UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

Date: September 9, 2012

SUBJECT: Propanil: In *Vitro* Rat Prostate Androgen Receptor Competitive Binding Assay

PC Code: 028201
Decision No.: N/A
Petition No.: N/A
Risk Assessment Type: N/A
TXR No.: 0051481
MRID No.: 48663401

DP Barcode: D397734
Registration No.: N/A
Regulatory Action: N/A
Case No.: N/A
CAS No.: 709-98-8
40 CFR: N/A

FROM: **Nancy McCarroll, Geneticist**
Risk Assessment Branch VI
Health Effects Division (7509 P)

THROUGH: **Felecia Fort, Chief**
Risk Assessment Branch VI
Health Effects Division (7509 P)

TO: **Joel Wolf, Risk Manager**
Risk Management and Implementation Branch 2
Pesticide Reevaluation Division (7508P)

And

Gregory Akerman, PhD, Executive Secretary
Endocrine Disruption Review Team
Health Effects Division (7509 P)

1 of 26

I. CONCLUSIONS

The *in vitro* rat prostate cytosol androgen receptor (AR) competitive binding assay (MRID 48663401) with propanil has been reviewed and classified as acceptable/non-guideline. **Propanil was found to be a weak binder to rat AR.**

II. BACKGROUND and ACTION REQUESTED

In May 2003, the Hazard Identification Assessment Review Committee (HIARC) determined that an *in vitro* androgen receptor binding assay was required to provide confirmation of the putative endocrine mode of action (MOA) for propanil. Further characterization of this potential MOA for propanil is necessary to adequately evaluate the risk to infants and children (TXR No. 0051852). In response, the Propanil Task Force II submitted the above study in support of the registration review. It is important to note that this action is independent of the endocrine screening part of the Endocrine Disruptor Screening Program (EDSP) developed by EPA. RAB VI was asked to review and prepare a DER for this study.

III. RESULTS AND DISCUSSION

The *in vitro* rat prostate cytosol androgen receptor (AR) competitive binding assay (MRID 48663401) for propanil has been reviewed. The DER is attached and the Executive Summary is as follows:

EXECUTIVE SUMMARY: In an androgen receptor (AR) binding assay (MRID 48663401) with propanil (99.6 % a.i.; Batch No. 02 Code Blue), ventral prostate cytosol isolated from Sprague Dawley rats was used as the source of AR to conduct a Saturation Binding Experiment and a Competitive Binding Experiment. The Saturation Binding Experiment was conducted to demonstrate that the AR isolated from rat prostate cytosol was present in reasonable numbers and was functioning with appropriate affinity for the radio-labeled reference androgen (^{3}H-R1881) prior to conducting the AR Competitive Binding Experiments. The Competitive Binding Assay was conducted to measure the binding of a single concentration of [^{3}H]-R1881 (final working concentration = 1 nM) in the presence of increasing concentrations of propanil (seven concentrations with logarithmic increases from 1nM to 1mM). Ethanol was used as a solvent for the reference and test materials. The assay included R1881 as the ligand reference standard, testosterone (1mM to 1 nM) as a strong positive control, and hydroxyflutamide (1 mM to 1 nM) as a weak positive control. The authors stated that corticosterone (1mM to 1 nM) was included in the study as the negative control; however, weak AR binding by corticosterone was reported in the EPA's Integrated Summary Report for the Validation of an Androgen Receptor Binding Assay as a Potential Screen in the Endocrine Disruptor Screening Program with a % relative binding affinity (%RBA) of 0.01%.

In the verification phase of testing, results from the saturation binding assay were acceptable and showed that the percent bound values were generally similar for the total specific (TSB) and nonspecific bound (NSB) [^{3}H]-R1881. The %CV was <20% for all samples with average values of 2.5% (TSB) and 8.4% (NSB). Ligand depletion ranged

from 1 to 9% and maximum binding (B_{max}) was 0.16 nM and the binding affinity (K_d) was 0.96 nM. Similarly, the concentration of [^{3}H]-R1881 (1 nM) used in this assay was within the linear region of the Scatchard plot.

The preliminary competitive binding experiment was also acceptable and showed that the percent inhibition of [^{3}H]-R1881 increased as the concentration of inert R1881, testosterone, hydroxyflutamide, or corticosterone increased; inhibition induced by these compounds was characterized as a sigmoidal concentration response. Mean IC_{50} values over the four replicate assays were 2.2 nM, 11.6 nM, 1.3 µM, and 14.4 µM for inert R1881, testosterone, hydroxyflutamide, and corticosterone, respectively. Based on these findings, the assay with propanil was performed and data from the single replicate assay indicated that the concentration that successfully displaced 50% of the reference androgen (mean IC_{50}) was 59 µM with a 95% confidence interval of 51 to 69 µM. The relative binding affinities (RBAs) for testosterone, hydroxyflutamide, corticosterone, or propanil were 21, 0.18, 0.01%, or 0.0035%, respectively. As these values indicate, the competitive binding results with propanil are much lower than those for the other inhibitors; the order of response is displayed as follows:

Testosterone > Hydroxyflutamide > corticosterone > propanil

Based on the mean IC_{50} of 59 µM with a 95% confidence interval of 51 to 69 µM, and a %RBA value of 0.0035%, it was concluded that propanil is a weak binder to the rat AR.

This study is classified as **acceptable/non-guideline.**

Primary Reviewer: Nancy McCarroll
RAB VI, Health Effects Division
Secondary Reviewer: Gregory Akerman, PhD
RAB VI, Health Effects Division

Signature: Nancy McCarroll
Date: 09/19/2012
Signature:
Date: 09/19/2012

Template version 08/2011

TXR#: 0051481

DATA EVALUATION RECORD

STUDY TYPE: Androgen Receptor Binding (Rat Prostate Cytosol); Non-guideline

PC CODE: 028201 **DP BARCODE:** 397734
CAS No.: 709-98-8

TEST MATERIAL (PURITY): Propanil (99.6 % a.i.; Batch No. 02 Code Blue)

SYNONYMS: 3', 4'-Dichloropropionanilide

CITATION: Thomas, J. A. (2008). In Vitro Rat Prostate Androgen Competitive Binding Assay of Propanil (CAS No. 709-98-8). WIL Research Laboratories, LLC, Ashland, OH. Project No.: WIL-141015, February 22, 2008. MRID 48663401. Unpublished.

