CeeTox

Androgenic Receptor Transactivation Activity in MDA-kb2

Final Report

AUTHOR(S): [redacted]

STUDY COMPLETION DATE: April 23, 2013

TEST FACILITY:
CeeTox, Inc.
4717 Campus Drive
Kalamazoo, MI 49008
USA

LABORATORY PROJECT ID: Study Number: 9070-100794ARTA
Human and Health Science No. HHSN273200900005C
NIEHS Contract No. N01ES00005

SPONSOR(S):
NIEHS
National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle Park, NC 27709 USA

STUDY MONITOR: [redacted]
(ILS, Inc, Durham, NC)

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

This page is intentionally left blank.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Study Number: 9070-100794ARTA

Study Title: Androgenic Receptor Transactivation Activity in MDA-kb2

I, the undersigned, hereby declare that this study was performed in accordance with the Environmental Protection Agency (EPA) Good Laboratory Practice (GLP) regulations (Title 40 Part 160 with the exception of section 160.113. Dose concentrations of test and control substances were not verified using analytical methods.

The study was conducted according to the procedures herein described and this report represents a true and accurate record of the results obtained. There were no deviations that impacted the quality or integrity of the study data. Any deviations that occurred during the course of the study were noted in this report, with the full write-ups included in the study binder.

[redacted] _____ 23 Apr 2013

Study Director Date

FLAGGING STATEMENT

This page is intentionally left blank.

QUALITY ASSURANCE STATEMENT

Study Title: Androgenic Receptor Transactivation Activity in MDA-kb2

Study Number: 9070-100794ARTA

In accordance with CeeTox, Inc.'s policies and Quality Assurance standard operating procedures for Good Laboratory Practice (GLP), the conduct of this study has been audited as follows:

Date(s) of Inspection/Audit	Inspection/Audit	Date(s) reported to Study Director	Date(s) reported to Management
03Jan2013	Draft Protocol Audit	03Jan2013	03Jan2013
05Feb2013	In-Process Audit (Test sub prep and dosing)	11Feb2013	11Feb2013
08Feb2013	In-Process Audit (PI assay)	11Feb2013	11Feb2013
15Feb2013	In-Process Audit (Luciferase assay)	18Feb2013	18Feb2013
15Mar2013	Data Binder	15Mar2013	15Mar2013
15Mar2013	Draft Report	15Mar2013	15Mar2013

The signature below indicates the summary table is an accurate representation of Quality Assurance's involvement with this study.

[redacted] 23Apr2013

Date

Quality Assurance Auditor
4717 Campus Drive
Kalamazoo, MI 49008

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
[redacted]	Study Director
	Laboratory Manager
	Cell Culture Scientist II
	Senior Scientist
	Associate Scientist I
	Research Associate
	Director of Project Management

Study Dates

Study initiation date: January 16, 2013
Experimental start date: February 5, 2013
Experimental termination date: February 15, 2013
Study termination date: April 23, 2013

Deviations from the Protocol

There were no deviations.

Other

All original data [including the original signed study protocol and all amendments, test substance information, observations, etc.] and the original final report will be transferred to the National Toxicology Program Archives following finalization of the study report to the address below:

NTP Archives
[redacted]
615 Davis Drive, Suite 300
Durham, NC 27713

TABLE OF CONTENTS

1.0 EXECUTIVE SUMMARY

1.1 Study Design

The purpose of this study was to analyze ensulizole, avobenzone, homosalate and padimate O for androgenic transactivation activity using the MDA-kb2 reporter cell line. The MDA-kb2 cell line was derived from a human breast cancer line transfected with an androgen receptor promoter linked to a luciferase gene. Consequently, the MDA-kb2 cell line can measure the ability of a test substance to induce (agonism) or antagonize Androgen Receptor (AR) mediated transactivation via luciferase gene expression. Cell viability was monitored by a two-read propidium iodide (PI) uptake assay.

Two runs were conducted on ensulizole, avobenzone, homosalate and padimate O. The final concentrations for ensulizole were: $10^{-7.5}$, 10^{-7},$10^{-6.5}$, 10^{-6}, $10^{-5.5}$, 10^{-5}, $10^{-4.5}$ and 10^{-4} M for runs 1 and 2 (05-February-2013 and 07-February-2013). The final concentrations for avobenzone, homosalate and padimate O were: $10^{-6.5}$, 10^{-6}, $10^{-5.5}$, 10^{-5}, $10^{-4.5}$, 10^{-4}, $10^{-3.5}$ and 10^{-3} M for runs 1 and 2 (05-February-2013 and 07-February-2013). Every run contained one agonist plate, one antagonist plate, and one cytotoxicity plate for each substance tested.

Solubility was determined by nephelometry for runs 1 and 2 (05-February-2013 and 07-February-2013).

For agonist plates, all concentrations were tested in replicates of 6/plate, with the addition of 2 replicates/plate that incorporated the antagonist nilutamide which is used as a CeeTox internal control. Replicates incorporating the nilutamide allow for the identification of non-specific (i.e., non-androgen receptor mediated) induction of the luciferase gene.

For antagonist plates, all test substance concentrations included four replicates with 1 nM DHT and four replicates with 1000 nM DHT. Replicates incorporating 1000 nM DHT allowed for the identification of assay interference.

For cytotoxicity plates, all concentrations were tested in replicates of 6/plate, with the addition of 2 replicates/plate that incorporated digitonin. Replicates incorporating digitonin allow for the identification of assay interference.

The duration of exposure was 24 hours. A complete concentration response curve for each of 3 reference compounds (dihydrotestosterone (DHT), nilutamide (NIL) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (p,p'-DDE)) was run each time the transcriptional activation assay was performed.

1.2 Results

Solubility was determined by nephelometry for runs 1 and 2 (05-February-2013 and 07-February-2013). Visual observations were also noted.

The top concentration for ensulizole in runs 1 and 2 (05-February-2013 and 07-February-2013) was 10^{-4} M. Precipitation was not observed in ensulizole.

The top concentration for avobenzone in runs 1 and 2 (05-February-2013 and 07-February-2013) was 10^{-3} M. Precipitation was observed at 10^{-4}, $10^{-3.5}$ and 10^{-3} M in avobenzone in runs 1 and 2 (05-February-2013 and 07-February-2013).

The top concentration for homosalate in runs 1 and 2 (05-February-2013 and 07-February-2013) was 10^{-3} M. Precipitation was observed at 10^{-4} M in homosalate in run 1 (05-February-2013) and at 10^{-4} and $10^{-3.5}$ M in run 2 (07-February-2013). Homosalate was also visually observed for precipitation in run 2 (07-February-2013) and noted as oily at the concentrations $10^{-3.5}$ and 10^{-3} M.

The top concentration for padimate O in runs 1 and 2 (05-February-2013 and 07-February-2013) was 10^{-3} M. Precipitation was observed at 10^{-4} M in padimate O in run 1 (05-February-2013) and at 10^{-4} and $10^{-3.5}$ M in run 2 (07-February-2013). Padimate O was also visually observed for precipitation in run 2 (07-February-2013) and noted as oily at the concentrations $10^{-3.5}$ and 10^{-3} M.

Cytotoxicity (≥20% reduction in cell viability) was observed in ensulizole at $10^{-4.5}$ M in the second run (07-February-2013). Cytotoxicity was noted in avobenzone at $10^{-4.5}$, 10^{-4}, $10^{-3.5}$ 10^{-3} M in runs 1 and 2 (05-February-2013 and 07-February-2013). Cytotoxicity was observed in homosalate at $10^{-3.5}$ and 10^{-3} M in the first run (05-February-2013). Cytotoxicity was observed in padimate O at 10^{-4}, $10^{-3.5}$, 10^{-3} M in the second run (07-February-2013).

In all independent runs of the agonist transcriptional activation assay, these test substances (ensulizole, avobenzone, homosalate and padimate O) did not result in an increase in luciferase activity at any of the viable soluble concentrations tested (RPC_{max}<20%).

In all independent runs of the antagonist transcriptional activation assay, ensulizole, avobenzone and padimate O did not result in a differential between the high antagonism and the low antagonism of greater than 50% at more than one viable soluble concentration.

In the antagonist transcriptional activation assay, homosalate did result in an average differential between the high antagonism and the low antagonism of greater than 50%. The average differential was 60% (28% run 1 and 92% run 2 at -4.5 M). A dose response of more than one viable soluble concentration was noted in run 2 (20%, 42%, 51% and 92% at concentrations 10^{-6}, $10^{-5.5}$, 10^{-5}, $10^{-4.5}$ M).

1.3 Conclusion

Ensulizole, avobenzone and padimate O do not demonstrate agonism or antagonism of AR mediated transactivation when tested in the MDA-kb2 cell model system. Homosalate does not demonstrate agonism, however there was a exposure dependent antagonism of AR-mediated transactivation when homosalate was tested in the MDA-kb2 cell model system.

2.0 INTRODUCTION

2.1 Purpose

The purpose of this study was to analyze ensulizole, avobenzone, homosalate and padimate O for androgenic transactivation activity using the MDA-kb2 reporter cell line. The MDA-kb2 cell line is derived from a human breast cancer line transfected with an androgen receptor promoter linked to a luciferase gene. Consequently, the MDA-kb2 cell line can measure the ability of a test substance to induce (agonism) or antagonize AR mediated transactivation via luciferase gene expression.

The MDA-kb2 cell line is derived from human breast cancer cells. These cells were transformed with an androgen responsive luciferase reporter plasmid driven by the mouse mammary tumor virus promoter (MMTV). The MMTV promoter was chosen for transformation because it is a robust viral promoter and is well characterized as being androgen responsive. Consequently, the MDA-kb2 cell line can measure the ability of a test substance to induce AR-mediated transactivation of luciferase gene expression, i.e., the cell line can be used to assess the ability of a test substance to act as an agonist of AR. Antagonism can be distinguished by the differential ellicited from the co-administration of the test article and AR agonist DHT at a high concentration (1000 nM) versus the co-administration of the test article and the AR agonist DHT at a low concentration (1 nM).

2.2 Regulatory Citations

Currently this assay has not been validated as part of the EDSP Tier 1 testing program and is not mandated.

3.0 MATERIALS AND METHODS

3.1 Test Substance

3.1.1 Test substance details

Test Substance Name:	2-Phenyl-5-benzimidazolesulfonic Acid (Ensulizole)
Test Substance Supplier:	Aldrich
CAS Number:	27503-81-7
Description:	White to off white powder
Solvent Used:	Dimethyl sulfoxide
Batch Number:	05117JE
Expiry Date:	Not provided
Purity:	99.6%
Molecular Formula:	$C_{13}H_{10}N_2O_3S$
Molecular Weight:	274.30 g/mol

Storage Conditions:	Room Temp. (eg. ambient)

Test Substance Name:	Butyl-methoxydibenzoylmethane (Avobenzone)
Test Substance Supplier:	Universal-Preserv-A-Chem, Inc.
CAS Number:	70356-09-1
Description:	Off white to yellowish crystalline powder
Solvent Used:	Dimethyl sulfoxide
Batch Number:	L802809
Expiry Date:	Not provided
Purity:	98.5%
Molecular Formula:	$C_{20}H_{22}O_3$
Molecular Weight:	310.39 g/mol
Storage Conditions:	Room Temp. (eg. ambient)

Test Substance Name:	3, 3, 5-Trimethlycyclohexyl Salicylate (Homosalate)
Test Substance Supplier:	Spectrum
CAS Number:	118-56-9
Description:	Colorless to light yellow liquid
Solvent Used:	Dimethyl sulfoxide
Batch Number:	YT0976
Expiry Date:	Not provided
Purity:	99.3%
Molecular Formula:	262.34 g/mol
Molecular Weight:	$C_{16}H_{22}O_3$
Storage Conditions:	Room Temp. (eg. ambient)

Test Substance Name:	2-Ethylhexyl-P-Dimethyl-Aminobenzoate (Padimate-O)
Test Substance Supplier:	Aldrich
CAS Number:	21245-02-3
Description:	Yellowish liquid
Solvent Used:	Dimethyl sulfoxide
Batch Number:	MKBF0590V
Expiry Date:	Not provided
Purity:	98.1%
Molecular Formula:	277.40 g/mol
Molecular Weight:	$C_{17}H_{27}NO_2$
Storage Conditions:	Room Temp. (eg. ambient)

Certificates of analysis for the test substances are presented in Appendix 2.

The reference compound DHT (CAS# 521-18-6) was purchased from Sigma Aldrich (Buchs, Switzerland) and was 100.0% pure. The catalog number was A8380 and the lot number was BCBF1698V.

The reference compound Nilutamide (CAS# 63612-50-0) was purchased from Sigma Aldrich (St. Louis, MO) and was 100% pure. The catalog number was N8534 and the lot number was 057K1157V.

The reference compound ppDDE (CAS# 72-55-9) was purchased from Sigma Aldrich (Milwaukee, WI) and was 98.6% pure. The catalog number was 123897 and the lot number was 11923EOV.

3.1.2 Vehicle selection

Dimethyl sulfoxide (DMSO) was selected as a suitable vehicle for test substances. Therefore, solutions with a test substance concentration of up to 10^{-3} M (the highest concentration tested) were prepared while limiting the final concentration of DMSO in the assay medium to 0.5% (v/v). Dihydrotestosterone, nilutamide, p,p'-DDE and test substances were all prepared on the day of dosing for the first run (05-February-2013). Dihydrotestosterone, nilutamide, p,p'-DDE prepared on February 5, 2013 were used for the second run (07-February-2013). Test substances were prepared on the day of dosing for the second run (07-February-2013).

3.2 Cell Line

3.2.1 Source

The stably transfected MDA-kb2 cell line was used in this study. The cell line was obtained from ATCC (Appendix 3). The cells were certified as Mycoplasma Free (Appendix 4).

3.2.2 Stability of the cell line

The stability of the cell line was monitored by the use of the following reference chemicals: dihydrotestosterone (DHT), nilutamide (Nil) and p,p'-DDE. A complete concentration response curve for each reference compound was run each time the transcriptional activation assay was performed (see Table 5).

3.2.3 Cell culture and plating conditions

Cells were maintained in Leibovitz's L-15 culture medium containing 10% fetal bovine serum, in an incubator at ~37°C without CO_2. The MDA-kb2 cell line is not contact inhibited and was grown to confluence. Cells were subcultivated at a 1:2 to 1:8 subcultivation ratio. The cells were suspended with complete medium and plated into wells of a 96-well cell culture plate at a density of ~1 X 10^4 cells/100 µL/well. The cells were then placed into an incubator without CO_2 at ~37°C overnight prior to chemical exposure.

3.3 Chemical Exposure and Assay Plate Organization

Each test substance was prepared for addition to the cell system by making a 400 mM stock. Dilutions were prepared in DMSO to 400x final target concentration. Ten microliter aliquots of the substance dilutions were added to 2 mL media in deep well plates and mixed to yield concentrations of test material 2-fold greater than the desired final concentration.

After the overnight post-seeding incubation, the plates were removed from the incubator and the media was aspirated. Fifty microliters of media and appropriate controls were added to the seeded plates. To achieve the final exposure concentrations each 2X solution was diluted 2-fold in the 96-well plate containing the cells and media and controls.

Agonist

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank*	DHT (10 nM)	VC**	VC	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Conc. 6	Conc. 7	Conc. 8
B	↓***	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	----------As above + antagonist (10 µM nilutamide)----------											
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

DHT = dihydrotestosterone
*Blank wells contain media only (no cells)
**Vehicle control (VC) wells contain cells and media + 0.5% (v/v) DMSO
***↓ Indicates the composition of the well is identical to the well directly above it

Antagonist

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank*	DHT (10 nM)	VC**	VC	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Conc. 6	Conc. 7	Conc. 8
B	↓***	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	----------As above + (1000 nM DHT instead of 1 nM DHT)----------											
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

*Blank wells contain media only (no cells)
**Vehicle control (VC) wells contain cells and media + 0.5% (v/v) DMSO
****↓ Indicates the composition of the well is identical to the well directly above it
Rows A-D are low agonist (1 nM DHT)
Rows E-H are high agonist (1000 nM DHT)

After adding the reference chemicals/test substances, the plates were incubated in an incubator at ~37°C without CO_2 for ~24 hours.

For the agonist plates, all concentrations were tested in replicates of 6/plate. In addition, for each concentration, 2 replicates/plate were prepared that incorporated the AR antagonist nilutamide. Replicates incorporating an AR antagonist allow for the identification of non-specific (i.e., non-AR-mediated) induction of the luciferase gene as true AR-mediated induction is inhibited by addition of an antagonist whereas non-specific induction is not.

For the antagonist plates, all concentrations were tested in replicates of 4/plate. Four replicates were co-administered 1 nM DHT and test article at each concentration. Four

replicates were co-administered 1000 nM DHT and test article at each concentration. Replicates incorporating 1000 nM DHT allowed for the identification of assay interference.

In view of the short-term nature of studies of this type, no analyses of stability, homogeneity or achieved concentration(s) were carried out on preparations of the test substance or positive control chemicals, either before or after the treatment phase. This was not considered to have affected the integrity of the study. For the reference control compounds, stability was demonstrated by an appropriate response in the assay system.

3.4 Assays

3.4.1 Cytotoxicity assay

Cell viability was monitored by a two-read propidium iodide (PI) uptake assay. PI was a light sensitive dye and all procedures were conducted under low light conditions. PI could not cross the plasma membrane of intact and viable cells. Cells that were dead or dying had weakened plasma membranes which allowed PI to enter the cytosol of the damaged cells. Once inside the cell, PI intercalated into DNA/RNA and yielded a fluorescent signal. In the two-read procedure, the first read was taken immediately after full exposure to controls and test articles. This measured "background" fluorescence. The cells were then lysed and a second read was taken. This read indicated cell death. The first read was then subtracted from the second read. The results of the subtracted reads were directly proportional to the viability of the cells. The control and test substance data were normalized to vehicle control to generate percent cell viability.

Cells were seeded as described in Section 3.2.3, with the exception that a black-walled 96-well cell culture plate was used. The cells were exposed to the test chemicals in replicates of 6 (rows A-F) while the last 2 rows (G and H) received 250 µM digitonin as a positive control for cell death. Following chemical exposure, the growth medium was removed and 50 µL of a PI working solution (44 µM in phosphate buffered saline) was added to each well. Background fluorescence was evaluated by measuring fluorescence immediately on a Packard Fusion fluorescence plate reader at an excitation wavelength of 544 nm and an emission wavelength of 612 nm for run 1 and on BioTek Synergy fluorescence plate reader at an excitation wavelength of 540 nm and an emission wavelength of 610 nm. Following this determination, 50 µL of a 2% (v/v) Triton X-100 solution was added to each well and the plate was incubated at room temperature for ~15 minutes to fully lyse all cells in the wells before measuring fluorescence at the same wavelengths on the same instrument.

The background-corrected fluorescence was calculated for each well by subtracting the results of the first read from the results of the second read. The change in cell viability was determined by comparing treated wells to the vehicle control wells. A ≥20% reduction in cell viability was considered evidence of cytotoxicity.

