appear to be very consistent throughout the course of this study, generally causing increased LDH levels (a measure of cytotoxicity) and decreased testosterone levels (a measure of effects on steroidogenesis) from exposure to either chemical for all of the assays for testosterone and LDH, as shown in Tables 5-15.

Table 15. Consistency of Positive Control Results in Sliced Testis Assay

	Response %I[a]	
Chemical	**Replicate 1**	**Replicate 2**
AG (50µM)-Positive Control	-65.4%	-65.5%
	-76.9%	-84.5%
	-69.5%	-68.6%
	-80.3%	-76.9%
	-74.9%	-84.8%
2,4-DNP (1000 µM)-Cytotoxic Control	-84.5%	-85.9%
	-90.2%	-90.5%
	-88.3%	-91.0%
	-91.3%	-89.9%

[a]Inhibitory Response as a % of Control = $100 - \{([T_{4\ Hr}] - [T_{0\ Hr}]_{Inhibitor\ with\ hCG})/([T_{4\ Hr}] - [T_{0\ Hr}]_{M\text{-}V\ Control\ with\ hCG}) \times 100\}$

The consistency of these controls is vital to the use of the assay to detect endocrine disruptors. The assay produced results that were very consistent between replicates for some chemicals, such as prochloraz, finasteride, ketoconazole, spironolactone, and flutamide, but the results for dimethoate, verapamil, vinclozolin, and atrazine were not as consistent. Perhaps the assay is not as sensitive for chemicals with these mechanisms of action, calcium channel blocker (verapamil) and StAR protein inhibitor (dimethoate). Or higher concentrations of the chemicals would result in more consistent response e.g. verapamil's highest concentration tested was 100 µM.

12.0 CONCLUSIONS

The 9 chemicals that were evaluated for anti-androgenic and/or cytotoxic effects in the *in vitro* sliced testis assay produced the following results:

Finasteride, a 5α-reductase inhibitor, converts testosterone to DHT in the testis and locally. DHT plays a major role in the perinatal determination of external male genitalia and

structures of the lower male reproductive tract. In this assay, finasteride caused concentration-related decreases in testosterone levels. LDH levels were increased at 1000 μM finasteride, with no effects at 10 or 100 μM. The assay response was opposite of what was expected.

Flutamide acts to inhibit the P450c17 enzyme, which converts 17α-hydroxypregnenolone to dehydroepiandrosterone (the precursor of androstenedione). Flutamide also produced significant decreases in testosterone concentrations at higher concentrations but was stimulatory at the lowest concentration of 10 μM. A concentration-related increase in LDH concentration was observed with flutamide, which may indicate it is cytotoxic to testicular cells.

Spironolactone acts by 17-α hydroxylase inhibition. Testosterone levels were decreased in a dose-related manner. LDH values increased in a concentration-related manner, although the values were within the control range. This may imply a possible cytotoxic action on the testicular cells.

Vinclozolin is a known antiandrogen and acts by binding to the AR. The results showed dose-related decreases in the testosterone concentrations at 10 and 100 μM, with variable effects at 1 μM. LDH levels were unaffected in the presence of vinclozolin.

Ketoconazole is a P450scc inhibitor. This enzyme converts cholesterol to pregnenolone at the beginning of the steroidogenesis pathway, so that testosterone levels should be reduced with increasing concentrations of ketoconazole. In fact, it decreased testosterone in a concentration-related manner, with the highest concentration giving the greatest decrease. LDH levels were significantly higher than the control values.

Verapamil is a calcium channel blocker and, as such, was not expected to significantly affect testosterone concentration. There was no measurable effect on testosterone concentrations at lower concentrations of verapamil, as would be expected. However, there was a 32% decrease in testosterone at the highest concentration of verapamil which was not significantly different from the control. LDH levels were unaffected.

Atrazine inhibits the steroidogenesis pathway prior to cholesterol synthesis and also acts at the level of the hypothalamus-pituitary to decrease or abolish the LH surge at ovulation in female rats. Atrazine decreased testosterone concentration at all 3 of its concentrations, but there was no concentration-dependent relationship (i.e., inhibition was greatest at the lowest concentration); it is considered negative in this assay. Atrazine exposure resulted in slightly higher LDH concentrations than in the controls.

Dimethoate is a StAR (steroid acute regulatory) protein inhibitor. Decreased testosterone concentration was observed at all 3 dimethoate concentrations in a clear, dose-response pattern. It had no effect on LDH levels. These results were clearer in Replicate 1 than Replicate 2.

Prochloraz (an aromatase inhibitor) produced concentration-related decreases in testosterone levels. The high dose significantly increased LDH and there was a significant linear component of trend.

Therefore, the assay identified all anti-androgenic chemicals regardless of sites or mechanisms of action. For these chemicals, effects on testosterone levels are clearly different in mechanism and potency from induction of increased LDH, which was used as a biomarker of cytotoxicity. Atrazine was interpreted as negative in this assay, which is consistent with its role in the central control of LH surges at ovulation in female rodents. The 2 positive control chemicals, AG for steroidogenesis and 2,4-DNP for cytotoxicity, were consistently and appropriately active for all test chemicals evaluated and between replicates per chemical.

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SOP Deviations

Deviation	Effect on Study	Reason
SOP LORET 080.02 specifies that the volume of media is not replaced. The entire volume of media was sampled and replaced.	None	The procedure was changed in the steroidogenesis optimization study.

Protocol Deviations

Deviation	Effect on Study	Reason
The technician accidently threw away the media supernatant from time point 3 for prochloraz, Replicate 1, before collecting enough for the composite sample (0.5 ml).	This will create a missing value.	Technician error.
The entire volume of media was sampled and replaced.	Prevented feedback inhibition on testosterone production.	This was changed in the optimization of the procedure.
Thirty rats were used instead of the six rats required by the protocol.	Improved variability results.	It was decided to use only the right testis from each animal to decrease variability and only 1-2 chemicals were tested in each run so more animals were necessary.
It was more than 30 days between the purity determinations and *in vitro* testing of atrazine, flutamide, and vinclozolin.	None. Purity determinations were only 20 days past 30 days.	The time from initial chemical testing until the chemical was used in the assay was longer than planned.
The concentration of 2,4-DNP was too high.	None. Formulation was diluted before use	Technical error.
The residual testing formulations were not sent back to the analytical lab since the cost of shipping and re-analysis was an issue.	None	This was a planned deviation.
The confidence intervals about the average were not calculated or reported.	None. Other measures of variability were used to assess results.	Statistical oversight.
DNP was not included in the assays for prochloraz.	None. The absence of a cytotoxicant control had no impact on interpreting the effect of prochloraz on the fragments.	It was a planned deviation and was not included because previous experiments suggested that it was not working as an effective cytotoxicant.

In the Study Director's professional opinion, these deviations did not affect the study integrity, performance, or interpretation, and are presented for completeness.

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Carol D. Sloan Date
Study Director

Appendix I

Analytical Chemistry Reports

Appendix II

Spreadsheets with Individual Data and Tables

Appendix III

Statistical Analysis

Appendix IV

Protocol and Amendments

Appendix V

QAPP and Amendments