SPONSOR: Propanil Task Force II, Washington, DC

EXECUTIVE SUMMARY: In an androgen receptor (AR) binding assay (MRID 48663401) with propanil (99.6 % a.i.; Batch No. 02 Code Blue), ventral prostate cytosol isolated from Sprague Dawley rats was used as the source of AR to conduct a Saturation Binding Experiment and a Competitive Binding Experiment. The Saturation Binding Experiment was conducted to demonstrate that the AR isolated from rat prostate cytosol was present in reasonable numbers and was functioning with appropriate affinity for the radio-labeled reference androgen (^{3}H-R1881) prior to conducting the AR Competitive Binding Experiments. The Competitive Binding Assay was conducted to measure the binding of a single concentration of [^{3}H]-R1881 (final working concentration = 1 nM) in the presence of increasing concentrations of propanil (seven concentrations with logarithmic increases from 1nM to 1mM). Ethanol was used as a solvent for the reference and test materials. The assay included R1881 as the ligand reference standard, testosterone (1mM to 1 nM) as a strong positive control, and hydroxyflutamide (1 mM to 1 nM) as a weak positive control. The authors stated that corticosterone (1mM to 1 nM) was included in the study as the negative control; however, weak AR binding by corticosterone was reported in the EPA's Integrated Summary Report for the Validation of an Androgen Receptor Binding Assay as a Potential Screen in the Endocrine Disruptor Screening Program with a % relative binding affinity (%RBA) of 0.01%.

In the verification phase of testing, results from the saturation binding assay were acceptable and showed that the percent bound values were generally similar for the total specific (TSB) and nonspecific bound (NSB) [^{3}H]-R1881. The %CV was <20% for all samples with average values of 2.5% (TSB) and 8.4% (NSB). Ligand depletion ranged from 1 to 9% and maximum binding (B_{max}) was 0.16 nM and the binding affinity (K_d) was 0.96 nM. Similarly, the concentration of [^{3}H]-R1881 (1 nM) used in this assay was within the linear region of the Scatchard plot.

The preliminary competitive binding experiment was also acceptable and showed that the percent inhibition of [^{3}H]-R1881 increased as the concentration of inert R1881, testosterone, hydroxyflutamide, or corticosterone increased; inhibition induced by these compounds was characterized as a sigmoidal concentration response. Mean IC_{50} values over the four replicate assays were 2.2 nM, 11.6 nM, 1.3 µM, and 14.4 µM for inert R1881, testosterone, hydroxyflutamide, and corticosterone, respectively. Based on these findings, the assay with propanil was performed and data from the single replicate assay indicated that the concentration that successfully displaced 50% of the reference androgen (mean IC_{50}) was 59 µM with a 95% confidence interval of 51 to 69 µM. The relative binding affinities (RBAs) for testosterone, hydroxyflutamide, corticosterone, or propanil were 21, 0.18, 0.01%, or 0.0035%, respectively. As these values indicate, the competitive binding results with propanil are much lower than those for the other inhibitors; the order of response is displayed as follows:

Testosterone > Hydroxyflutamide > corticosterone > propanil

Based on the mean IC_{50} of 59 µM with a 95% confidence interval of 51 to 69 µM, and a %RBA value of 0.0035%, it was concluded that propanil is a weak binder to the rat AR.

This study is classified as **acceptable/non-guideline.**

COMPLIANCE: Signed and dated Data Confidentiality statements were provided; a Quality Assurance statement was not included. The study was not conducted in compliance with GLP standards.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test facility: WIL Research Laboratories, LLC

Location:	Ashland , OH
Study Director:	J.A. Thomas
Other Personnel:	C. MacElrevey
Study Period:	Not reported

2. Test substance: Propanil

Description:	Solid in an amber glass bottle
Source:	Carolina Research Center (Snow Camp, NC)
Batch #:	02 Code Blue (Sample 02-10A)
Purity:	99.6%
Solubility:	Soluble in ethanol up to 30 mM
Stability:	Not reported but the expiration date of May 16, 2009 was listed.
Storage conditions:	Ambient temperature upon arrival; -20°C for 30 mM stock solution.
CAS #:	709-98-8

3. Non-labeled ligand: Inert R1881

Supplier:	Perkin-Elmer, Waltham, MA
Batch #:	WIL REF # CP#08-139
Purity:	>97%
CAS #:	965-93-5

4. Radioactive ligand: [^{3}H]-R1881

Supplier:	Perkin-Elmer, Waltham, MA
Batch #:	WIL REF # CP#08-140
Radiochemical purity:	98%
Specific activity:	72 Ci/mmol
Concentration in stock:	100 nM

5. Positive controls:

	Testosterone	Hydroxyflutamide
Supplier:	Sigma-Aldrich, St. Louis, MO	LKT Laboratories, St. Paul, MN
Batch #:	WIL REF # CP#08-134	WIL REF # CP#08-138
Purity:	≥98%	98%
CAS # :	58-22-0	52806-53-8

6. **Negative control:** Corticosterone

Supplier:	LKT Laboratories, St. Paul, MN
Batch #:	WIL REF # CP#08-137
Purity:	Not reported
CAS #:	50-22-6

6. **Solvent/vehicle control:**

Justification for choice of solvent: 100% Ethanol (ETOH) was selected as the solvent for all of the above as well as the test material because it is the recommended solvent for this test system and the test material formed a clear solution with no precipitate in ETOH and it remained in solution after incubation at room temperature for 1.5 hours.
Final concentration: ≈3%

B. METHODS

1. **Preparation of Rat Ventral Prostate Cytosol:** The rat ventral prostate tissue was prepared from 22 male Sprague-Dawley rats. The rats were ≈12.5 weeks of age and weighed 364-411g at time of receipt. Rats were purchased from Charles River Laboratories (Raleigh, NC) and were judged to be in good health. They were fed PMI Nutrition International, Inc. Certified Rodent Lab Diet 5002 and water *ad libitum* and held in quarantine for 1 week. After the quarantine period, the rats were anesthetized with isoflurane and castrated. Approximately 24 hours after castration, the rats were euthanized by carbon dioxide inhalation and the prostrate was removed, weighed, trimmed of all fat and placed in ice-cold TEDG buffer [10 mM TRIS buffer (pH 7.4), 10% glycerol, 1 mM sodium molybdate, 1.5 mM ethylenediaminetetra-acetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT)] until homogenization. Prostates were pooled, homogenized in 10 ml of buffer/g of tissue, and centrifuged for 30 min at 30,000 x *g* at 4°C. The supernatant (cytosolic fractions) was pooled, aliquoted in 3-mL volumes, flash frozen in liquid nitrogen, and stored at approximately -70°C. Protein concentration of the cytosol was determined on the initial day of preparation and subsequently on each day the cytosol was used for the AR binding assays. As shown below, protein concentrations ranged from 5.07 to 6.43 mg/mL using the Bradford Protein assay.