3.4.2 Precipitation assessment

Solubility limits were determined by Nephelometry. A 96-well clear bottom plate containing 200 µL of every test concentration in cell culture media was evaluated using the Nepheloskan. Nephelometry measured the particulate light scattering. Visual observations were also noted.

3.4.3 Transcriptional activation assay

A luciferase assay was performed as described in CeeTox Standard Operating Protocol SOP 2041 using the reagents listed below. Luciferase assay reagent was prepared as described in CeeTox SOP 2041 (proprietary information).

Reagent	Supplier	Catalog #
Trisma Base	Sigma	T6066
Magnesium Chloride	Sigma	M2393
EDTA	Sigma	E5134
Dithiothreitol	Sigma	D0632
ATP	Sigma	A2383
Coenzyme A	Sigma	C3019
AMP	Sigma	A1752
Luciferin	Promega	E160E
Glycerol	Sigma	G5516
Triton-X100	Sigma	T8787
Bovine Serum Albumin	Sigma	A9418
CDTA	Sigma	32869

3.5 Agonist Transcriptional Activation Assay Data Analysis and Interpretation

In order to determine the relative transcriptional activity as compared to the positive control (PC), 10 nM DHT, the luminescence data from each plate were analyzed according to the steps outlined below. Wells incorporating nilutamide were analyzed in an identical fashion to wells not incorporating nilutamide, except that the data were normalized by subtracting the mean value for the nilutamide-containing vehicle control (VC) wells. Data was analyzed using Microsoft Excel.

1. Any cytotoxic concentrations (as defined in Section 3.4.1) were excluded from data analysis.
2. The mean value for the VC wells was calculated.
3. The mean value for the VC wells was subtracted from each well to normalize the data.
4. The mean value for the normalized PC wells was calculated.

5. The normalized value for each well was divided by the mean value of the normalized PC wells (with the normalized mean of the PC wells being defined as 100% relative transcriptional activity). The final value for each well is the relative transcriptional activity for that well compared to the mean normalized PC response.

The data were then interpreted in according to the following steps (analyzed using Microsoft Excel):

1. Where appropriate, $LogPC_{50}$, $LogPC_{10}$, $LogEC_{50}$ and Hill slope values were calculated.
2. For the test substance, the maximum response relative to the positive control (RPC_{Max}) was determined. In each individual run of the transcriptional activation assay, if RPC_{max} was less than 20%, the test substance was considered to have given a negative response for AR agonism.
3. For each individual run of the transcriptional activation assay, the acceptability of the data was evaluated using the following criteria:
    - The mean normalized luciferase signal of the PC (10 nM DHT) should be at least 4-fold that of the mean VC on each plate.
    - The results of the reference compounds, nilutamide and DHT, should be within the acceptable ranges.
4. If the acceptability criteria outlined above were met, that run of the transcriptional activation assay was considered to be definitive
5. The test substance was considered negative if RPC_{Max} was <20% in at least 2 definitive runs of the transcriptional activation assay. The test substance was considered positive if RPC_{Max} was ≥20% in at least 2 definitive runs of the transcriptional activation assay.

3.6 Antagonist Transcriptional Activation Assay Data Analysis and Interpretation

In order to determine the relative transcriptional activity as compared to the positive control (PC), 10 nM DHT, the luminescence data from each plate were analyzed according to the steps outlined below. Wells incorporating 1 nM DHT were analyzed in an identical fashion to wells incorporating 1000 nM DHT, except that the data was normalized to the induced control with 1 nM DHT or 1000 nM DHT, respectively.

1. Any cytotoxic concentrations (as defined in Section 3.4.1) were excluded from data analysis.
2. The mean value for the VC wells was calculated.
3. The mean value for the VC wells was subtracted from each well to normalize the data.
4. The mean value for the induced control with 1 nM DHT was calculated.
5. The mean value for the induced control with 1000 nM DHT was calculated.
6. The wells dosed with test or control substance and 1 nM DHT were normalized to the mean value for the induced control with 1 nM DHT.

7. The wells dosed with test or control substance and 1000 nM DHT were normalized to the mean value for the induced control with 1000 nM DHT.
8. Averages of antagonist % maximal induction control were calculated (test or control substance a with 1 nM DHT).
9. Averages of high agonist control % maximal induction control were calculated (test or control substance a with 1000 nM DHT).
10. Differentials were calculated (averages of high agonist control % maximal induction control minus averages of antagonist % maximal induction control).

The data were then interpreted according to the following steps:

1. Where appropriate, RICMax, Differential IC_{50}, Differential IC_{30}, $LogEC_{50}$ and Hill slope values were calculated.
2. If the differential between the high antagonism and the low antagonism was greater then 50% and had a dose response (more than one data point), than the test substance was considered positive.
3. If the differential between the high antagonism and the low antagonism was less then 50% and did not have a dose response (more than one data point), than the test substance was considered negative.
4. For each individual run of the transcriptional activation assay, the acceptability of the data was evaluated using the following criteria:
    - The mean normalized luciferase signal of the PC (10 nM DHT) should have been at least 4-fold that of the negative control on each plate.
5. If the acceptability criteria outlined above were met, that run of the transcriptional activation assay was considered to be definitive.

4.0 RESULTS AND DISCUSSION

4.1 Concentration Range for the Test Substance

Two runs were conducted on ensulizole, avobenzone, homosalate and padimate O. The final concentrations for ensulizole were: $10^{-7.5}$, 10^{-7},$10^{-6.5}$, 10^{-6}, $10^{-5.5}$, 10^{-5}, $10^{-4.5}$ and 10^{-4} M for runs 1 and 2 (05-February-2013 and 07-February-2013). The final concentrations for avobenzone, homosalate and padimate O were: $10^{-6.5}$, 10^{-6}, $10^{-5.5}$, 10^{-5}, $10^{-4.5}$, 10^{-4}, $10^{-3.5}$ and 10^{-3} M for runs 1 and 2 (05-February-2013 and 07-February-2013).

4.2 Transcriptional Activation Assay Acceptance Criteria

In all valid independent runs of the assay, the mean luciferase activity of the PC (10 nM DHT) was greater than 4-fold that of the mean luciferase activity of the VC on each plate.

Test article data and data from the 3 reference compounds were excluded from evaluation and interpretation in instances of excessive cytotoxicity or precipitation observed in the valid independent runs.

4.3 Transcriptional Activation Assay Results

Two runs were conducted on ensulizole, avobenzone, homosalate and padimate O.

In all independent runs of the agonist transcriptional activation assay, these test substances (ensulizole, avobenzone, homosalate and padimate O) did not result in an increase in luciferase activity at any of the viable soluble concentrations tested (RPC_{max}<20%).

In all independent runs of the antagonist transcriptional activation assay, ensulizole, avobenzone and padimate O did not result in a differential between the high antagonism and the low antagonism of greater than 50% at more than one viable soluble concentration.

In the antagonist transcriptional activation assay, homosalate did result in an average differential between the high antagonism and the low antagonism of greater than 50%. The average differential was 60% (28% run 1 and 92% run 2 at -4.5 M). A dose response of more than one viable soluble concentration was noted in run 2 (20%, 42%, 51% and 92% at concentrations 10^{-6}, $10^{-5.5}$, 10^{-5} and $10^{-4.5}$ M).

4.4 Discussion

The suitable top concentration of ensulizole for use in the transcriptional activation assays was 10^{-4} M. The second run (07-February-2013) demonstrated a dip below the cytotoxicity limit (23% reduction in viability at $10^{-4.5}$ M) but then returned to viable at 10^{-4} M.

The suitable top concentration of avobenzone for use in the transcriptional activation assays was 10^{-5} M. Precipitation as observed by nephelometry was noted at the concentration10^{-4} M and higher. Cytotoxicity (≥20% reduction) was demonstrated at the concentration $10^{-4.5}$ M and higher.

The suitable top concentration of homosalate for use in the transcriptional activation assays was $10^{-4.5}$ M. Precipitation as observed by nephelometry in conjunction with visual observation was noted at the concentration10^{-4} M and higher. The first run (05-February-2013) demonstrated a dip below the cytotoxicity limit at the two highest concentrations (24% and 22% reduction in viability at $10^{-3.5}$ and 10^{-3} M, respectively).

The suitable top concentration of padimate O for use in the transcriptional activation assays was $10^{-4.5}$ M. Precipitation as observed by nephelometry in conjunction with visual observation was noted at the concentration 10^{-4} M and higher. The second run (07-February-2013) demonstrated a dip below the cytotoxicity limit at the three highest concentrations (21%, 24% and 24% reduction in viability at 10^{-4} M, $10^{-3.5}$, and 10^{-3} M, respectively).

The suitable top concentration of DHT for use in the transcriptional activation assays was 10^{-8} M. No precipitation was observed at any concentration tested. The second run (07-

February-2013) demonstrated a dip below the cytotoxicity limit at $10^{-8.5}$ M (48% reduction in viability).

The suitable top concentration of Nilutamide for use in the transcriptional activation assays was 10^{-5} M. A peak suggesting precipitation was observed in one of the two replicates for the first run (05-February-2013) at 10^{-4} M. The first run (05-February-2013) demonstrated a dip below the cytotoxicity limit at the two highest concentrations (21% and 41% reduction in viability at $10^{-4.5}$ and 10^{-4} M, respectively). The second run (07-February-2013) demonstrated a dip below the cytotoxicity limit at the two highest concentrations (31% and 42% reduction in viability at $10^{-4.5}$ and 10^{-4} M, respectively).

The suitable top concentration of ppDDE for use in the transcriptional activation assays was $10^{-4.5}$ M. Precipitation as observed by nephelometry was noted at the concentration 10^{-4} M. Cytotoxicity (≥20% reduction) was demonstrated at the concentration 10^{-4} M.

In all independent runs of the transcriptional activation assay, ensulizole, avobenzone, homosalate and padimate O did not result in an increase in luciferase activity in agonism plates at any of the viable soluble concentrations tested (RPC_{max}<20%).

In all independent runs of the transcriptional activation assay, ensulizole, avobenzone and padimate O did not result in a differential on the antagonism plates greater than 50% for two or more viable soluble doses.

In the antagonist transcriptional activation assay, homosalate did result in an average differential between the high antagonism and the low antagonism of greater than 50%. The average differential was 60% (28% run 1 and 92% run 2 at -4.5 M). A dose response of more than one viable soluble concentration was noted in run 2 (20%, 42%, 51% and 92% at concentrations 10^{-6}, $10^{-5.5}$, 10^{-5}, $10^{-4.5}$ M).

5.0 CONCLUSIONS

Ensulizole, avobenzone and padimate O did not demonstrate agonism or antagonism of AR-mediated transactivation when tested in the MDA-kb2 cell model system. Homosalate did not demonstrate agonism, however there was an exposure dependent antagonism of AR-mediated transactivation when tested in the MDA-kb2 cell model system.

6.0 REFERENCES

Wilson, VS., Bobseine, K., Lambright, CR., and Gray, LE., Jr. (2002). A novel cell line, MDA-kb2, which stably expresses an androgen and glucocorticoid-responsive reporter for detection of hormone receptor agonists and antagonists. *Toxicol. Sci.* **66**, 69-81.

TABLES SECTION

TABLE 1 Results of 1st Valid Transcriptional Activation Assay Agonist

Chemical	Concentration (M)	RTA (% of PC)		RTA with Nil (% of PC)		Cell Viability (% of VC)		Precipitation	
		Mean	SD	Value 1	Value 2	Mean	SD	Value 1	Value 2
Ensulizole	-7.5	0	1	1	0	98	4	137	165
	-7	0	1	0	0	100	3	143	152
	-6.5	0	1	1	0	95	3	175	157
	-6	1	1	0	1	94	2	148	156
	-5.5	2	1	3	2	97	3	172	150
	-5	1	1	3	2	93	2	177	195
	-4.5	1	1	0	2	93	1	253	160
	-4	2	2	1	0	87	2	415	158
Avobenzone	-6.5	1	0	-2	0	99	3	133	133
	-6	0	0	0	0	97	2	166	113
	-5.5	1	1	1	1	95	3	112	108
	-5	0	1	-2	-1	87	3	125	95
	-4.5	*	*	*	*	**72	3	220	251
	-4	*	*	*	*	**63	2	#6370	#6140
	-3.5	*	*	*	*	**48	3	#17346	#16898
	-3	*	*	*	*	**44	1	#18323	#17912
Homosalate	-6.5	0	1	0	1	100	4	113	170
	-6	1	1	1	-1	100	5	133	163
	-5.5	1	1	2	1	100	2	117	179
	-5	0	0	0	0	97	2	138	156
	-4.5	1	1	2	3	98	4	300	289
	-4	0	1	2	2	90	8	#742	#582
	-3.5	*	*	*	*	**76	15	#386	#604
	-3	*	*	*	*	**78	10	#318	#249
Padimate O	-6.5	0	1	1	-1	102	3	124	124
	-6	1	1	0	1	100	4	140	120
	-5.5	1	1	2	1	102	4	127	170
	-5	0	1	1	0	98	2	136	118
	-4.5	1	1	2	1	98	4	297	302
	-4	1	1	0	1	98	3	#625	#669
	-3.5	1	1	-1	0	98	2	#382	#422
	-3	1	1	2	0	91	4	#273	#249

RTA = Relative Transcriptional Activation
PC = Positive Control (10 nM DHT)
VC = Vehicle Control
SD = Standard Deviation
# = Precipitation
* = data not evaluated due to observed Cytotoxicity
** = Cytotoxicity observed

TABLE 1 Results of 1st Valid Transcriptional Activation Assay Agonist (Continued)

Chemical	Concentration (M)	RTA (% of PC)		RTA with Nil (% of PC)		Cell Viability (% of VC)		Precipitation	
		Mean	SD	Value 1	Value 2	Mean	SD	Value1	Value2
DHT	-11.5	0	1	-1	-2	99	2	109	147
	-11	1	1	-1	-1	96	2	122	187
	-10.5	6	2	1	0	95	2	117	195
	-10	23	7	0	0	96	1	107	225
	-9.5	75	20	5	2	97	4	205	236
	-9	98	14	5	7	98	1	175	174
	-8.5	116	30	30	18	98	2	103	143
	-8	106	14	76	62	95	2	122	132
Nil	-7.5	-1	1	-1	1	97	6	170	215
	-7.0	0	1	0	2	96	4	198	195
	-6.5	0	1	0	2	98	5	180	245
	-6.0	0	1	-1	0	96	5	159	138
	-5.5	2	2	4	4	95	5	148	265
	-5.0	3	2	5	3	93	6	180	202
	-4.5	*	*	*	*	**79	3	149	173
	-4.0	*	*	*	*	**59	1	156	861
ppDDE	-7.5	0	1	0	-1	99	4	181	465
	-7.0	0	1	0	-2	97	4	174	171
	-6.5	1	0	0	0	97	3	362	554
	-6.0	0	1	0	0	94	3	123	213
	-5.5	2	1	0	0	95	5	227	190
	-5.0	3	1	0	0	91	3	202	321
	-4.5	4	1	3	0	87	3	448	440
	-4.0	*	*	*	*	**73	3	#5421	#5261

RTA = Relative Transcriptional Activation
PC = Positive Control (10 nM DHT)
VC = Vehicle Control
SD = Standard Deviation
# = Precipitation
* = data not evaluated due to observed Cytotoxicity
** = Cytotoxicity observed
shaded areas not evaluated

TABLE 2 Results of 1st Valid Transcriptional Activation Assay Antagonist

Chemical	Concentration (LogM)	Low Agonist Maximal Induction Antagonism (%)		High Agonist Maximum Induction (1000 nM DHT) (%)		Differential
		Mean	SD	Mean	SD	
10 nM DHT		115	36	89	15	
VC		0	0	107	14	
Induced Controls (1nM DHT)		100	13	93	20	
Ensulizole	-7.5	129	18	79	12	-50
	-7.0	104	20	82	16	-21
	-6.5	107	21	117	25	10
	-6.0	118	27	121	6	3
	-5.5	153	22	102	16	-51
	-5.0	136	36	117	17	-19
	-4.5	137	15	144	7	7
	-4.0	144	38	110	21	-34
10 nM DHT		89	23	97	4	
VC		0	2	97	22	
Induced Controls (1nM DHT)		100	29	103	5	
Avobenzone	-6.5	102	18	95	10	-7
	-6.0	83	14	114	12	31
	-5.5	119	24	106	10	-13
	-5.0	76	13	87	12	12
	-4.5	*	*	*	*	*
	-4.0	*	*	*	*	*
	-3.5	*	*	*	*	*
	-3.0	*	*	*	*	*
10 nM DHT		126	25	90	16	
VC		0	1	97	21	
Induced Controls (1nM DHT)		100	11	103	24	
Homosalate	-6.5	121	22	93	7	-28
	-6.0	122	13	99	32	-23
	-5.5	112	39	115	20	2
	-5.0	109	10	114	25	5
	-4.5	88	9	116	11	28
	-4.0	28	6	113	16	#86
	-3.5	*	*	*	*	*
	-3.0	*	*	*	*	*
10 nM DHT		118	39	100	22	
VC		0	1	91	6	
Induced Controls (1nM DHT)		100	8	109	38	
Padimate O	-6.5	119	19	84	21	-36
	-6.0	109	30	77	18	-32
	-5.5	144	10	85	12	-59
	-5.0	133	38	99	24	-34
	-4.5	157	24	118	15	-39
	-4.0	145	24	104	20	-41
	-3.5	157	15	102	17	-55
	-3.0	153	45	93	14	-60

VC = Vehicle Control
SD = Standard Deviation
* = data not evaluated due to observed Cytotoxicity, Cytotoxicity values shown on corresponding agonist table
shaded areas = does not apply
= differential > 50

TABLE 2 Results of 1st Valid Transcriptional Activation Assay Antagonist (Continued)

Chemical	Concentration (LogM)	Low Agonist Maximal Induction Antagonism (%)		High Agonist Maximum Induction (1000 nM DHT) (%)		Differential
		Mean	SD	Mean	SD	
10 nM DHT		122	69	110	12	
VC		0	1	94	18	
Induced Controls (1nM DHT)		100	22	106	12	
DHT	-11.5	107	27	102	22	-6
	-11	109	14	117	20	8
	-10.5	141	36	129	26	-12
	-10	146	33	119	29	-27
	-9.5	139	30	133	7	-6
	-9	135	18	148	21	13
	-8.5	145	21	137	23	-7
	-8	181	35	130	20	-51
10 nM DHT		124	24	103	6	
VC		0	2	101	13	
Induced Controls (1nM DHT)		100	14	99	19	
Nil	-7.5	77	19	90	6	12
	-7.0	59	12	86	9	27
	-6.5	50	7	87	19	37
	-6.0	17	4	113	12	#96
	-5.5	12	2	124	17	#112
	-5.0	9	3	104	17	#95
	-4.5	*	*	*	*	*
	-4.0	*	*	*	*	*
10 nM DHT		60	16	87	44	
VC		0	0	94	32	
Induced Controls (1nM DHT)		100	12	106	16	
ppDDE	-7.5	96	6	94	34	-2
	-7.0	145	34	130	51	-15
	-6.5	125	26	143	57	18
	-6.0	123	16	183	83	#61
	-5.5	120	17	206	107	#86
	-5.0	81	21	222	85	#140
	-4.5	46	4	155	27	#110
	-4.0	*	*	*	*	*