Protein Analysis Results			
Assay Description	Assay Date	Protein stock concentration (mg/mL, measured)	Protein final working concentration (mg/mL, measured)
Day of Cytosol Preparation	27 February2008	5.14	NA
Saturation Binding Assay	28 February 2008	5.07	3.93
Control Chemical Evaluation Rep 1	1 April 2008	5.14	3.81
Control Chemical Evaluation Rep 2	3 April 2008	*	4.78
Control Chemical Evaluation Rep 3	8 April 2008	6.43	4.41
Assay with Propanil	10 April 2008	5.15	4.10

* = Concentration of protein stock not measured on 3 April 2008 NA = Not applicable

Data were derived from the study report p. 26, MRID 48663401.

2. Androgen Receptor Binding Assays (The following information was extracted from the Study Report.)

a. General Method:

The prepared TEDG buffer stock solutions of [^{3}H]-R1881 and triamcinolone acetonide (a synthetic corticosteroid) were added to all test tubes. Triamcinolone acetonide was added to saturate the progesterone receptors (PR) in order to prevent [^{3}H]-R1881 from binding to the PR in the cytosol preparations. For non-specific binding (NSB) samples, a 100-fold molar excess of unlabeled (inert) R1881, ranging from 25 to 1000 nM, was added to triplicate tubes containing 0.25 to 10.0 nM [^{3}H]-R1881 (See scheme presented below). For the total binding only [^{3}H]-R1881 at the above nM concentrations was added and for the total counts samples, [^{3}H]-R1881 was added in 7.5, 15, 21, 30 or 45 µL volumes of a 10 nM solution and in 7.5, 15 or 30 µL volumes of a 100 nM solution. The solvent (ETOH) was evaporated from all tubes until dry. Test or control compound was added to each tube where appropriate and samples were chilled on ice for 5 minutes. Prostate cytosol in 300-µL volumes and diluted to the desired concentration (1.2 mg protein/300 µL in low salt TEDG buffer), was added to the ice-cold tubes. Samples were mixed and refrigerated at 4°C for 20 hours. Following incubation, 100 µL from each tube was transferred to new tubes, mixed with hydroxyapatite slurry (equilibrated with 50 mM Tris buffer, pH 7.4), held in an ice water bath, and mixed every 5 min. for an additional 20 minutes. Reaction mixtures were held at 4°C, centrifuged, and washed. Pellets were mixed and repeatedly washed in Tris buffer followed by ethanol. Duplicate aliquots (0.5 mL) of the ethanol supernatant fraction containing the bound fraction of the [^{3}H]-R1881 were transferred to 20-mL scintillation vials, mixed with 14 mL of Optifluor scintillation cocktail and counted for ^{3}H using liquid scintillation counting. Samples were counted for 10 minutes or until a 2-sigma error of 1% was achieved.

b. Androgen Receptor Binding Assay:

The activity and specificity of the AR in the rat prostate homogenate was tested prior to use in the competitive binding assays. Using the method described above, concentrations of [^{3}H]-R1881 (0.25- 10.0 nM) in the presence of 100-fold excess of unlabeled (inert) R1881 were used for the NSB determination. Ligand depletion was assessed by adding [^{3}H]-R1881 (0.25- 10.0 nM) directly to the scintillation vials to determine total binding (TB) in the absence of unlaheled (inert) R1881 and total counts (TCs) were determined from vials containing 7.5, 15, 21, 30 or 45 µL volumes of a 10 mM solution and in 7.5, 15 or 30 µL volumes of a 100 mM solution of [^{3}H]-R1881 (See scheme presented below).

8

Saturation Assay Sample Preparation			
Sample ID	^{3}H-R1881	R1881	Replicate Tubes
	(Final Concentration, nM)		
NSB1	0.25	25	3
NSB2	0.5	50	3
NSB3	0.7	70	3
NSB4	1.0	100	3
NSB5	1.5	150	3
NSB6	2.5	250	3
NSB7	5.0	500	3
NSB8	10.0	1000	3
TB1	0.25	--	3
TB2	0.5	--	3
TB3	0.7	--	3
TB4	1.0	--	3
TB5	1.5	--	3
TB6	2.5	--	3
TB7	5.0	--	3
TB8	10.0	--	3
TC1	7.5 µL of 10 nM	--	3
TC2	15 µL of 10 nM	--	3
TC3	21 µL of 10 nM	--	3
TC4	30 µL of 10 nM	--	3
TC5	45 µL of 10 nM	--	3
TC6	7.5 µL of 100 nM	--	3
TC7	15 µL of 100 nM	--	3
TC8	30 µL of 100 nM	--	3

NSB = Non-Specific Binding
TB = Total Binding
TC = Total Counts
"--" = Not Applicable

Data were derived from the study report, p19, MRID 48663401.

From these data, the density of functional ARs in the cytosol preparation (B_{max}) and the dissociation constant (K_d) for binding of the [^{3}H]-R1881 ligand to the AR were determined. Following characterization of the ligand binding properties, the verification of the competitive

AR binding was conducted using a set of reference chemicals.

c. Competitive binding assays:

The Competitive Binding Experiment was performed according to the "ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays (NIH Publication No: 03-4503)" document. Using the Androgen Receptor Binding Assay General Method, the Competitive Binding Experiment measures the binding of a single concentration of [^{3}H]-R1881 (adjusted specific activity of 72 Ci/mmol) to the AR in the presence of increasing concentrations of a test substance. In order to optimize the assay and insure consistency between experiments, the receptor concentration in the cytosolic protein homogenate was standardized prior to performance of the assay with the test substance. Consequently, validation assays were conducted before the test chemical assay was performed.

1. Reference chemicals experiment:

A series of verification assays were conducted as previously described with three reference control chemicals: Testosterone (Positive Control with a **strong** binding affinity, 10^{-3} to 10^{-9} M), Hydroxyflutamide (Positive Control with a **weak** binding affinity, 10^{-3} to 10^{-9} M) and Corticosterone (Negative Control with a **nominal** binding affinity, 10^{-3} and 10^{-4} M). The solvent alone and 10^{-6} M inert R 1881 were included. Three independent trials were performed. In the third trial, five additional concentrations of corticosterone were evaluated (total concentration range: 10^{-3} to 10^{-9} M). The organizational scheme for these assays is shown below:

10

Competition Assay Sample Organization, Days 1-3				
Sample ID	Competitor	Replicate tubes	Description	Final Concentration (M)
TC	None	1	^{3}H-R1881 added directly to scintillation vial to determine Total Counts	NA
VEH	EtOH	1	Assay with Vehicle only	NA
TSB	None	1	Assay with no vehicle or competitor to determine Total Specific Binding	NA
NSB	Radioinert R1881	2	Assay with excess Radioinert R1881 to determine Non-Specific Binding of Tracer (^{3}H-R1881)	1×10^{-6}
S1	Radioinert R1881	2	Assay with unlabeled reference androgen (Radioinert R1881) to determine IC_{50}	1×10^{-7}
S2	Radioinert R1881	2	Assay with unlabeled reference androgen (Radioinert R1881) to determine IC_{50}	1×10^{-8}
S3	Radioinert R1881	2	Assay with unlabeled reference androgen (Radioinert R1881) to determine IC_{50}	1×10^{-9}
S4	Radioinert R1881	2	Assay with unlabeled reference androgen (Radioinert R1881) to determine IC_{50}	1×10^{-10}
S5	Radioinert R1881	2	Assay with unlabeled reference androgen (Radioinert R1881) to determine IC_{50}	1×10^{-11}
T1	Testosterone	2	Assay with positive control chemical 1	1×10^{-3}
T2	Testosterone	2	Assay with positive control chemical 1	1×10^{-4}
T3	Testosterone	2	Assay with positive control chemical 1	1×10^{-5}
T4	Testosterone	2	Assay with positive control chemical 1	1×10^{-6}
T5	Testosterone	2	Assay with positive control chemical 1	1×10^{-7}

11

Competition Assay Sample Organization, Days 1-3 (continued)				
Sample ID	Competitor	Replicate tubes	Description	Final Concentration (M)
T6	Testosterone	2	Assay with positive control chemical 1	1×10^{-8}
T7	Testosterone	2	Assay with positive control chemical 1	1×10^{-9}
H1	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-3}
H2	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-4}
H3	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-5}
H4	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-6}
H5	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-7}
H6	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-8}
H7	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-9}
C1	Corticosterone	2	Assay with negative control chemical	1×10^{-3}
C2	Corticosterone	2	Assay with negative control chemical	1×10^{-4}
VEH	EtOH	1	Assay with Vehicle Only	NA
TSB	None	1	Assay with no vehicle or competitor to determine Total Specific Binding	NA
NSB	Radioinert R1881	2	Assay with excess Radioinert R1881 to determine Non-Specific Binding of Tracer (^{3}H-R1881)	1×10^{-6}
TC	None	1	^{3}H-R1881 added directly to scintillation vial to determine Total Counts	NA
NA = Not applicable				

Data were extracted from the study report, pp. 20 and 21, MRID 48663401.

2. Test chemical experiment:

After the verification and preliminary phase of the analysis was completed, the test material was evaluated in a single trial using triplicate samples at each test material concentration. A summary of the assay conditions for the Competitive Binding Experiment with propanil is presented in Table 1. Ethanol was used as a vehicle, based on the solubility of the test material in this solvent. The test material was evaluated over a concentration range of 10^{-3} to 10^{-9} M along with the solvent (ETOH), positive controls (testosterone and hydroxyflutamide, 10^{-3} to 10^{-9} M), the negative control (corticosterone, 10^{-3} and 10^{-4} M), and internal assay controls. Triplicate samples were prepared for the test material dilutions and duplicate samples for the positive and negative controls. Based on the findings of the preliminary work, the concentration of [^{3}H]-R1881 used in this assay was 1 nM.

TABLE 1. Summary of Conditions for Competitive Binding Experiment with Propanil		
Source of receptor		Rat ventral prostate cytosol
Concentration of radioligand		1 nM
Optimization of receptor concentration		Sufficient to bind 5% of 1.0 nM radioligand
Concentration of test substance (as serial dilutions)		10^{-10} to 10^{-3} mM
Incubation Temperature		4°C
Incubation time		20 hours
Composition of assay buffer	Tris	0.01 M (pH 7.4)
	EDTA	1.5 mM
	Sodium molybdate	1 mM
	Glycerol	10%
	Phenylmethylsulfonyl fluoride	1 mM
	DTT	1 mM

The assay scheme for the competitive binding assay with propanil is presented below:

Competition Assay Sample Preparation, Day 4				
Sample ID	Competitor	Replicate tubes	Description	Final Concentration (M)
VEH	EtOH	1	Assay with Vehicle Only	NA
TSB	None	1	Assay with no vehicle or competitor to determine Total Specific Binding	NA
NSB	Radioinert R1881	2	Assay with excess Radioinert R1881 to determine Non-Specific Binding of Tracer (^{3}H-R1881)	1×10^{-6}
S1	Radioinert R1881	2	Assay with unlabeled reference androgen (Radioinert R1881) to determine IC_{50}	1×10^{-7}
S2	Radioinert R1881	2	Assay with unlabeled reference androgen (Radioinert R1881) to determine IC_{50}	1×10^{-8}
S3	Radioinert R1881	2	Assay with unlabeled reference androgen (Radioinert R1881) to determine IC_{50}	1×10^{-9}
S4	Radioinert R1881	2	Assay with unlabeled reference androgen (Radioinert R1881) to determine IC_{50}	1×10^{-10}
S5	Radioinert R1881	2	Assay with unlabeled reference androgen (Radioinert R1881) to determine IC_{50}	1×10^{-11}
T1	Testosterone	2	Assay with positive control chemical 1	1×10^{-3}
T2	Testosterone	2	Assay with positive control chemical 1	1×10^{-4}
T3	Testosterone	2	Assay with positive control chemical 1	1×10^{-5}
T4	Testosterone	2	Assay with positive control chemical 1	1×10^{-6}
T5	Testosterone	2	Assay with positive control chemical 1	1×10^{-7}
T6	Testosterone	2	Assay with positive control chemical 1	1×10^{-8}
T7	Testosterone	2	Assay with positive control chemical 1	1×10^{-9}
NA = Not applicable				

Competition Assay Sample Preparation, Day 4 (continued)				
Sample ID	Competitor	Replicate tubes	Description	Final Concentration (M)
H1	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-3}
H2	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-4}
H3	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-5}
H4	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-6}
H5	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-7}
H6	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-8}
H7	Hydroxy-flutamide	2	Assay with positive control chemical 2	1×10^{-9}
C1	Corticosterone	2	Assay with negative control chemical	1×10^{-3}
C2	Corticosterone	2	Assay with negative control chemical	1×10^{-4}
P1	Propanil	3	Assay with test chemical	1×10^{-3}
P2	Propanil	3	Assay with test chemical	1×10^{-4}
P3	Propanil	3	Assay with test chemical	1×10^{-5}
P4	Propanil	3	Assay with test chemical	1×10^{-6}
P5	Propanil	3	Assay with test chemical	1×10^{-7}
P6	Propanil	3	Assay with test chemical	1×10^{-8}
P7	Propanil	3	Assay with test chemical	1×10^{-9}
VEH	EtOH	1	Assay with Vehicle Only	NA
TSB	None	1	Assay with no vehicle or competitor to determine Total Specific Binding	NA
NSB	Radioinert R1881	2	Assay with excess Radioinert R1881 to determine Non-Specific Binding of Tracer (^{3}H-R1881)	1×10^{-6}
NA = Not applicable				

d. **Data analysis**: AR binding was calculated from the average disintegrations per minute (dpm)/mL of the ETOH extract. Total sample dmps were then calculated by multiplying the average dpm/mL by the full ETOH extraction volume (1.5 mL). Specific binding was calculated as the TB-NSB.