VC = Vehicle Control
SD = Standard Deviation
* = data not evaluated due to observed Cytotoxicity, Cytotoxicity values shown on corresponding agonist table
shaded areas = does not apply
= differential > 50

TABLE 3 Results of 2nd Valid Transcriptional Activation Assay Agonist

Chemical	Concentration (M)	RTA (% of PC)		RTA with Nil (% of PC)		Cell Viability (% of VC)		Precipitation	
		Mean	SD	Value 1	Value 2	Mean	SD	Value 1	Value 2
Ensulizole	-7.5	0	1	0	0	105	16	109	139
	-7	-1	1	0	-1	97	15	103	122
	-6.5	0	0	0	1	96	13	138	93
	-6	0	1	0	-1	97	18	109	122
	-5.5	1	1	1	4	100	14	149	86
	-5	1	0	1	3	103	16	350	253
	-4.5	*	*	*	*	**77	11	339	543
	-4	1	1	2	1	107	21	490	189
Avobenzone	-6.5	2	5	1	0	99	18	113	***14797
	-6	0	1	0	0	93	25	112	106
	-5.5	0	0	1	0	97	25	108	105
	-5	0	1	-1	-1	85	20	115	105
	-4.5	*	*	*	*	**61	5	220	234
	-4	*	*	*	*	**50	0	#7039	#6882
	-3.5	*	*	*	*	**46	9	#18642	#18736
	-3	*	*	*	*	**50	11	#19685	#19518
Homosalate	-6.5	0	1	-2	-1	101	13	123	102
	-6	1	1	-1	-1	102	13	132	109
	-5.5	0	0	1	0	101	16	106	114
	-5	0	0	0	0	106	13	115	119
	-4.5	2	0	0	2	105	20	255	256
	-4	1	1	0	1	89	11	#1530	#1492
	-3.5	1	1	0	0	80	54	#882	#530
	-3	0	1	3	-1	83	26	#7237	#259
Padimate O	-6.5	0	0	0	-1	94	14	105	92
	-6	0	1	2	0	87	2	100	92
	-5.5	1	1	0	0	85	17	101	135
	-5	1	1	0	-1	87	15	130	100
	-4.5	2	1	2	2	89	20	321	330
	-4	*	*	*	*	**79	10	#1105	#1125
	-3.5	*	*	*	*	**76	12	#526	#872
	-3	*	*	*	*	**76	6	#258	#356

RTA = Relative Transcriptional Activation
PC = Positive Control (10 nM DHT)
VC = Vehicle Control
SD = Standard Deviation
# = Precipitation
* = data not evaluated due to observed Cytotoxicity
** = Cytotoxicity observed
*** = bubble

TABLE 3 Results of 2nd Valid Transcriptional Activation Assay Agonist (Continued)

Chemical	Concentration (M)	RTA (% of PC)		RTA with Nil (% of PC)		Cell Viability (% of VC)		Precipitation	
		Mean	SD	Value 1	Value 2	Mean	SD	Value1	Value2
DHT	-11.5	0	1	0	-1	90	6	154	185
	-11	1	1	1	-1	85	8	149	124
	-10.5	4	1	0	0	90	8	184	124
	-10	22	6	0	0	88	13	116	201
	-9.5	74	27	2	1	89	11	166	220
	-9	104	17	4	3	92	7	117	132
	-8.5	*	*	*	*	**52	2	126	116
	-8	127	44	38	29	104	25	120	115
Nil	-7.5	0	0	0	0	98	4	126	216
	-7.0	0	1	0	0	100	4	170	188
	-6.5	-1	1	0	-1	118	53	103	124
	-6.0	0	1	0	1	104	6	121	121
	-5.5	2	1	3	2	99	12	115	159
	-5.0	3	1	3	4	109	10	92	115
	-4.5	*	*	*	*	**69	7	104	97
	-4.0	*	*	*	*	**58	5	161	107
ppDDE	-7.5	0	0	0	0	97	4	106	186
	-7.0	0	0	0	0	96	11	95	95
	-6.5	1	1	1	0	97	10	83	102
	-6.0	1	0	1	0	101	6	106	103
	-5.5	1	1	0	3	101	10	153	94
	-5.0	2	1	2	3	101	10	112	143
	-4.5	3	1	3	3	98	3	267	300
	-4.0	*	*	*	*	*77	9	#5064	#4709

RTA = Relative Transcriptional Activation
PC = Positive Control (10 nM DHT)
VC = Vehicle Control
SD = Standard Deviation
# = Precipitation
* = data not evaluated due to observed Cytotoxicity
** = Cytotoxicity observed

TABLE 4 Results of 2nd Valid Transcriptional Activation Assay Antagonist

Chemical	Concentration (LogM)	Low Agonist Maximal Induction Antagonism (%)		High Agonist Maximum Induction (1000 nM DHT) (%)		Differential
		Mean	SD	Mean	SD	
10 nM DHT		125	17	97	18	
VC		0	0	104	13	
Induced Controls (1nM DHT)		100	23	96	14	
Ensulizole	-7.5	106	22	100	7	-5
	-7.0	95	32	97	16	2
	-6.5	109	11	126	25	17
	-6.0	110	30	122	33	12
	-5.5	125	20	136	15	11
	-5.0	145	20	135	28	-9
	-4.5	*	*	*	*	*
	-4.0	116	35	127	19	11
10 nM DHT						
VC		0	0	108	29	
Induced Controls (1nM DHT)		100	37	92	6	
Avobenzone	-6.5	86	17	97	19	11
	-6.0	90	18	99	22	9
	-5.5	99	15	107	16	7
	-5.0	79	22	85	8	6
	-4.5	*	*	*	*	*
	-4.0	*	*	*	*	*
	-3.5	*	*	*	*	*
	-3.0	*	*	*	*	*
10 nM DHT		115	32	110	17	
VC		0	0	100	15	
Induced Controls (1nM DHT)		100	31	100	18	
Homosalate	-6.5	99	30	134	26	35
	-6.0	96	22	117	33	20
	-5.5	81	8	123	27	42
	-5.0	91	20	142	16	#51
	-4.5	55	6	147	27	#92
	-4.0	24	3	126	11	#102
	-3.5	6	2	49	12	#43
	-3.0	11	1	83	16	#72
10 nM DHT		91	44	84	14	
VC		0	1	94	14	
Induced Controls (1nM DHT)		100	15	106	15	
Padimate O	-6.5	84	8	94	13	10
	-6.0	102	23	95	7	-6
	-5.5	121	5	117	21	-4
	-5.0	123	20	125	17	1
	-4.5	125	19	136	34	11
	-4.0	*	*	*	*	*
	-3.5	*	*	*	*	*
	-3.0	*	*	*	*	*

VC = Vehicle Control
SD = Standard Deviation
* = data not evaluated due to observed Cytotoxicity, Cytotoxicity values shown on corresponding agonist table
shaded areas = does not apply
= differential > 50

TABLE 4 Results of 2nd Valid Transcriptional Activation Assay Antagonist (Continued)

Chemical	Concentration (LogM)	Low Agonist Maximal Induction Antagonism (%)		High Agonist Maximum Induction (1000 nM DHT) (%)		Differential
		Mean	SD	Mean	SD	
10 nM DHT						
VC		0	0	91	33	
Induced Controls (1nM DHT)		100	20	109	29	
DHT	-11.5	89	9	101	21	12
	-11	96	9	98	18	1
	-10.5	125	29	125	25	0
	-10	121	9	120	27	0
	-9.5	122	23	134	23	12
	-9	125	19	136	11	11
	-8.5	*	*	*	*	*
	-8	166	28	100	37	-66
10 nM DHT		152	50	119	35	
VC		0	1	105	28	
Induced Controls (1nM DHT)		100	17	95	18	
Nil	-7.5	94	33	91	7	-3
	-7.0	90	17	109	33	19
	-6.5	52	10	106	6	#54
	-6.0	17	5	108	9	#91
	-5.5	9	3	124	22	#115
	-5.0	6	1	122	10	#116
	-4.5	*	*	*	*	*
	-4.0	*	*	*	*	*
10 nM DHT		121	30	95	29	
VC		0	1	104	20	
Induced Controls (1nM DHT)		100	29	96	28	
ppDDE	-7.5	106	20	89	20	-17
	-7.0	117	16	113	25	-4
	-6.5	117	14	117	27	0
	-6.0	106	39	110	18	4
	-5.5	86	7	113	22	27
	-5.0	64	8	118	22	#54
	-4.5	27	7	117	15	#90
	-4.0	*	*	*	*	*

VC = Vehicle Control
SD = Standard Deviation
* = data not evaluated due to observed Cytotoxicity, Cytotoxicity values shown on corresponding agonist table
shaded areas = does not apply
= differential > 50

TABLE 5 $LogPC_{50}$, $LogPC_{10}$, $LogEC_{50}$ and Hill Slope Values for the Reference Chemicals

Agonist

Name	$LogPC_{50}$		$LogPC_{10}$		$LogEC_{50}$		Hill Slope	
	1st Valid Assay	2nd Valid Assay	1st Valid Assay	2nd ValidAssay	1st Valid Assay	2nd Valid Assay	1st Valid Assay	2nd ValidAssay
DHT	-9.7	-9.7	-10.4	-10.3	-9.7	-9.6	1.7	1.4
Nil	-	-	-	-	-	-	-	-
ppDDE	-	-	-	-	-	-	-	-

PC = Positive Control (10 nM DHT)

Antagonist

Name	Differential IC_{50}		Differential IC_{30}		$LogEC_{50}$		Hill Slope	
	1st Valid Assay	2nd ValidAssay	1st Valid Assay	2nd ValidAssay	1st Valid Assay	2nd Valid Assay	1st Valid Assay	2nd Valid Assay
DHT	-	-	-	-	-	-	-	-
Nil	-6.4	-6.6	-6.8	-6.8	-6.5	-6.5	-0.8	-1.5
ppDDE	-6.1	-5.1	-6.4	-5.4	-4.7	-4.9	-1.3	-1.1

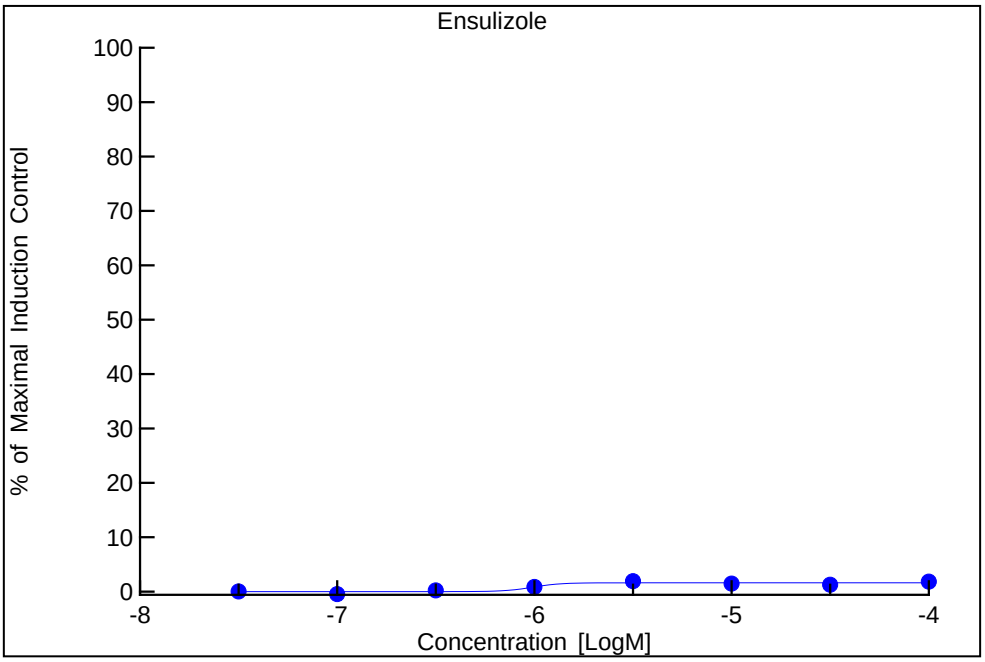
Differential = High agonist minus low agonist
Differential IC_{50} = concentration at which the high agonist minus low agonist is 50%

Name	Relative Inhibitory Concentration Max (RICMax)	
	1st Valid Assay	2nd Valid Assay
DHT	-	-
Nil	91.1	93.7
ppDDE	54.4	73.3

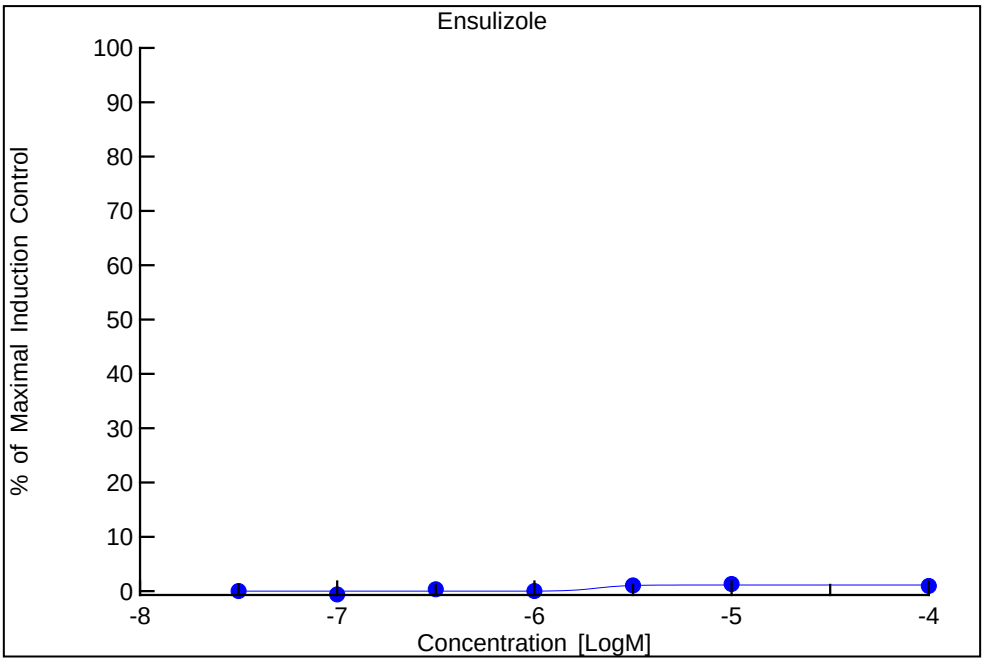
FIGURES SECTION

FIGURE 1 Ensulizole – Agonist

05Feb2013



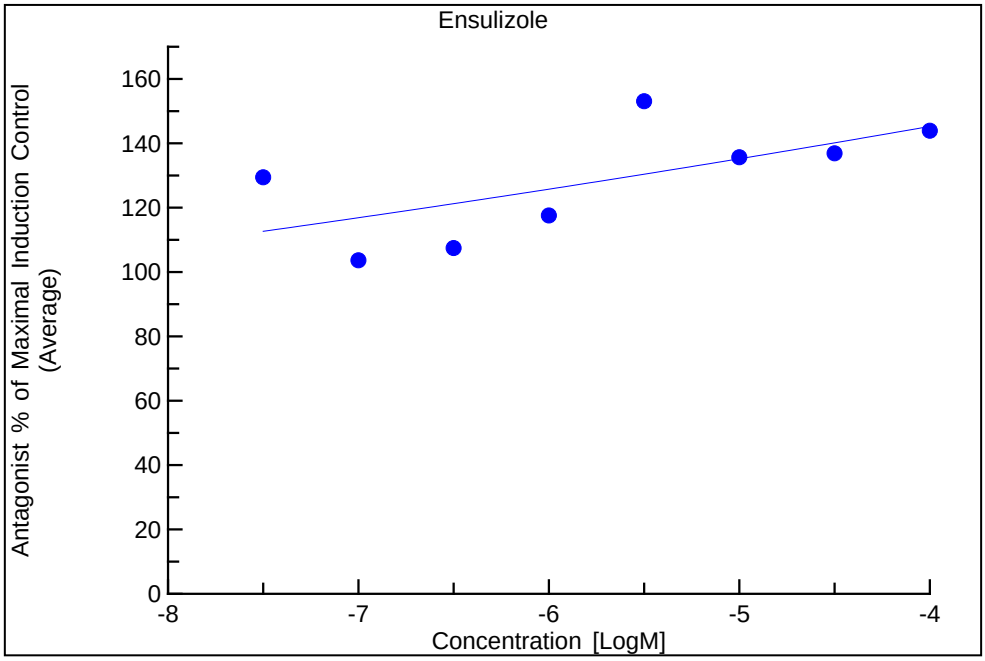
07Feb2013

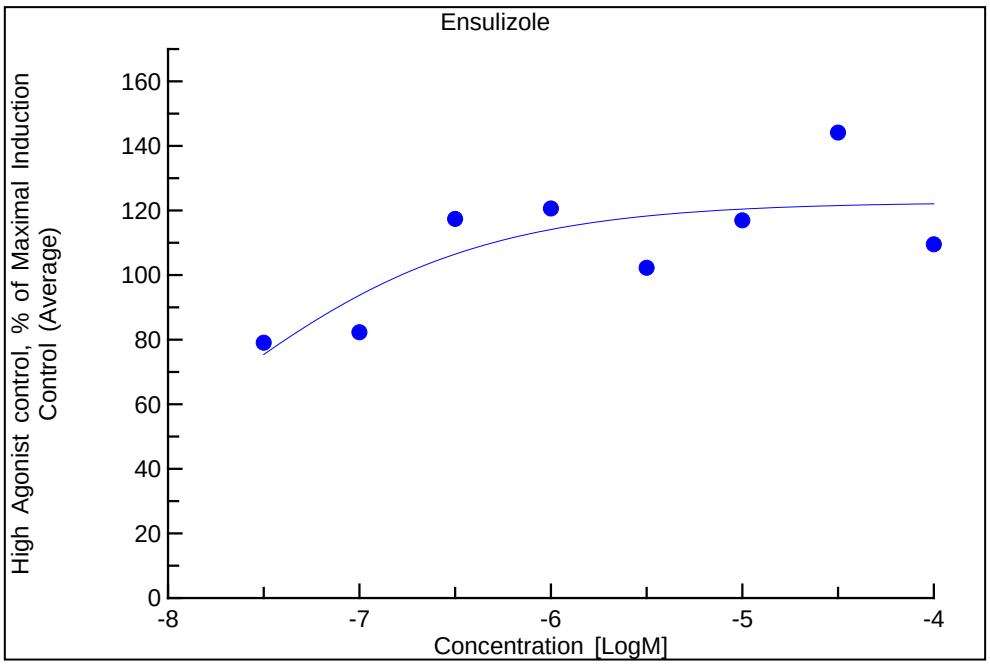


The two separate graphs represent the data (Means±Standard Deviation) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