1. Saturation binding analysis:

For the saturation binding assay, the total dmp values were converted to nanomolar concentrations of [^{3}H]-R1881 based on the manufacture's reported specific activity (72 Ci/mmol) and a final volume of 300 µL. Ligand depletion was analyzed by calculating the dpm ratio of total binding (TB) to total counts (TC) for each concentration of [^{3}H]-R1881.

15

The initial saturation binding curve was generated by plotting the final molar concentration of total [^{3}H]-R1881bound (y axis) versus the molar concentration of [^{3}H]-R1881 added to the samples (x axis). The hyperbolic curve was then fit to the following equation for one-site binding using GraphPad Prism (Ver. 5, GraphPad Software, Inc., San Diego, CA):

$$\text{Specific Binding} = \frac{B_{max} \times X}{X + K_d}$$

$$\text{Non-specific Binding} = NS \times X$$

Where X = The test material and NS = The slope of the linear NSB

Estimates of the K_d (dissociation constant for the binding affinity of the ligand to the receptor) and the B_{max} (maximal binding capacity of the actual number of receptors) were determined using GraphPad Prism version 5.

For visual analysis of the non-linear ligand binding data, a Scatchard plot analysis was also performed on the saturation data plotting bound ligand (x axis) versus bound/free ligand (y axis).

Performance standards for the saturation binding assay included: 1) NSB should not be >50% of the TB; 2) TC for [^{3}H]-R1881 bound should not exceed 10% of the total concentration present; and 3) the K_d should falls within the range of 0.81 to 0.97nM.

2. Competitive binding analysis:

For the competitive binding assay, the amounts of radioactivity (dpm) were directly compared without conversion to concentration. Each sample was corrected for NSB. The corrected values were then divided by the average dpm of the vehicle control samples to yield the fraction bound. Ligand depletion was evaluated by calculating the ratio between average dpm in the vehicle control and the TC samples. The influence of the vehicle was assessed by the ratio between vehicle control and TB samples. A single solvent (ETOH) was used to solubilize all compounds. Specific binding (% bound) was calculated as shown below:

$$\%\ \text{Bound} = \frac{\text{TB (in presence of the inhibitor)} - \text{NSB}}{\text{Average TB in Solvent Control}} \times 100$$

Intra-assay variability was assessed by calculating the standard error of the mean (SEM) and %CV for the duplicate or triplicate (propanil) % bound values. SEM and %CV values were also used to assess inter-assay variability of mean value of duplicate samples from each individual competition assay.

To generate a competitive binding curve, the percent [^{3}H]-R1881bound (y-axis) was

plotted versus the log of the M concentration of the inhibitor (x-axis) using GraphPad Prism for curve fitting.

The inhibition concentration (IC_{50}) for each inhibitor was determined using Graph Pad Prism to fit the resultant sigmoidal curve. The curve was fit to the following 4 parameter equation to determine the IC50 (inflection point), slope, and upper and lower asymptotes:

$$Y= \frac{\text{Bottom} + [\text{Top} - \text{Bottom}]}{(1 + 10^{((\text{LogIC50-X}) \times \text{Hill Slope})})}$$

The percent relative binding affinity (RBA) was determined as:

$$\%\ \text{RBA} = \frac{IC_{50}\ (\text{Inert R1881})}{IC_{50}\ (\text{Competitor})} \times 100$$

The overall IC50 reported for each chemical was the arithmetic mean of the IC50 values reported for each individual assay and includes the SEM and %CV. Coefficient of determination values (R^2) were reported

Competitive binding assay performance standards included:

Assay validity: The assay was considered valid if the following criteria were met: 1) Inhibition must be greater than 50% at the highest positive control concentration in at least 3 runs; 2) the binding curve for the positive control compounds must be S-shaped, with a slope ≈-1, 3) NSB should be <10% of the total ligand concentration; 4) the solvent control should not alter the binding curves in the control and the test article concentrations; and 5) the %CV for the positive controls, should be ≥20% between replicates and ≥25% between runs (days).

Positive response: The test material will be classified as negative (non-binder) for AR bindings if >25% of [^{3}H]-R1881 is displaced at any concentration of the test material. Binding will be considered equivocal if >25% but <50% of [^{3}H]-R1881 is displaced at any concentration of the test material. Positive inhibition will be indicated if >50% of [^{3}H]-R1881 is displaced. Inhibition may alternately reported as 'weak" or strong'

II. RESULTS (The following was stated in the study report)

17

A. **SATURATION BINDING EXPERIMENT**: A single saturation binding assay was performed and classified as acceptable (*i.e.*, saturation was achieved within the range of concentrations tested; NSB was <10% of the total ligand concentration; ligand depletion was <10%; and the K_d was within the range of 0.81 to 0.97 nM). As shown in the following Table, the % bound values from triplicate samples were generally similar for the specific and nonspecific bound [^{3}H]-R1881. The %CV was <20% for all samples with average values of 2.5% (TBS) and 8.4% (NBS).

Intra-Assay Variability of the Concentration of ^{3}H-R1881 Bound to the Androgen Receptor

Sample Type	Initial [^{3}H-R1881] (nM)	Bound [^{3}H-R1881] (nM)			Overall		
		Tube 1	Tube 2	Tube 3	Mean	SEM	%CV
TB	0.25	0.0274	0.0259	0.0256	2.63%	0.58%	3.60%
	0.50	0.0532	0.0527	0.0513	5.24%	0.43%	1.89%
	0.70	0.0714	0.0701	0.0688	7.01%	0.48%	1.80%
	1.00	0.0902	0.0912	0.0852	8.89%	1.09%	3.64%
	1.50	0.1080	0.1066	0.1035	10.60%	0.71%	2.17%
	2.50	0.1220	0.1273	0.1212	12.35%	0.95%	2.69%
	5.00	0.1362	0.1421	0.1392	13.92%	0.80%	2.14%
	10.00	0.1531	0.1496	0.1559	15.29%	0.80%	2.04%
NSB	0.25	0.0023	0.0029	0.0022	0.24%	0.77%	15.68%
	0.50	0.0027	0.0029	0.0025	0.27%	0.43%	8.40%
	0.70	0.0026	0.0028	0.0032	0.29%	0.53%	9.90%
	1.00	0.0037	0.0039	0.0045	0.41%	0.70%	10.92%
	0.50	0.0039	0.0038	0.0046	0.41%	0.66%	10.30%
	2.50	0.0054	0.0054	0.0056	0.55%	0.18%	2.48%
	5.00	0.0072	0.0085	0.0073	0.77%	0.82%	9.40%
	10.00	0.0119	0.0124	0.0129	1.24%	0.44%	3.97%