FIGURE 2 Ensulizole – Antagonist

05Feb2013

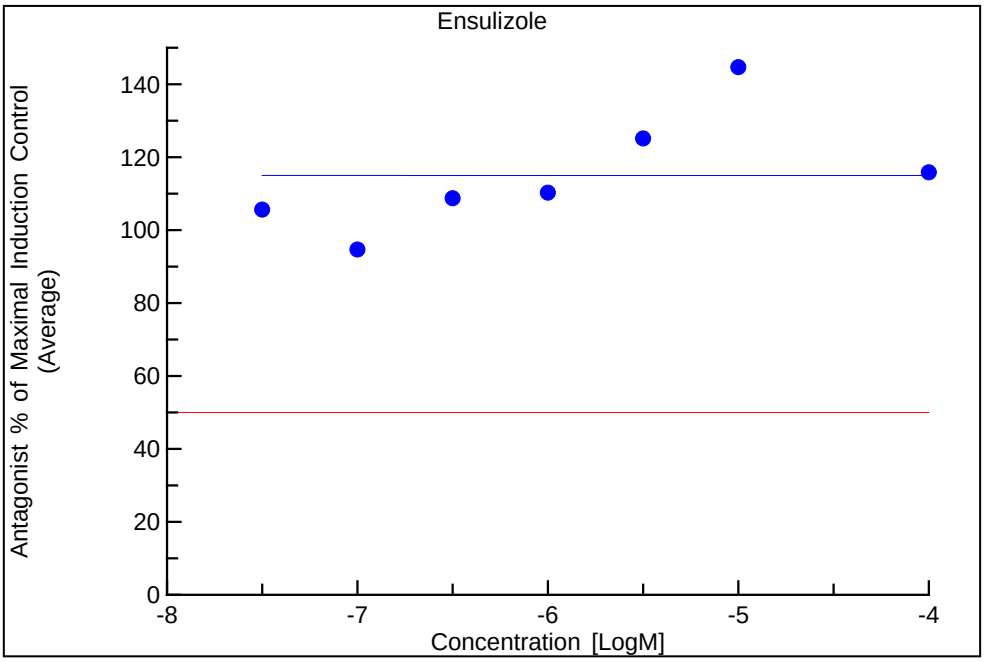


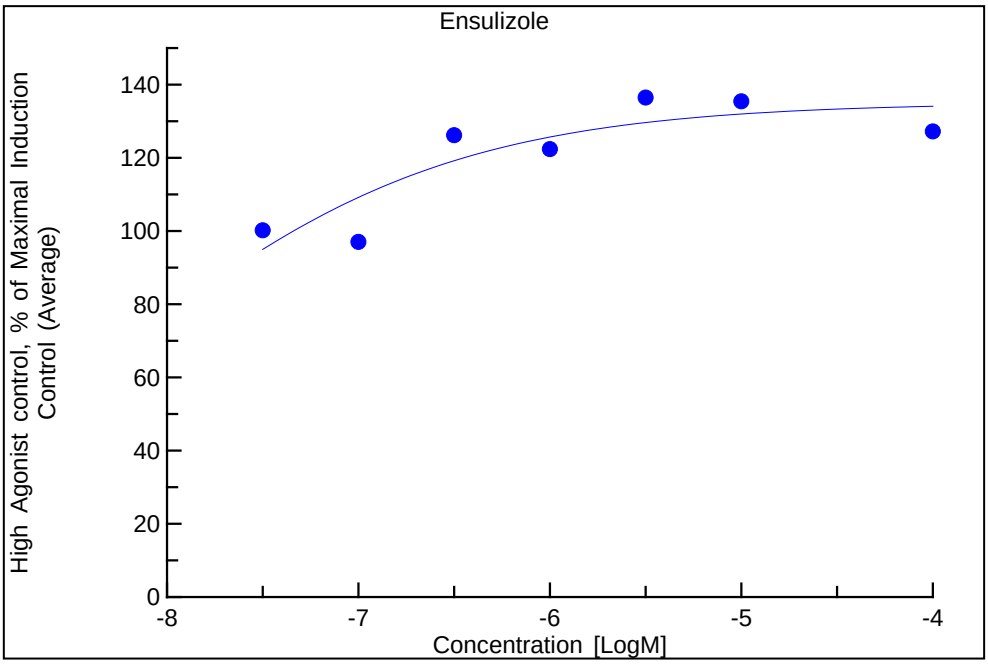


The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT.

FIGURE 2 Ensulizole – Antagonist (Continued)

07Feb2013

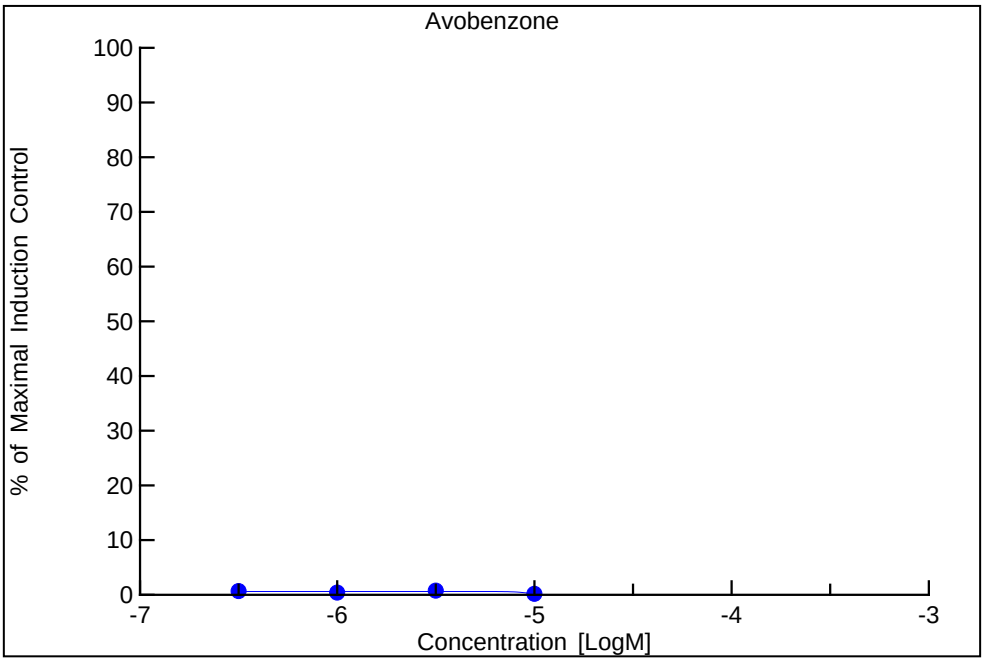




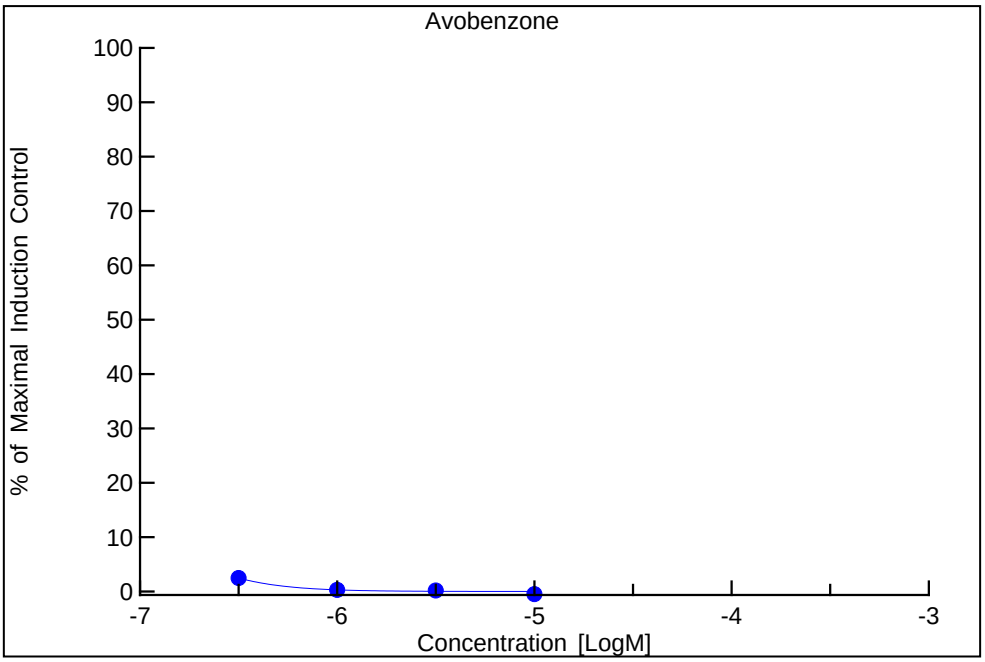
The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT.

FIGURE 3 Avobenzone – Agonist

05Feb2013



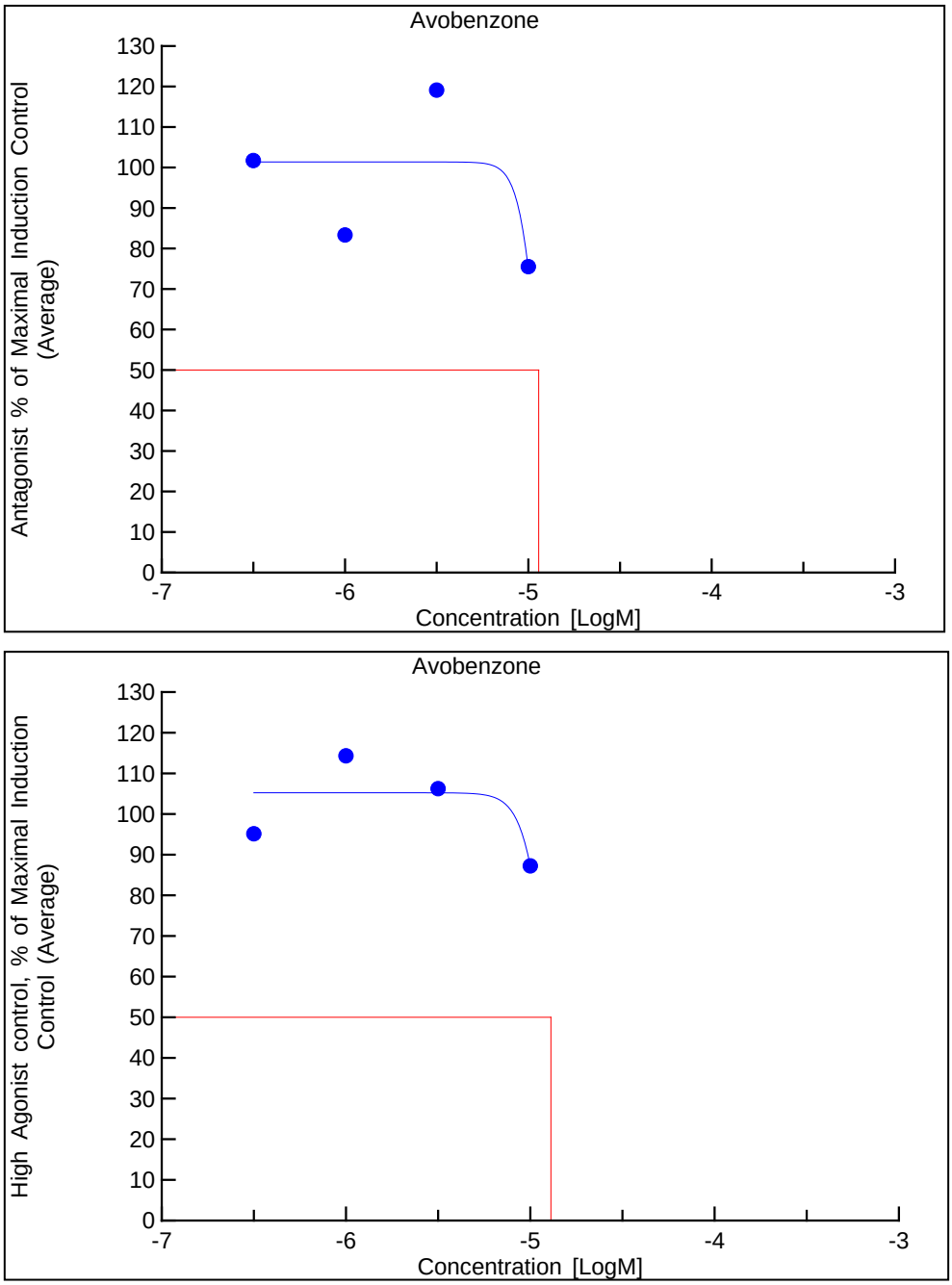
07Feb2013



The two separate graphs represent the data (Means±Standard Deviation) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration). The limit of cyotoxicity was -5.0 logM in run one.

FIGURE 4 Avobenzone – Antagonist

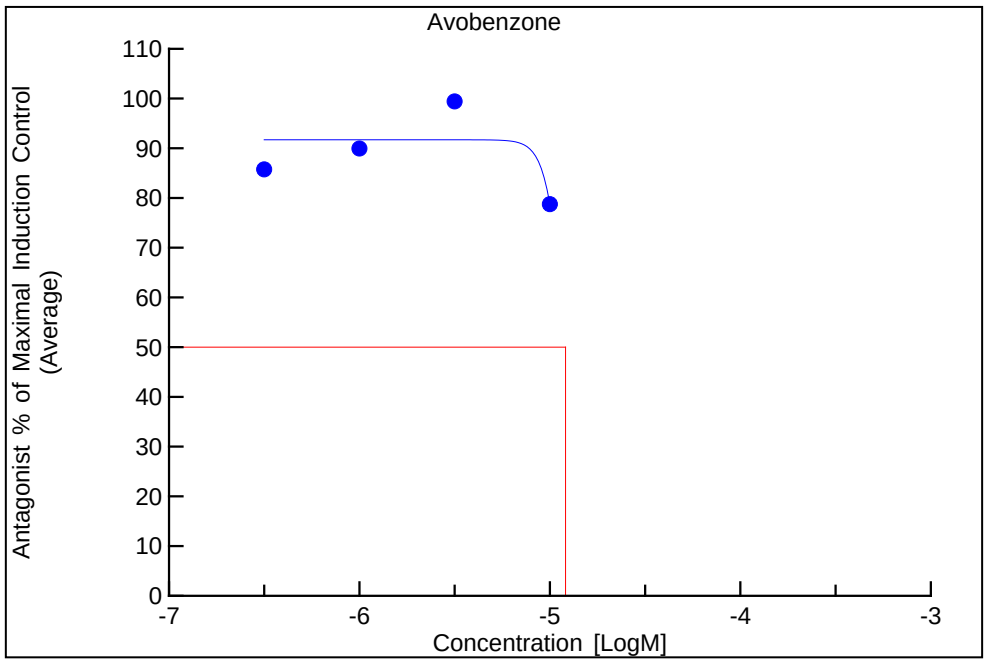
05Feb2013

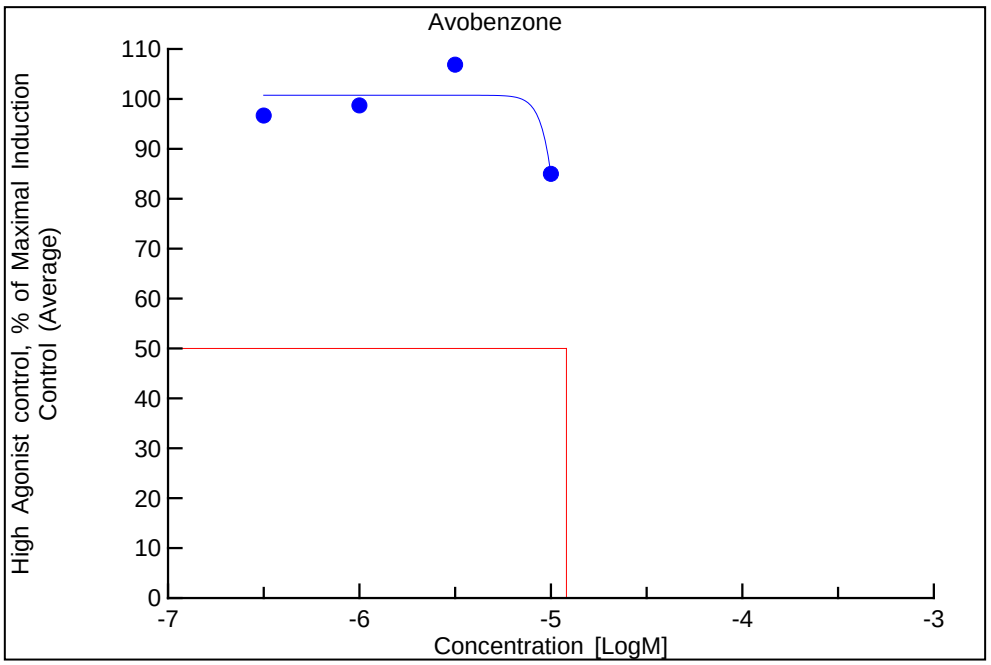


The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT. The limit of cyotoxicity was -5.0 logM.