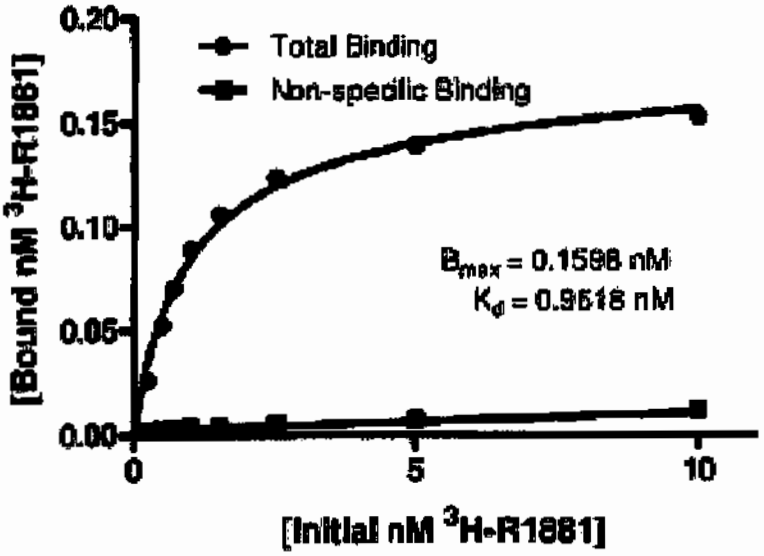
TB = Total Binding
NSB = Non-Specific Binding

Data were extracted from the study report, p. 28, MRID 48663401

18

Similarly, ligand depletion ranged from 1 to 9%. As shown in this Figure 1, saturation of the AR occurred at concentrations of [^{3}H]-R1881 above 5 nM. Figure 1 shows that the maximum binding (B_{max}) was 0.16 nM and the K_d was 0.96 nM. The determined Kd value for [^{3}H]-R1881 is within the range presented in the EPA Validation Report for the AR Binding Assay (0.8121-0.9698 nM)[1].

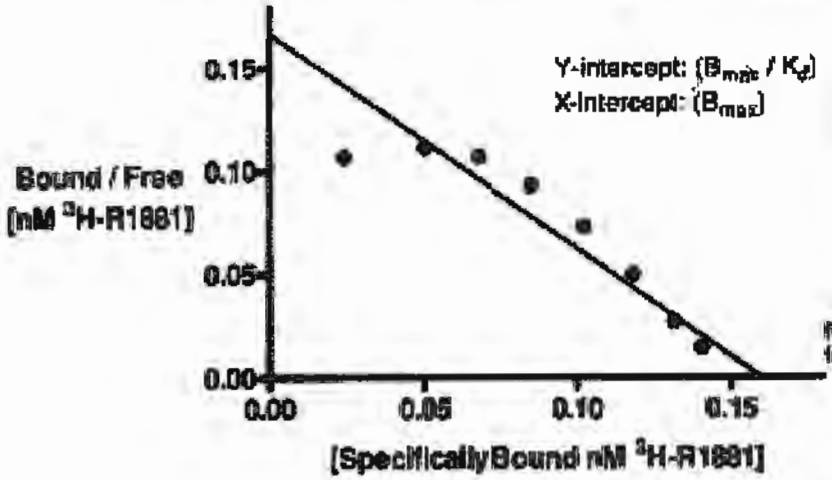
Figure 1. Saturation Assay: Binding of ^{3}H-R1881 to the Androgen Receptor



A clear deviation from linearity was observed when the saturation binding data were presented as a Scatchard plot (Figure 2). The study author suggests that the deviation is indicative of ligand or receptor heterogeneity which may result from dimerization of the AR upon binding to the ligand. It should be noted that Bmax and Kd values were calculated using nonlinear regression (as discussed above) and were not determined from the Scatchard plot. The plot does show that the concentration of [^{3}H]-R1881 (1nM) used in this assay was within the linear region of the Scatchard plot.

1 U.S. EPA (2007). Integrated Summary Report for the Validation of an Androgen Receptor Binding Assay as a Potential Screen in the Endocrine Disruptor Screening Program. U.S. EPA Office of Science Coordination and Policy, Wash. D.C. Nov. 7, 2007.

Figure 2. Saturation Assay: Scatchard Plot



nM Bound	Bound/Free
0.023858	0.106697
0.049639	0.111350
0.067228	0.106725
0.084805	0.093075
0.101915	0.073111
0.118055	0.049679
0.131485	0.027052
0.140455	0.014285

Free = Initial - Non-specific - Specific for each concentration.

B. COMPETITIVE BINDING EXPERIMENT: Three independent runs of the competitive binding experiments were performed and classified as acceptable (*i.e.*, inhibition of [^{3}H]-R1881 was <50% at the highest positive control concentration; the binding curve for the positive control compounds was S-shaped, with a slope of ≈-1; NSB was <50% of the total ligand concentration; and ETOH did not alter the binding curves of the controls or the test material). Figures 3 thru 6 show that the percent inhibition of 1 nM [^{3}H]-R1881 increases as the concentration of inert R1881 and as the reference chemicals testosterone, hydroxyflutamide, or corticosterone increases; inhibition induced by these compounds was characterized as a sigmoidal concentration response. The mean values are presented in the following Table and graphically illustrated in Figures 4- 6; these findings indicate that the mean IC_{50} values over the 4 days of testing were 2.2 nM (inert R1881), 11.6 nM (testosterone), 1.3 μM (hydroxyflutamide), and 14.4 μM (corticosterone). The %RBA, as shown below for testosterone, hydroxyflutamide and corticosterone were 21, 0.18 and 0.01%, respectively.

Graphic results for propanil are presented in Figure 7. In agreement with the above findings, the percent inhibition of [^{3}H]-R1881 increased as the concentration of propanil increased and the induced inhibition was characterized as a sigmoidal concentration response. The mean IC_{50} value for the single assay was 59 μM with a 95% confidence interval of 51 to 69 μM. The %RBA for propanil was 0.0035%. The comparative binding activity levels was displayed by the test material and the controls as shown below and presented graphically in Figure 8.

IC_{50} and Slope Results											
Test Chemical	Day	Log [IC_{50}]	s.e. log [IC_{50}]	IC_{50} (nM)	Slope	R^2	% RBA	Overall			
								IC_{50} (nM)	SEM	%CV	% RBA
R1881	1	-8.69	0.039	2.04	-1.032	0.9981	NA	2.21	0.23	15.70%	NA
	2	-8.57	0.026	2.73	-1.111	0.9991					
	3	-8.70	0.027	2.00	-0.924	0.9992					
	4	-8.69	0.032	2.07	-0.980	0.9988					
Testosterone	1	-8.04	0.042	9.06	-0.904	0.9986	22.51%	12.64	1.48	41.63%	21.00%
	2	-8.14	0.050	7.23	-0.786	0.9991	37.71%				
	3	-7.75	0.027	17.70	-1.066	0.9985	11.29%				
	4	-7.78	0.028	16.55	-0.976	0.9987	12.48%				
Hydroxy-flutamide	1	-5.86	0.024	1375	-0.849	0.9991	0.15%	1287.53	8.71	24.27%	0.18%
	2	-5.85	0.029	1423	-0.766	0.9989	0.19%				
	3	-6.08	0.288	828	-0.467	0.9559	0.24%				
	4	-5.82	0.212	1524	-0.562	0.9660	0.14%				
Corticosterone	3	-4.84	0.060	14450	-0.898	0.9956	0.01%				
Propanil	4	-4.23	0.032	59140	-1.120	0.9961	0.0035%				

Data were extracted from the study report p.34, MRID 48663401.

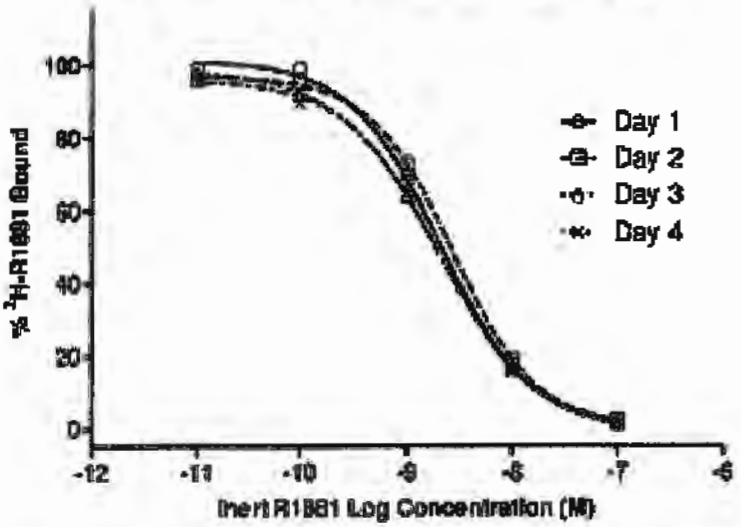
Binding order as ranked by %RBA:

Testosterone > Hydroxyflutamide > Corticosterone > Propanil

The study author stated that the order of AR binding for the first three chemicals is supported by the data in the ICCVAM document (NIH Publication No. 03-4503, May 2003).

21

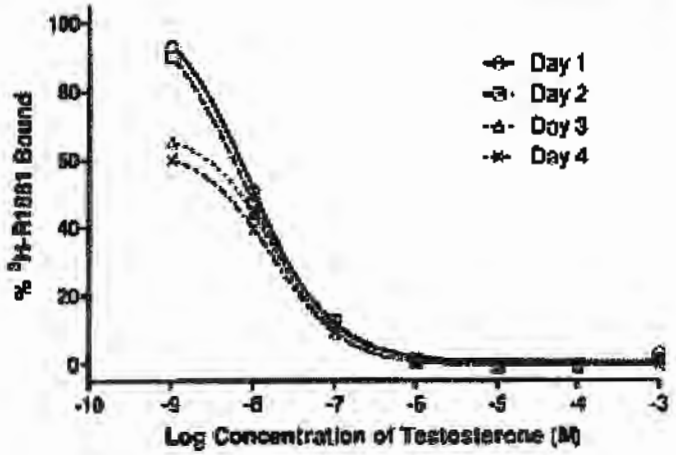
Figure 3. Competition Assay: Standard Curve (inert R1881), Days 1-4



	Day 1	Day 2	Day 3	Day 4
BOTTOM	0.4611	0.5987	-1.524	-0.6429
TOP	101.2	96.88	98.21	95.93
LOGIC50	-8.690	-8.565	-8.699	-8.665
HILLSLOPE	-1.092	-1.111	-0.9244	-0.9798
IC50	2.043e-009	2.725e-009	1.998e-009	2.086e-009
Span	100.8	96.29	99.74	96.57

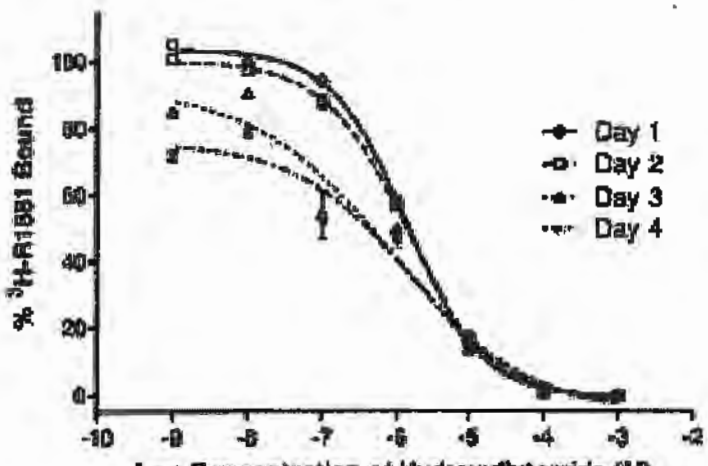
22

Figure 4. Competition Assay: Testosterone Days 1-4



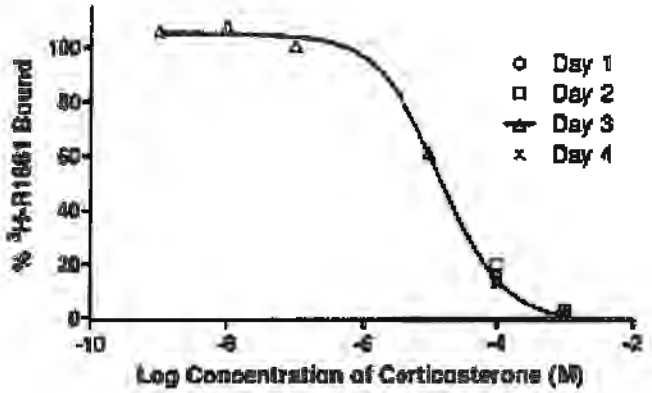
	Day 1	Day 2	Day 3	Day 4
BOTTOM	0.6102	-0.2650	0.01401	-0.2003
TOP	105.8	109.2	66.28	64.05
LOGIC50	-8.043	-8.141	-7.752	-7.781
HILLSLOPE	-0.9044	-0.7855	-1.056	-0.9764
IC50	9.053e-009	7.227e-009	1.770e-008	1.655e-008
Span	105.2	109.5	66.27	64.25