FIGURE 4 Avobenzone – Antagonist (Continued)

07Feb2013

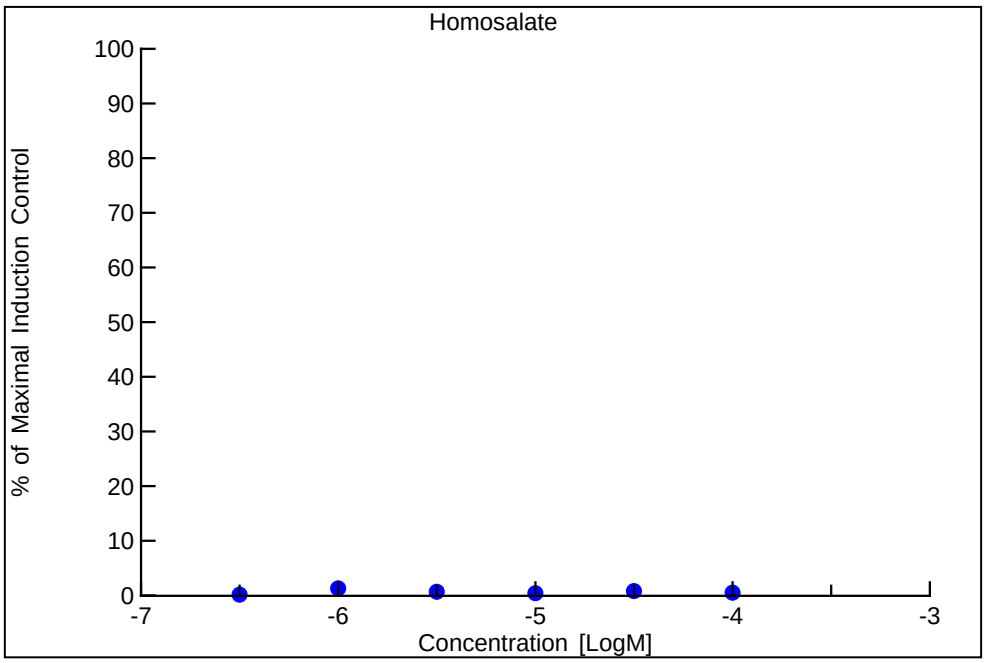




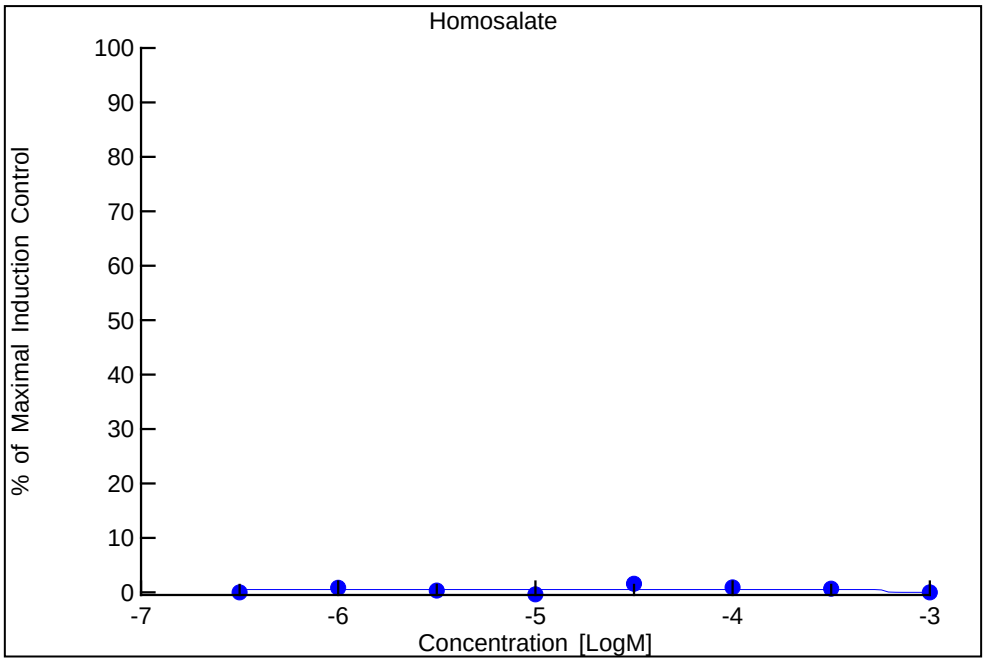
The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT. The limit of cyotoxicity was -5.0 logM

FIGURE 5 Homosalate – Agonist

05Feb2013



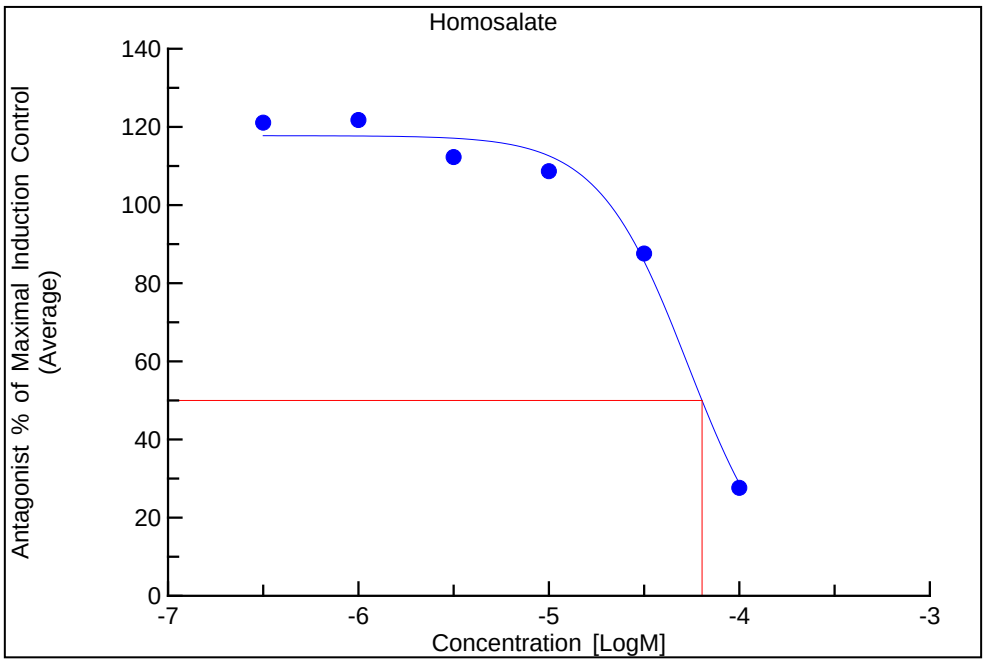
07Feb2013

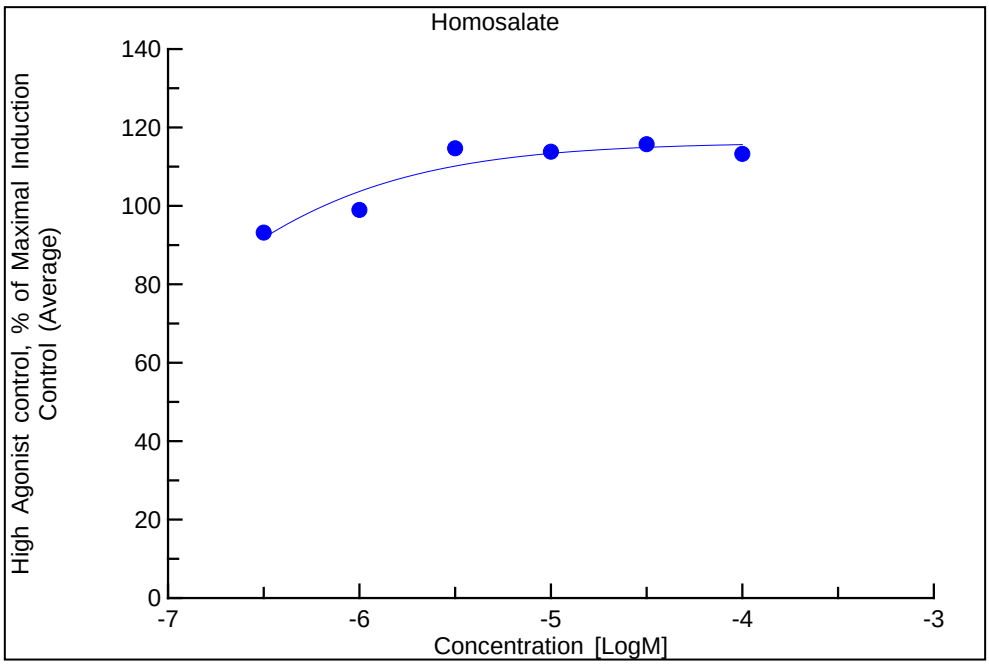


The two separate graphs represent the data (Means±Standard Deviation) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration). The limit of cytotoxicity was -4.0 logM for run one.

FIGURE 6 Homosalate– Antagonist

05Feb2013

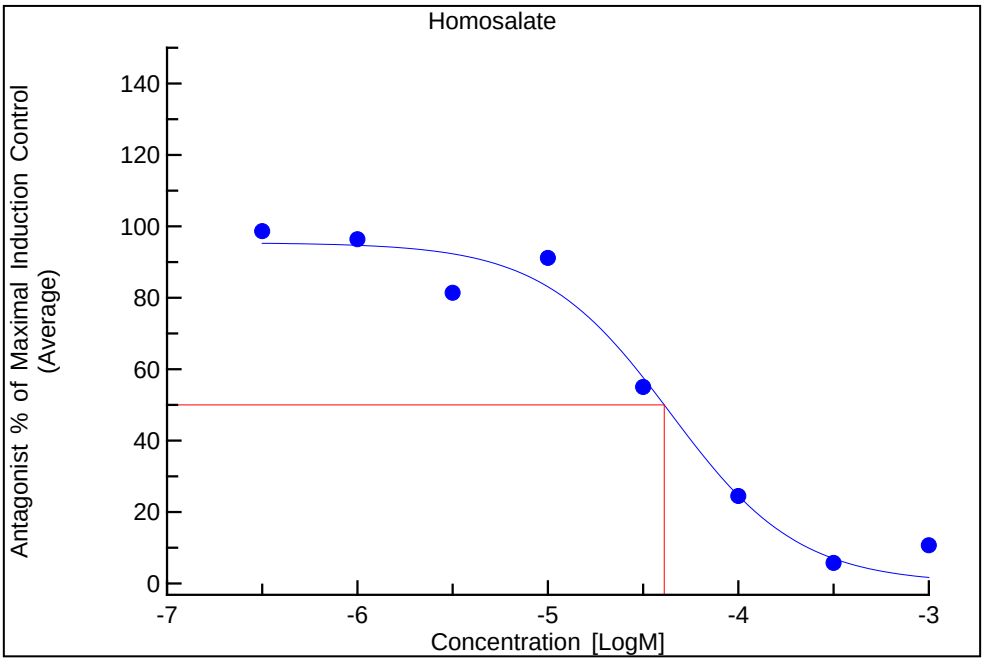


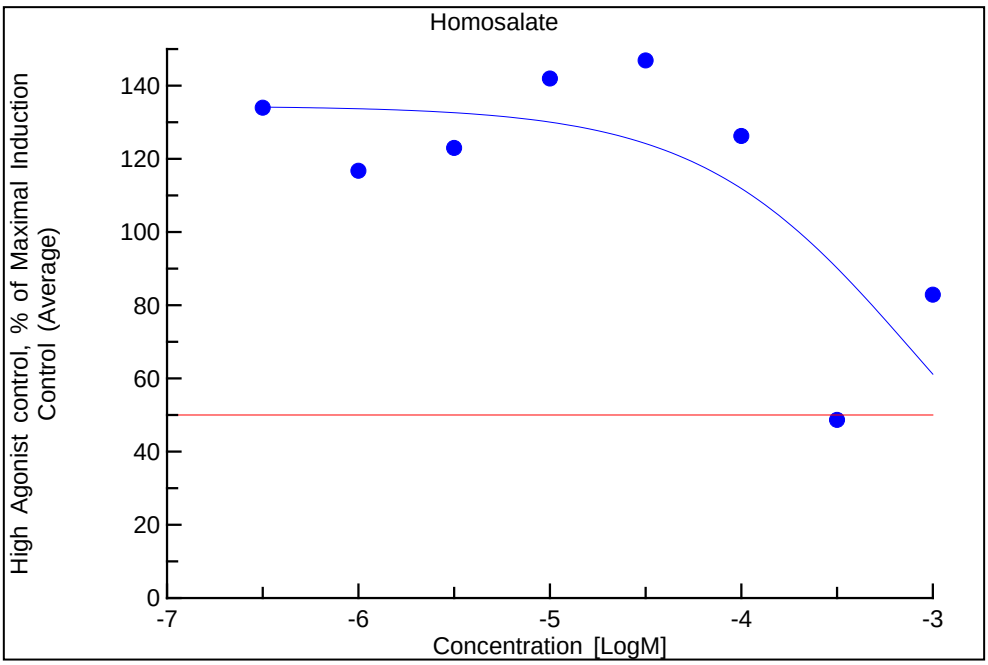


The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT. The limit of cytotoxicity was -4.0 logM for run one.

FIGURE 6 Homosalate – Antagonist (Continued)

07Feb2013

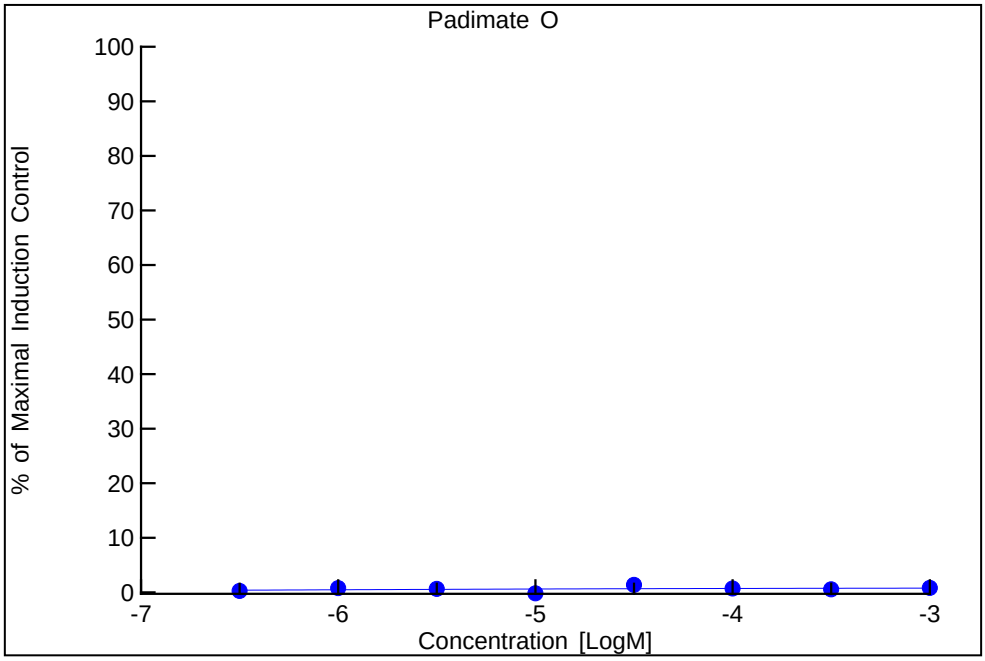




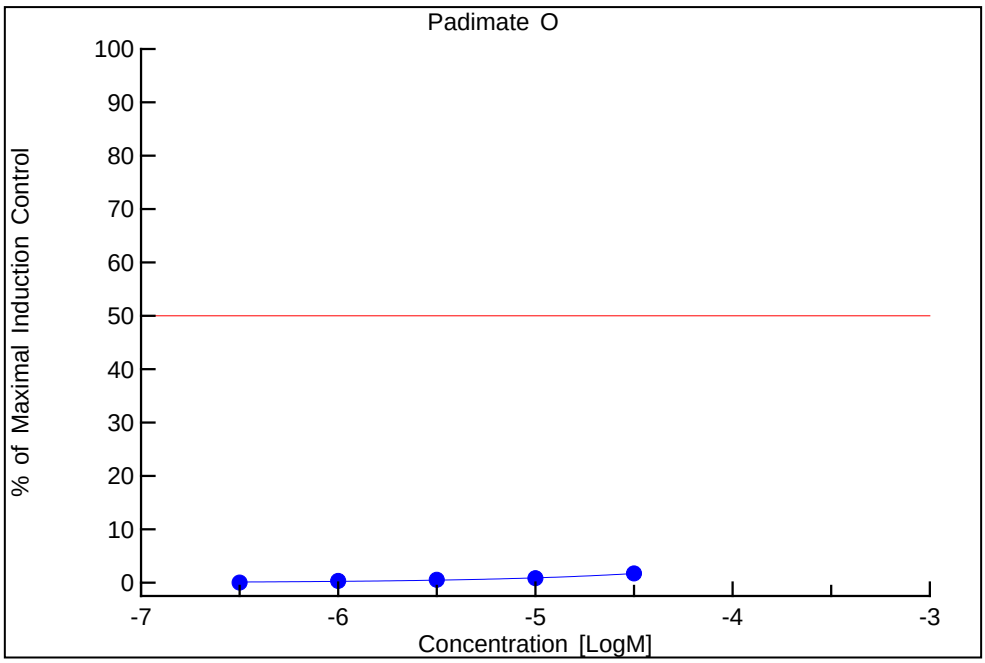
The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT..

FIGURE 7 Padimate O – Agonist

05Feb2013



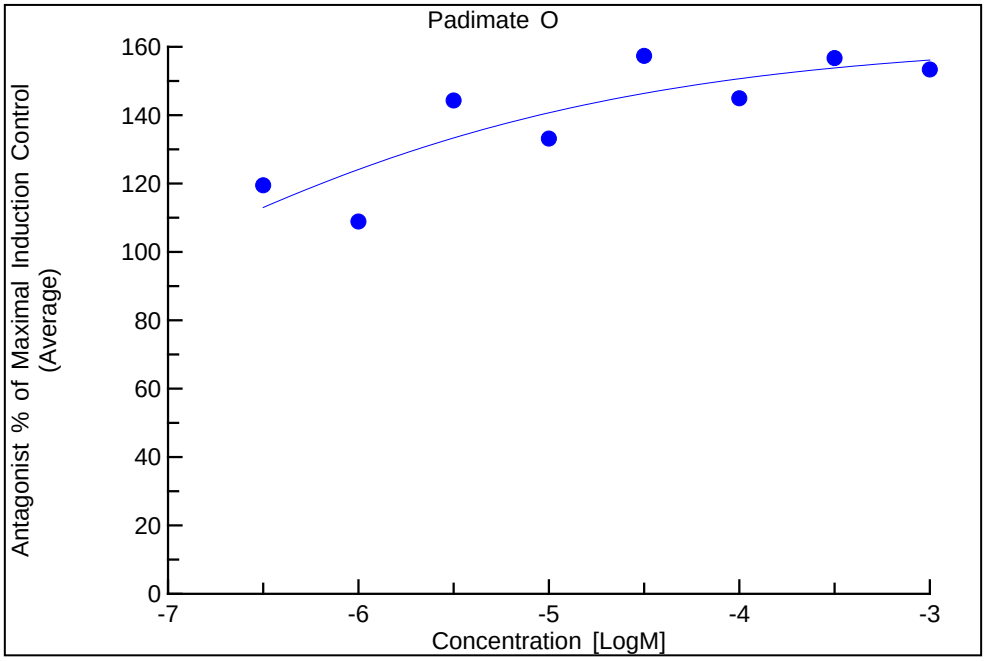
07Feb2013

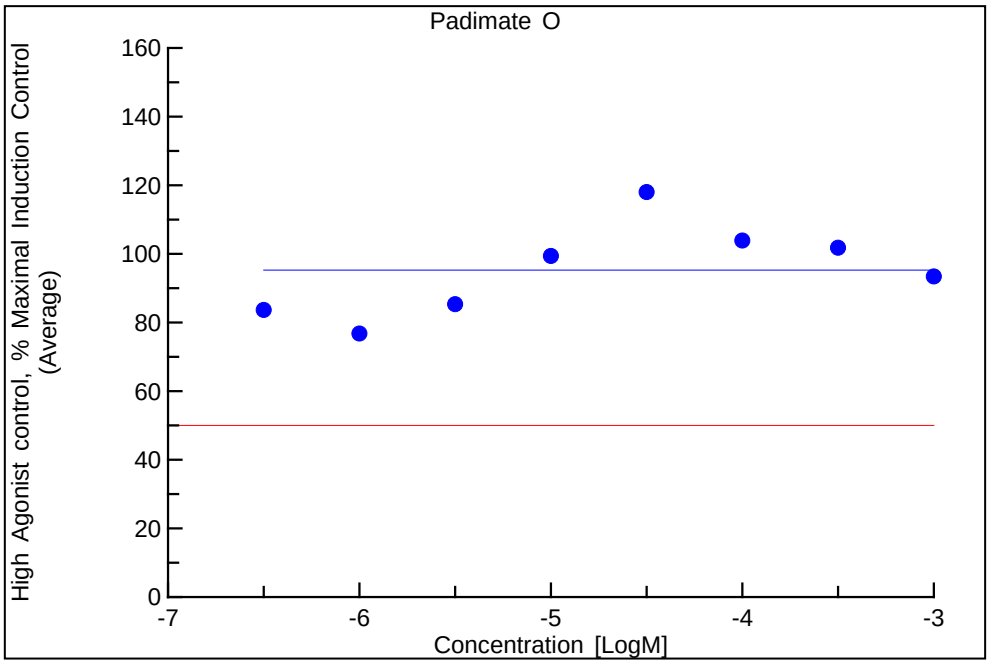


The two separate graphs represent the data (Means±Standard Deviation) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration). The cytotoxicity limit for run two was -4.5 logM.

FIGURE 8 Padimate O – Antagonist

05Feb2013

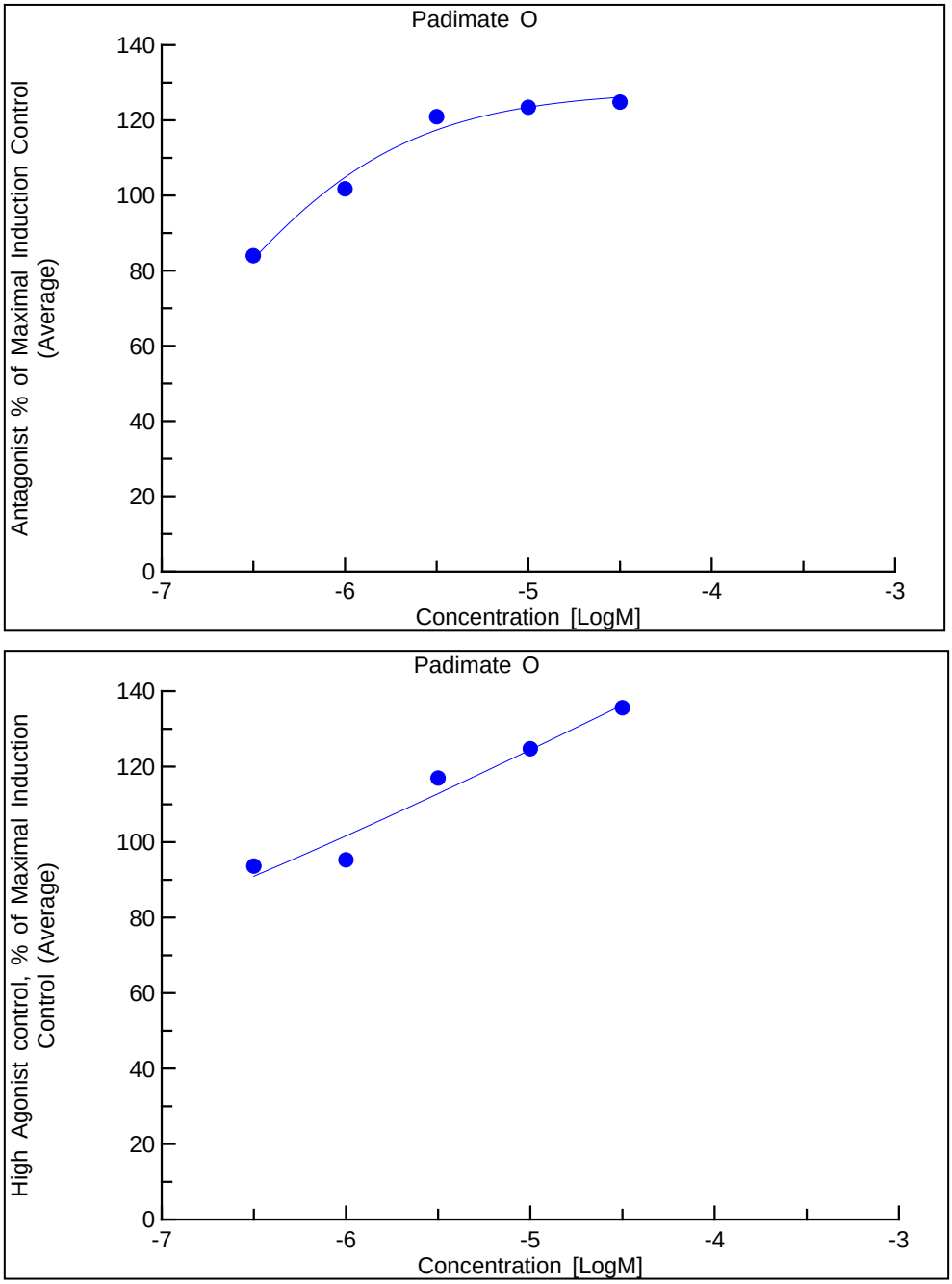




The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT.

FIGURE 8 Padimate O – Antagonist (Continued)

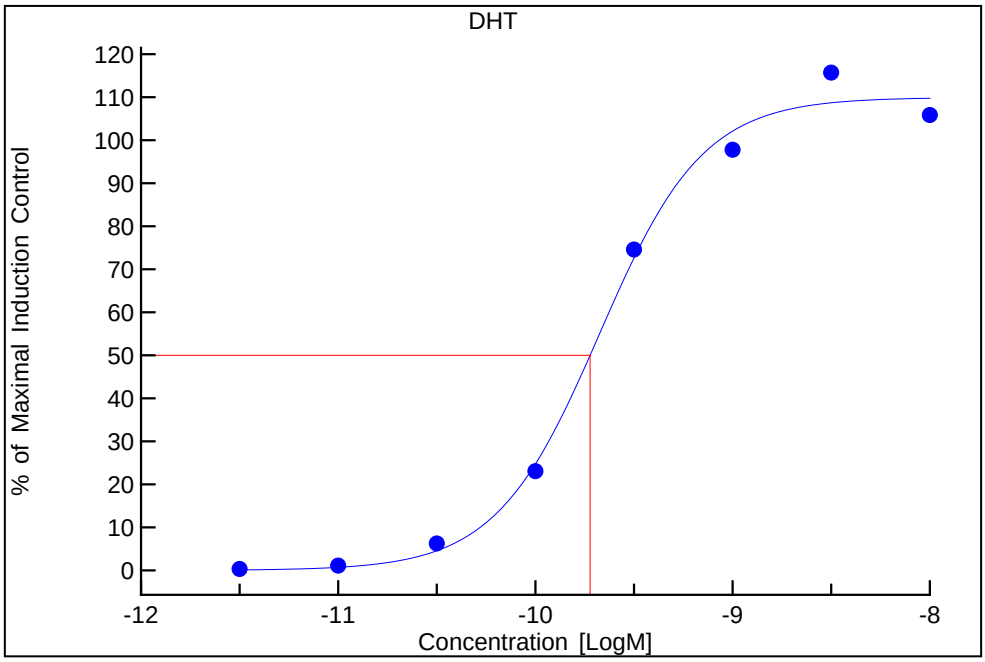
07Feb2013



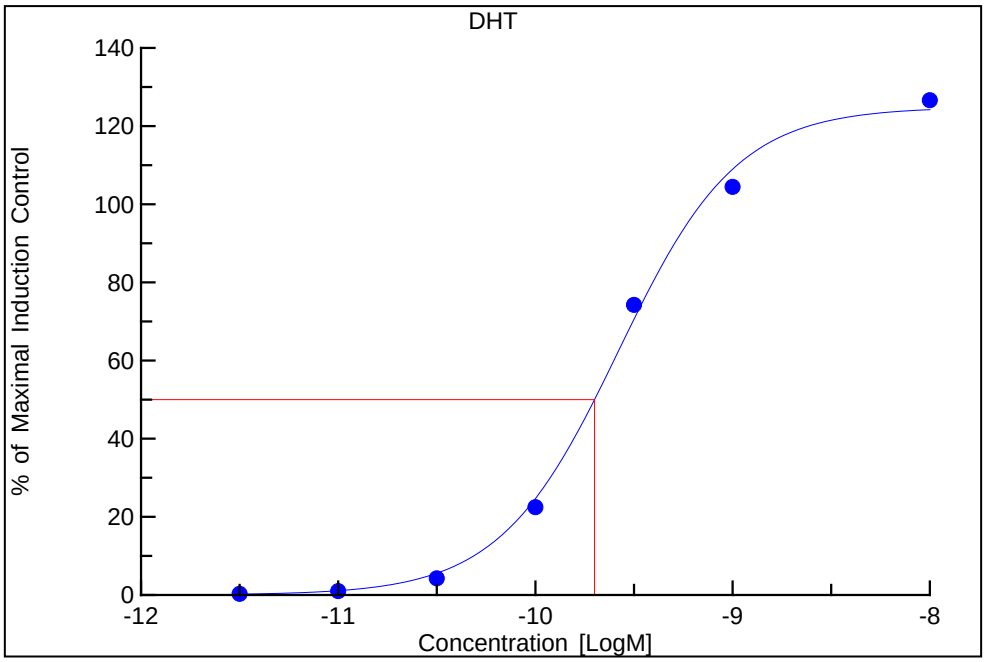
The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT. The cytotoxicity limit for run two was -4.5 logM.

FIGURE 9 DHT – Agonist

05Feb2013



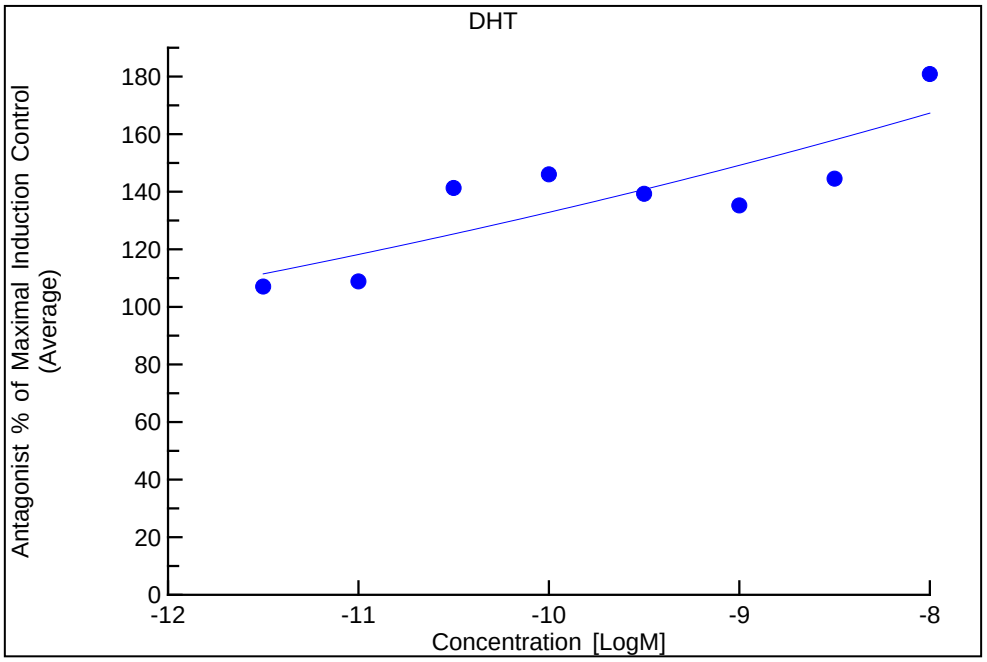
07Feb2013

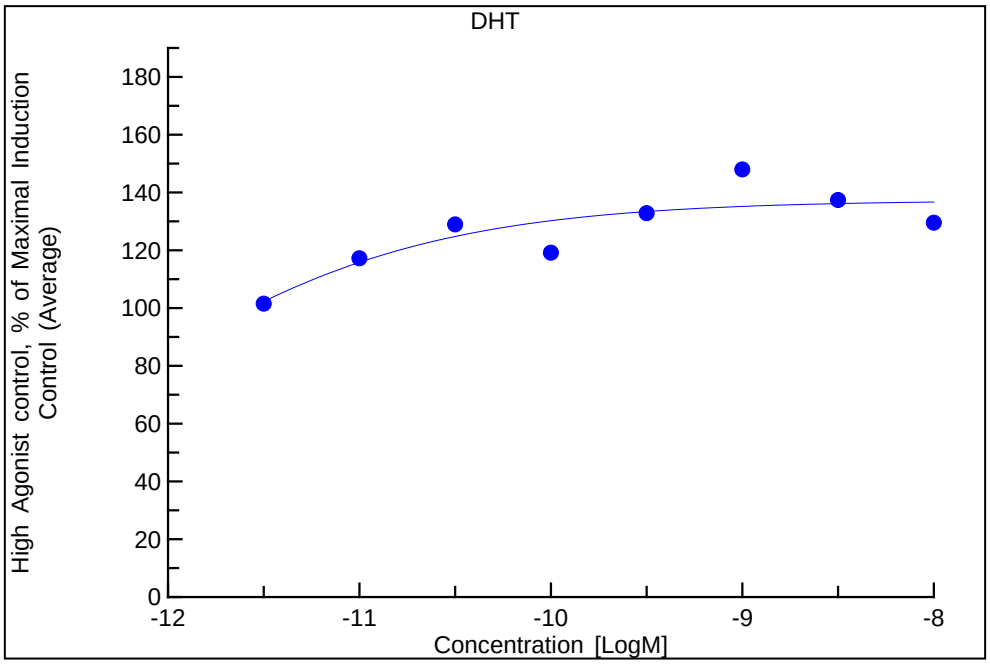


The two separate graphs represent the data (Means±Standard Deviation) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration).

FIGURE 10 DHT – Antagonist

05Feb2013

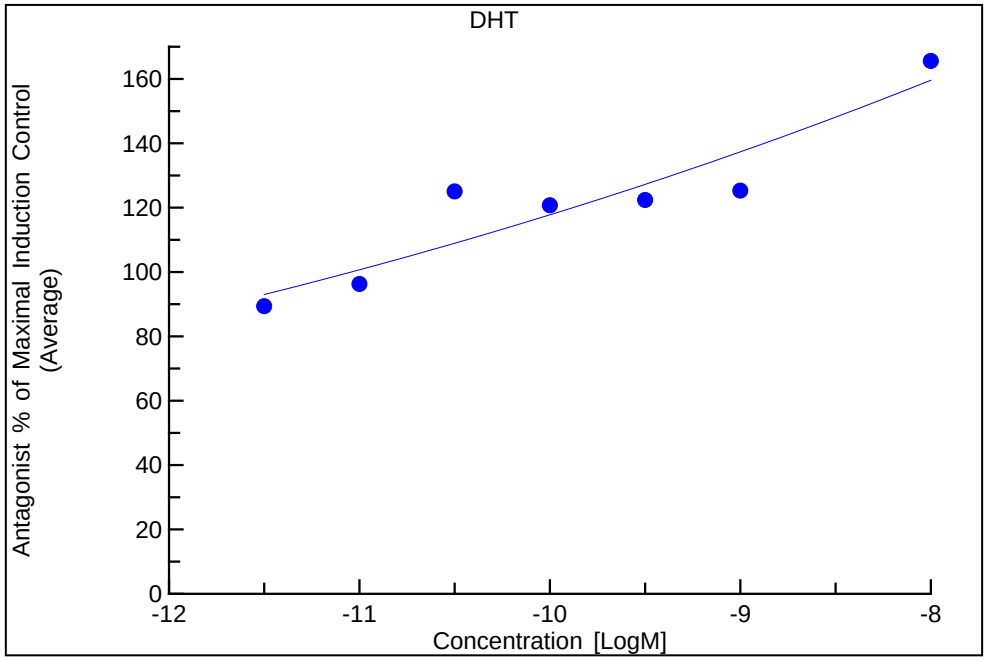


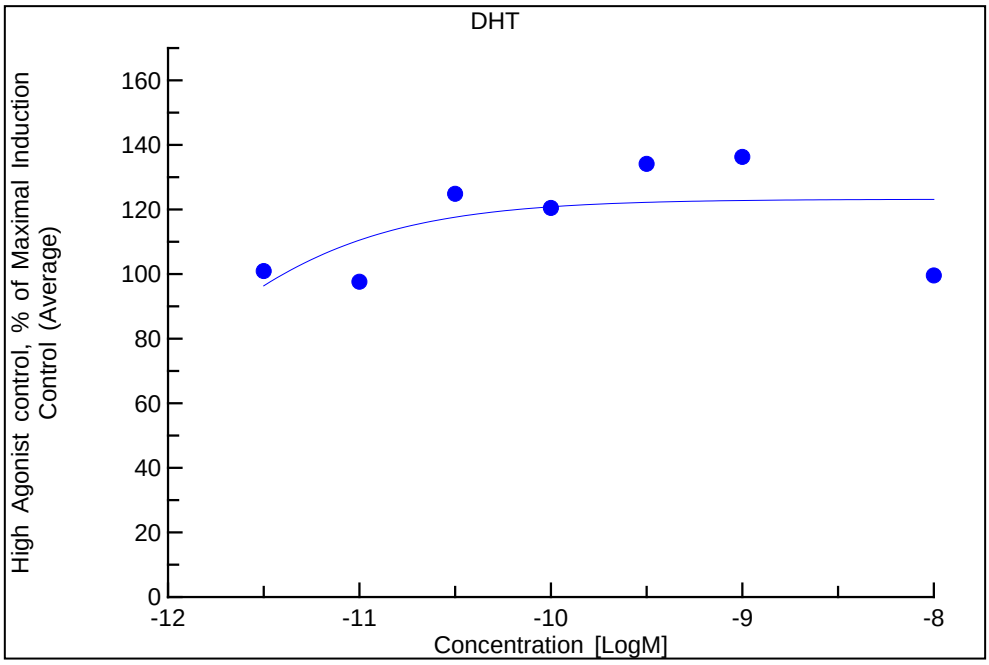


The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT.