Figure 5. Competition Assay: Hydroxyflutamide Days 1-4



	Day 1	Day 2	Day 3	Day 4
BOTTOM	-0.6235	-1.526	-6.033	-3.902
TOP	103.7	100.3	92.31	75.73
LOGIC50	-5.862	-5.847	-6.062	-5.817
HILLSLOPE	-0.8485	-0.7657	-0.4672	-0.5623
IC50	1.375e-006	1.423e-006	8.680e-007	1.524e-006
Span	104.4	101.8	98.34	79.63

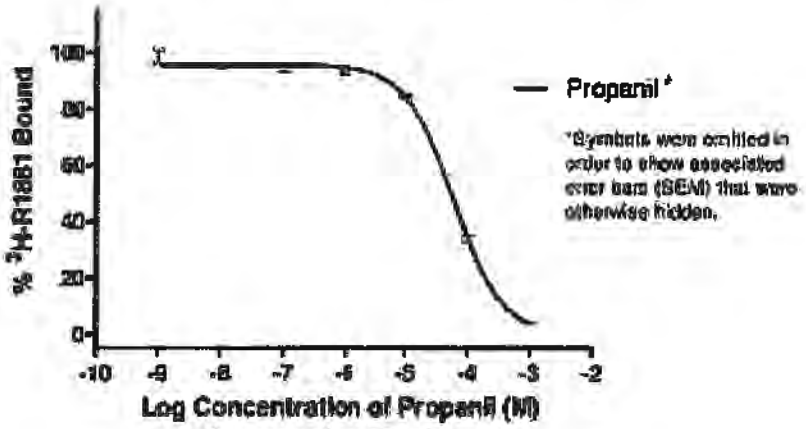
Figure 6. Competition Assay: Corticosterone Days 1-4



Day 3

BOTTOM	[illegible]
TOP	105.2
LOGIC50	-4.840
HILLSLOPE	-0.8962
IC50	1.445e-005
Span	107.0

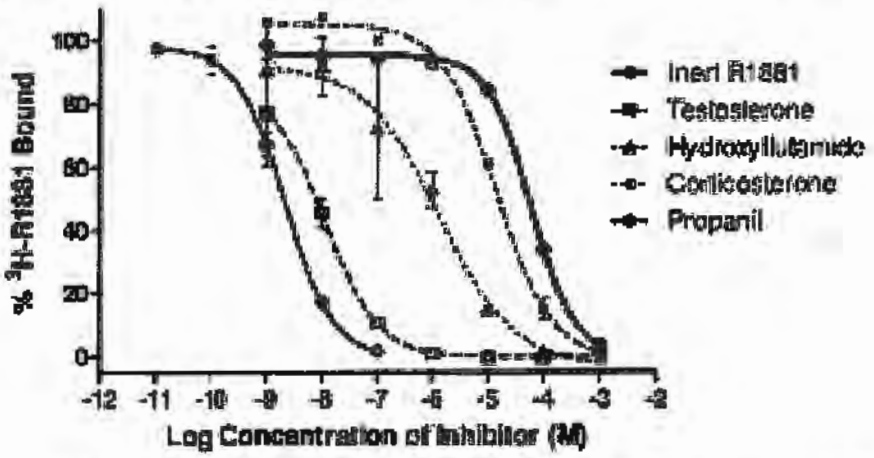
Figure 7. Competition Assay: Propanil Day 4



Summary of best fit values

BOTTOM	-0.09236
TOP	95.54
LOGIC50	-4.229
HILLSLOPE	-1.129
IC50	5.914e-005
Span	95.63

Figure 6. Competition Assay:Summary of Mean Results



	Standard Curve	Testosterone	Hydroxyflutamide	Corticosterone	Propanil
BOTTOM	-0.2425	0.02387	-3.004	-1.070	-0.09236
TOP	97.93	85.47	92.15	105.3	95.54
LOGIC50	-8.658	-7.937	-5.872	-4.949	-4.228
HILLSLOPE	-1.011	-0.9112	-0.6492	-0.6740	-1.120
IC50	2.199e-009	1.157e-008	1.343e-006	1.496e-005	5.914e-005
Span	98.16	85.45	95.16	106.4	95.63

III. DISCUSSION AND CONCLUSIONS

A. **INVESTIGATOR'S CONCLUSIONS:** The investigator concluded that the rat prostate cytosol was successfully prepared and characterized, then used to determine the potential of propanil to competitively bind to the AR. "Results from these experiments suggest that propanil is a very weak, but viable substrate for the rat AR. Propanil displaced 97% of the reference androgen when present in million-fold excess. The concentration that successfully displaced 50% of the reference androgen (IC_{50}) was 59 µM in this study. This corresponds to a %RBA of 0.0035%, 1 order of magnitude lower than the value measured for corticosterone".

B. **REVIEWERS' COMMENTS:** The reviewers agree that the rat cytosolic preparations contained AR in sufficient numbers and with adequate affinity to be used in AR competitive binding assays. All three reference control chemicals used in the validation of the Competitive Binding Experiment produced results that were consistent with the values generated either by ICCVAM or EPA for these chemicals:

25

Percent Relative Binding Affinity (% RBA)		
Test Chemical	**Reference Values**	**Current Assay**
Testosterone	0.45-125 (34%)[a]	21%
Hydroxyflutamide	0.00143-1.75 (0.585%)[a]	0.18%
Corticosterone	0.000068%[a]	0.01%
	0.018%[b]	
Propanil	-	0.0035%

[a] Value reported by ICCVAM (2003)[2].
[b] Value reported by EPA (2007)[3].

These data provide confidence in the ability of the test system to detect agents that competitively bind to the AR. Accordingly, the concentration of propanil that successfully displaced 50% (IC_{50}) of the reference androgen (R1881) was 59 µM in this study. This corresponds to a %RBA of 0.0035%, which is one order of magnitude lower than the value measured for corticosterone in the current assay and the reference value provided by EPA. However, the propanil %RBA is two orders of magnitude (515 X) higher than the ICCVAM reference %RBA value for corticosterone. Based on these considerations and in conjunction with the data indicating that propanil displaced 97% of the reference androgen with a %RBA value of at least one magnitude lower than corticosterone, we concluded that propanil is a very weak AR binder in this well-conducted test system. This study is, therefore, classified as **acceptable/non-guideline**.

C. <u>STUDY DEFICIENCIES</u>: None

[2] ICCVAM Background Review Document. Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Androgen Receptor Binding Assays. NIH Publication 03-4506, October 2002.
[3] U.S. EPA (2007). Integrated Summary Report for the Validation of an Androgen Receptor Binding Assay as a Potential Screen in the Endocrine Disruptor Screening Program. U.S. EPA Office of Science Coordination and Policy, Wash. D.C. Nov. 7, 2007.