FIGURE 10 DHT – Antagonist (Continued)

07Feb2013

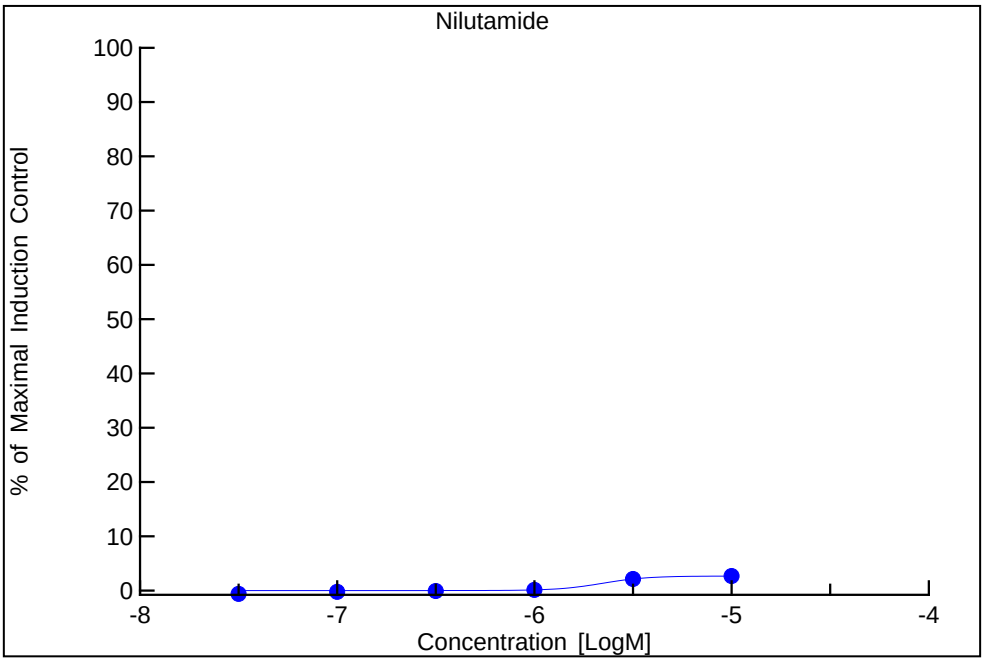




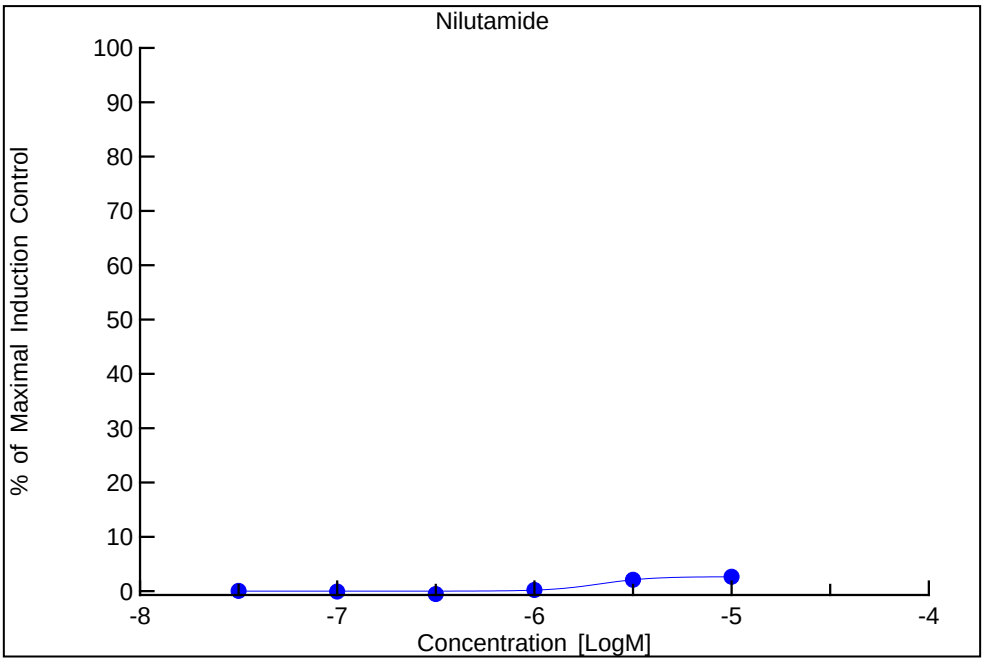
The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT.

FIGURE 11 Nilutamide – Agonist

05Feb2013



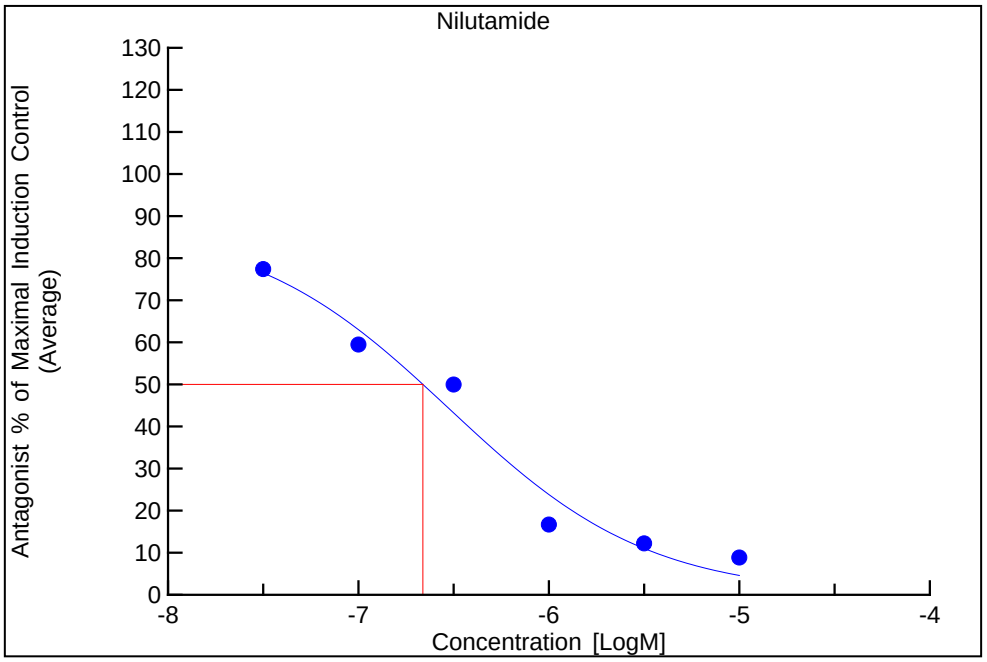
07Feb2013

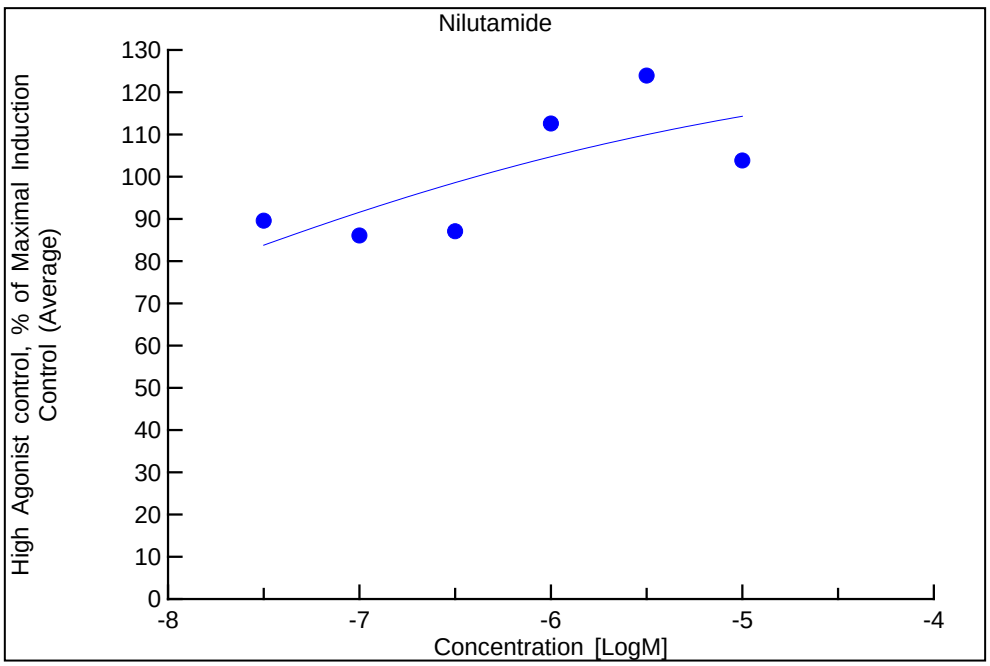


The two separate graphs represent the data (Means±Standard Deviation) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration). The cytotoxicity limit is -5 logM.

FIGURE 12 Nilutamide – Antagonist

05Feb2013

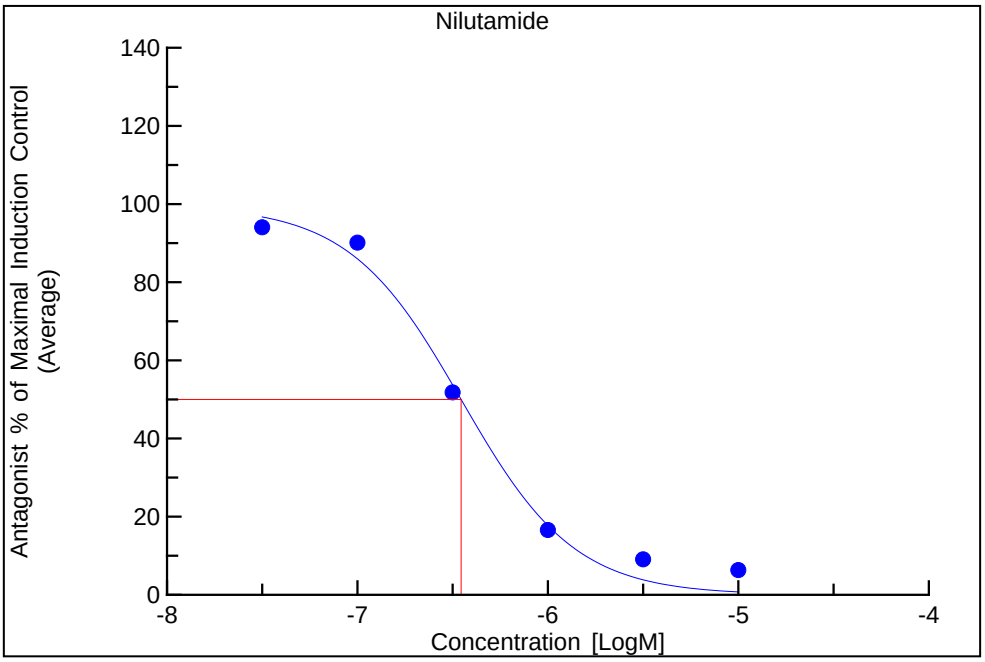


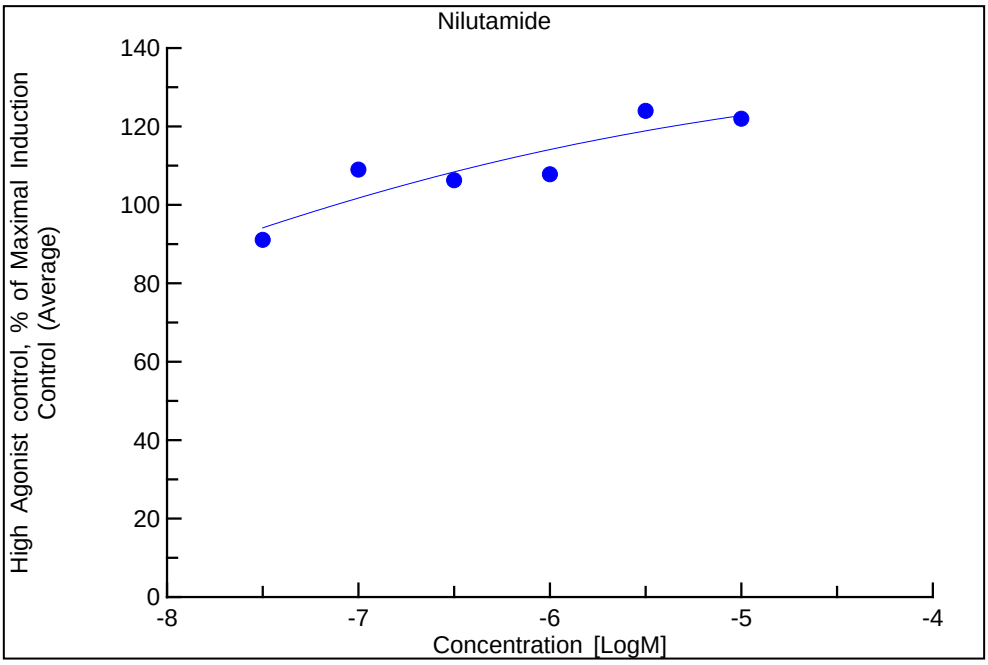


The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT. The cytotoxicity limit is -5 logM.

FIGURE 12 Nilutamide – Antagonist (Continued)

07Feb2013

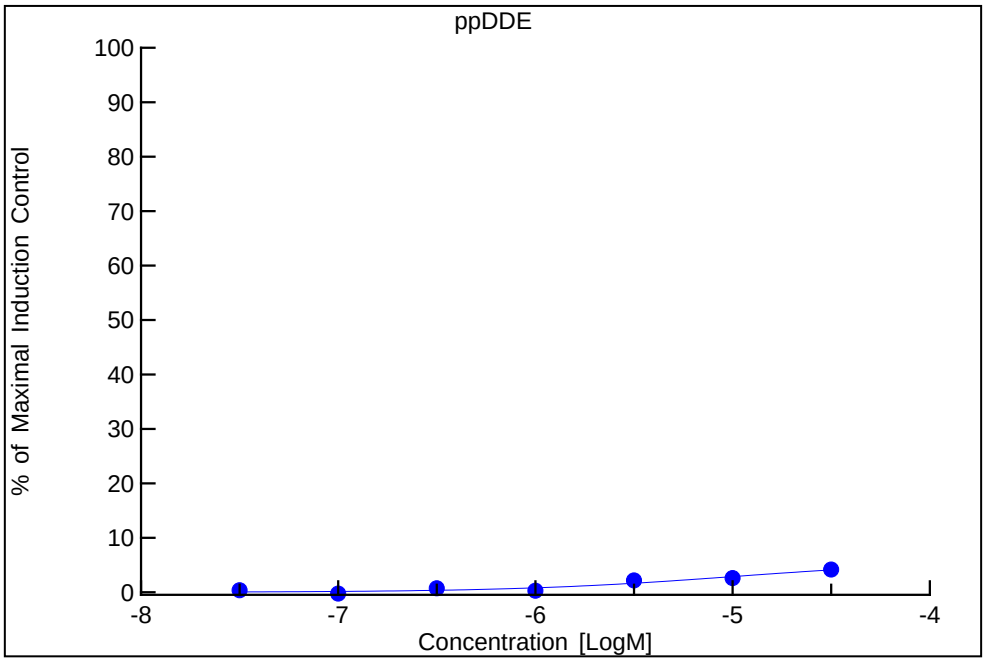




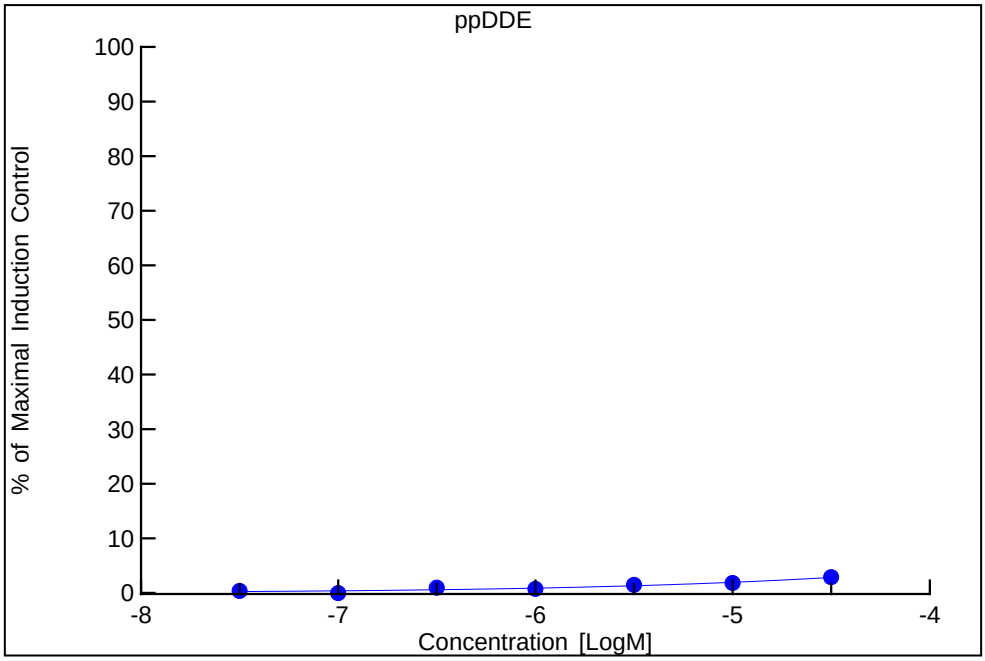
The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT. The cytotoxicity limit is -5 logM.

FIGURE 13 ppDDE – Agonist

05Feb2013



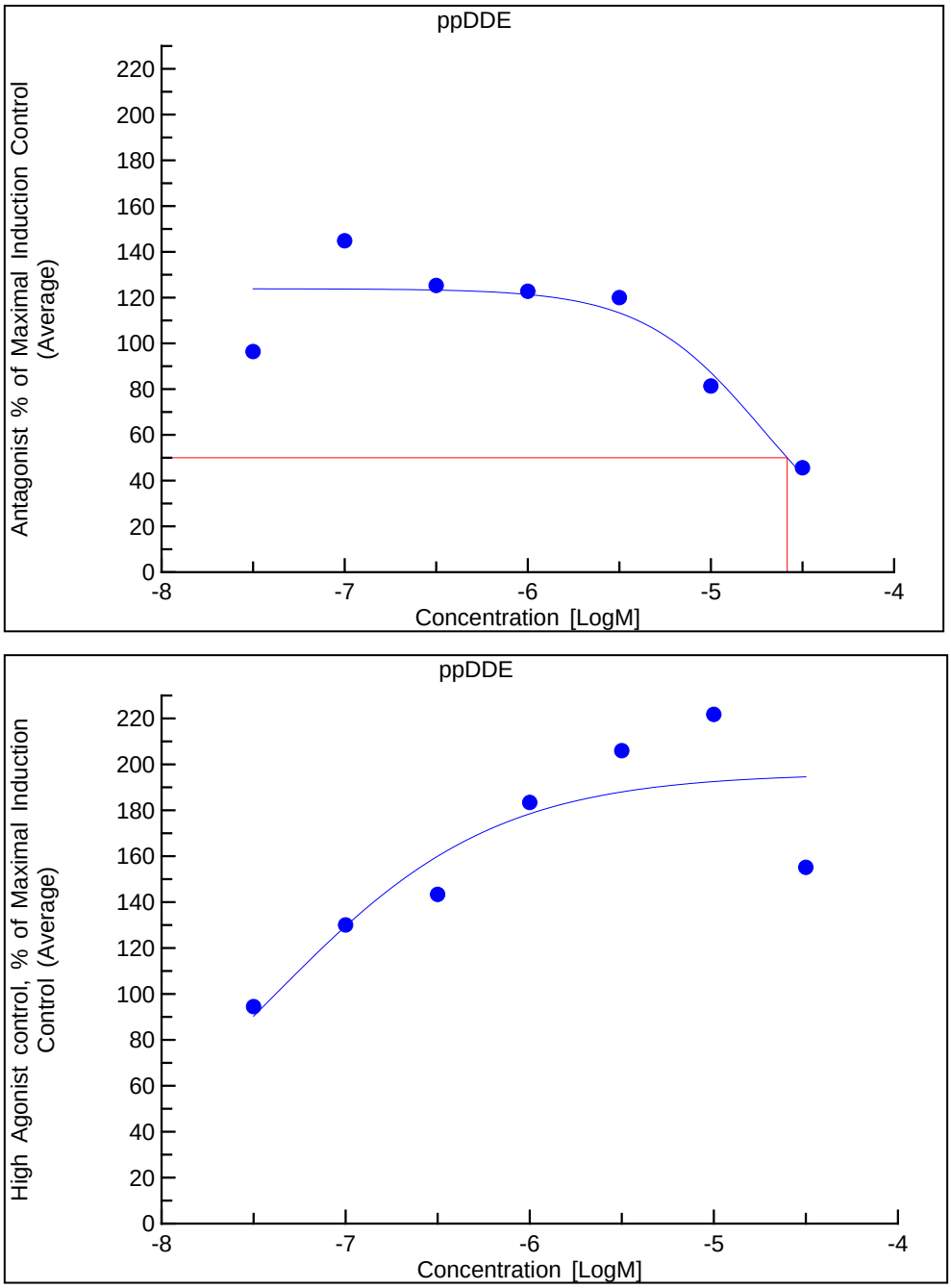
07Feb2013



The two separate graphs represent the data (Means±Standard Deviation) from the two different independent runs of the assay in the absence of antagonist (n =6/concentration). The cytotoxicity limit is -4.5 logM.

FIGURE 14 ppDDE – Antagonist

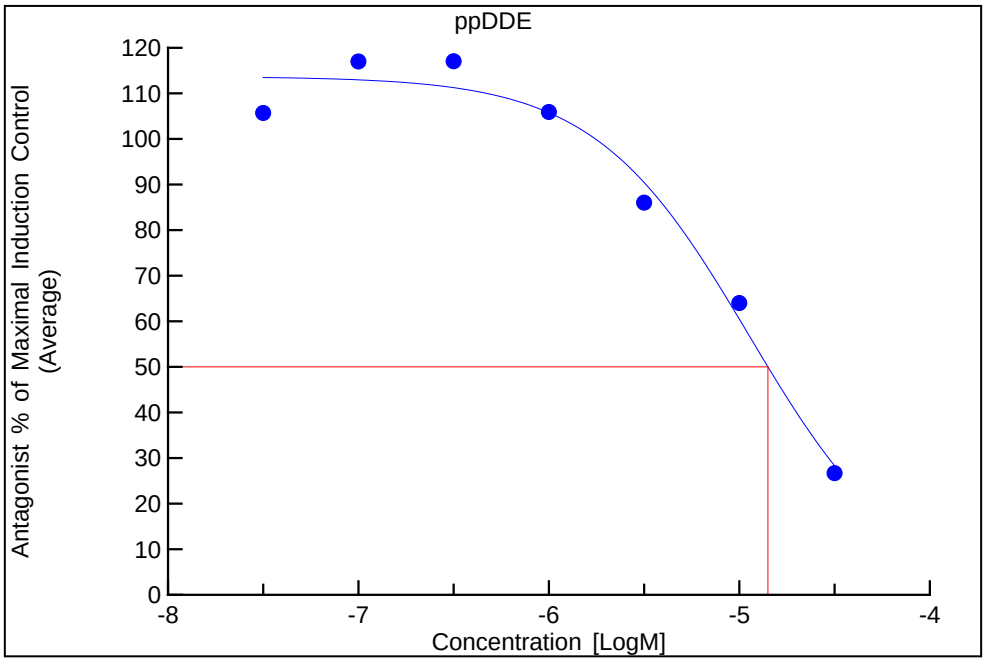
05Feb2013

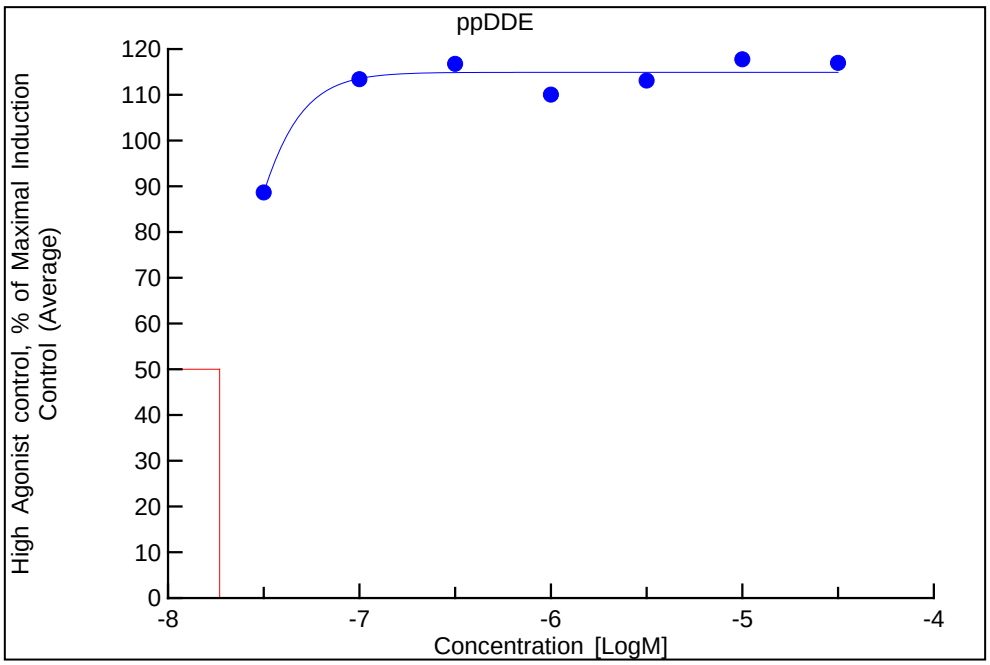


The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT. The cytotoxicity limit is -4.5 logM.

FIGURE 14 ppDDE – Antagonist (Continued)

07Feb2013





The top graph represents the cells co-dosed with 1 nM DHT. The bottom graph represents the cells co-dosed with 1000 nM DHT. The cytotoxicity limit is -4.5 logM.

APPENDICES SECTION

APPENDIX 1 Data Spreadsheets

APPENDIX 1 Raw and Normalized Luminescence Data

VALID RUN 1
February 5, 2013

					Concentration [LogM]							
Ensulizole (Ag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	100	20000	400	500	300	250	550	650	900	750	800	350
B	50	21900	700	150	600	300	550	650	1000	750	1000	1400
C	0	17150	500	650	750	700	700	550	1250	700	550	1100
D	50	21500	450	700	500	450	550	1000	700	1050	1150	1200
E	0	24600	650	650	350	600	450	750	900	1300	800	750
F	0	22550	800	400	800	400	750	750	900	550	550	750
G	0	*16250*	*350*	*400*	*600*	*400*	*750*	*450*	*1100*	*1000*	*550*	*650*
H	0	*8950*	*550*	*500*	*450*	*550*	*500*	*650*	*900*	*800*	*800*	*550*

					Concentration [LogM]							
Ensulizole (Antag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	50	9750	450	13150	18350	12350	15250	12600	17800	16500	23250	17600
B	0	17150	450	15100	22500	14650	12100	18300	24700	15800	20150	15700
C	0	21500	350	13850	16400	19150	17250	21900	23300	20700	18550	27450
D	50	20000	450	17350	19200	15400	19150	16800	24300	27050	18800	24050
E	*50*	*23950*	*26850*	*23000*	*17100*	*14800*	*26200*	*26200*	*17800*	*29850*	*32700*	*24450*
F	*0*	*21650*	*26450*	*26200*	*15450*	*19650*	*32150*	*27150*	*25950*	*25300*	*32000*	*31250*
G	*0*	*18500*	*22350*	*18350*	*17500*	*16900*	*28150*	*28750*	*24550*	*21250*	*30350*	*20450*
H	*0*	*16500*	*20350*	*16450*	*21450*	*23000*	*18850*	*26100*	*23700*	*28550*	*33900*	*22250*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 7, 2013

					Concentration [LogM]							
Ensulizole (Ag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	0	19700	600	300	450	300	700	250	500	1000	750	600
B	0	31100	400	550	500	550	750	800	950	850	1200	1050
C	0	26750	650	600	600	350	700	800	950	1050	850	950
D	0	42400	500	550	550	150	600	550	800	750	700	1150
E	0	28050	850	500	250	550	500	450	1100	850	750	550
F	0	25400	400	450	850	250	500	350	650	900	300	500
G	*0*	*11950*	*350*	*650*	*650*	*500*	*550*	*450*	*850*	*900*	*1200*	*1250*
H	*0*	*11950*	*900*	*450*	*450*	*350*	*800*	*400*	*1650*	*1350*	*800*	*850*

					Concentration [LogM]							
Ensulizole (Antag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	0	21550	400	14150	16500	11350	21400	14750	24150	31100	22600	16650
B	0	28100	450	20600	20400	20700	22950	22700	26000	34350	32000	19350
C	0	28750	550	24700	23000	27100	20050	24250	31050	24600	29150	27650
D	0	24100	450	23200	27300	19200	25300	29250	21750	28700	36350	31800
E	*0*	*29350*	*27750*	*24900*	*29150*	*24000*	*30350*	*23600*	*42950*	*29400*	*31950*	*32150*
F	*50*	*33450*	*24950*	*32650*	*30250*	*30750*	*32950*	*35150*	*37450*	*35100*	*34900*	*42150*
G	*0*	*24850*	*32750*	*25300*	*25900*	*31550*	*45500*	*32900*	*33000*	*48100*	*35800*	*30550*
H	*0*	*21950*	*31350*	*25350*	*27400*	*22900*	*32650*	*45600*	*39450*	*39100*	*34400*	*37750*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
February 5, 2013

					Concentration [LogM]							
Avobenzone (Ag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	0	23000	250	400	650	600	300	350	500	300	50	150
B	0	22800	550	800	550	400	750	550	450	200	100	100
C	0	26600	200	200	500	350	400	500	400	50	150	250
D	50	23350	300	400	400	600	900	300	250	200	50	100
E	0	24850	250	200	650	500	600	400	450	150	100	200
F	0	22550	450	1000	700	600	600	650	350	0	100	150
G	*50*	*16700*	*450*	*750*	*150*	*450*	*750*	*150*	*100*	*50*	*200*	*200*
H	*0*	*10100*	*550*	*450*	*450*	*600*	*850*	*300*	*100*	*200*	*300*	*150*

					Concentration [LogM]							
Avobenzone (Antag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	0	12150	1000	15650	16950	13650	20900	13550	3850	300	100	50
B	0	21200	450	16750	20250	18600	24700	17850	6150	450	300	250
C	0	20950	400	18400	24550	14700	28800	12350	3750	200	50	150
D	0	15300	350	27550	17900	18700	18500	15950	3600	250	250	200
E	*0*	*20650*	*25100*	*21750*	*21450*	*26650*	*21450*	*15450*	*4200*	*500*	*100*	*100*
F	*0*	*20200*	*21200*	*22450*	*18800*	*21950*	*19650*	*20550*	*3200*	*250*	*50*	*150*
G	*0*	*20850*	*19850*	*21750*	*21600*	*21550*	*23050*	*17150*	*3950*	*350*	*300*	*300*
H	*50*	*19250*	*14350*	*20050*	*17450*	*24700*	*24150*	*19750*	*3800*	*500*	*300*	*300*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 7, 2013

					Concentration [LogM]							
Avobenzone (Ag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	0	25550	650	800	600	350	600	750	250	150	50	300
B	0	34350	850	550	450	700	550	200	150	150	100	50
C	50	39450	550	450	4850	1400	600	450	350	200	0	50
D	0	34800	550	300	1050	300	500	300	100	50	100	150
E	0	35350	450	600	650	400	600	400	200	150	250	150
F	50	27700	400	450	500	700	800	250	200	50	50	0
G	*0*	*12650*	*600*	*700*	*900*	*350*	*700*	*250*	*150*	*200*	*150*	*0*
H	*0*	*22400*	*250*	*450*	*600*	*650*	*350*	*200*	*100*	*0*	*100*	*200*

					Concentration [LogM]							
Avobenzone (Antag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	0	14550	500	20500	16250	17200	20750	13450	3700	350	50	250
B	0	42500	500	22400	24000	22550	30050	22900	5250	0	250	0
C	0	30450	700	20850	25950	27300	25450	19400	7550	400	250	150
D	50	25250	450	39800	22900	26300	26700	26250	5250	250	50	150
E	*50*	*25950*	*26650*	*32550*	*43100*	*24850*	*44150*	*30950*	*9350*	*250*	*50*	*400*
F	*0*	*37950*	*49250*	*34300*	*29050*	*42200*	*31350*	*26550*	*7600*	*350*	*350*	*50*
G	*0*	*38050*	*32850*	*32050*	*29100*	*32800*	*39650*	*33050*	*7750*	*50*	*100*	*150*
H	*0*	*26050*	*42650*	*29700*	*34150*	*38350*	*34300*	*28750*	*3900*	*250*	*200*	*250*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
February 5, 2013

					Concentration [LogM]							
Homosalate (Ag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	0	21150	400	500	600	500	550	600	600	550	600	300
B	50	26900	400	500	650	1000	400	550	950	750	300	250
C	0	23150	500	800	400	1300	1000	550	900	350	450	200
D	0	27000	350	550	400	850	1000	600	550	600	350	650
E	0	29100	400	800	750	600	550	700	650	1000	600	450
F	50	27250	500	700	600	900	700	800	750	700	450	750
G	*0*	*19200*	*450*	*550*	*500*	*600*	*850*	*500*	*1000*	*800*	*450*	*600*
H	*50*	*9700*	*500*	*150*	*600*	*200*	*600*	*450*	*1150*	*900*	*450*	*1200*

					Concentration [LogM]							
Homosalate (Antag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	0	18900	300	17900	18300	19850	17950	22300	15500	6200	3350	5200
B	0	24950	600	17700	20650	22000	24850	19450	18900	6050	2800	4100
C	0	29050	250	17500	23600	25250	12400	18300	15600	5550	2600	700
D	0	20850	350	21700	27700	23650	28600	21100	15700	3950	3450	2900
E	*0*	*18600*	*26200*	*30250*	*23050*	*16250*	*25250*	*31350*	*25600*	*22700*	*30050*	*25150*
F	*0*	*20100*	*24950*	*20350*	*19500*	*33450*	*31900*	*24200*	*30700*	*29950*	*9700*	*31800*
G	*50*	*26400*	*24000*	*26500*	*21700*	*22000*	*28200*	*30700*	*25600*	*28750*	*13950*	*22350*
H	*0*	*19100*	*15650*	*18400*	*22650*	*20500*	*21250*	*19550*	*25650*	*23900*	*17750*	*17250*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 7, 2013

					Concentration [LogM]							
Homosalate (Ag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	0	25500	250	500	300	450	650	350	800	550	1050	450
B	0	29250	500	650	400	600	500	400	850	850	500	250
C	0	29150	500	250	300	800	600	400	1000	650	400	750
D	0	19150	400	550	700	600	450	400	800	500	450	600
E	0	25450	500	400	500	850	400	350	850	800	450	350
F	50	22650	600	350	500	650	600	250	750	700	850	300
G	*100*	*11150*	*900*	*400*	*250*	*350*	*800*	*750*	*600*	*700*	*600*	*1350*
H	*0*	*10400*	*850*	*400*	*450*	*500*	*750*	*750*	*1150*	*950*	*650*	*400*

					Concentration [LogM]							
Homosalate (Antag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	0	21700	750	16400	19450	20550	25700	18250	13850	6250	1500	3550
B	0	33750	650	30250	38800	33200	20950	24500	17650	7400	2450	3250
C	0	29050	500	36250	24800	30250	21350	27500	15050	8400	2800	3550
D	50	42000	650	27700	26100	22700	22500	30800	15450	6950	2000	3750
E	*50*	*33500*	*32900*	*21700*	*29650*	*32200*	*27450*	*40000*	*40200*	*32800*	*11300*	*24600*
F	*0*	*37500*	*24850*	*29950*	*44900*	*21100*	*33800*	*47000*	*43150*	*38050*	*12450*	*30200*
G	*50*	*26350*	*25400*	*30800*	*44400*	*40850*	*34700*	*39650*	*33300*	*34550*	*15250*	*21200*
H	*0*	*29600*	*32450*	*33300*	*35150*	*40450*	*45700*	*36450*	*52050*	*39950*	*18600*	*20300*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
February 5, 2013

					Concentration [LogM]							
Padimate-O (Ag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	0	20350	550	600	500	450	500	250	900	700	800	1050
B	50	22900	550	250	500	700	500	450	500	800	650	600
C	0	28950	850	600	550	650	500	400	1250	400	950	550
D	50	19600	200	350	450	750	1100	500	1000	900	450	750
E	0	19850	550	800	900	1000	900	950	900	600	500	650
F	0	26800	550	650	700	750	600	450	550	800	650	750
G	*0*	*9350*	*450*	*250*	*550*	*400*	*850*	*700*	*800*	*500*	*250*	*800*
H	*50*	*8200*	*450*	*500*	*150*	*700*	*550*	*450*	*700*	*700*	*500*	*300*

					Concentration [LogM]							
Padimate-O (Antag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	0	13500	500	13600	13600	20300	21700	16150	23650	22450	22700	17850
B	0	17600	650	16000	16900	14100	21150	18000	25550	23850	19100	23500
C	0	12150	500	13550	17500	17100	20400	14900	22000	19600	23450	29600
D	50	24100	500	14200	20100	10750	18550	26600	17800	16250	23400	15850
E	*50*	*19300*	*22300*	*17700*	*17750*	*23850*	*23950*	*30650*	*28800*	*29450*	*28200*	*22050*
F	*0*	*26650*	*21650*	*29350*	*26550*	*19650*	*17500*	*23050*	*31950*	*19100*	*26500*	*24700*
G	*0*	*29850*	*19650*	*20000*	*20650*	*16100*	*21400*	*24200*	*27950*	*23150*	*22800*	*17850*
H	*0*	*19650*	*23050*	*36950*	*15150*	*14100*	*18800*	*16850*	*23400*	*27250*	*19500*	*24600*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 7, 2013

					Concentration [LogM]							
Padimate-O (Ag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	50	18750	600	350	450	700	350	250	950	750	650	250
B	50	23250	800	650	600	750	850	700	700	650	650	1100
C	0	22800	600	400	400	350	650	900	700	800	700	350
D	0	27100	550	350	600	550	800	1000	1150	1000	300	350
E	0	37100	350	700	600	850	650	700	1050	550	450	600
F	50	37450	600	500	600	550	800	1050	1500	800	150	600
G	*0*	*18250*	*800*	*550*	*650*	*1000*	*650*	*500*	*1150*	*450*	*550*	*400*
H	*50*	*25950*	*700*	*300*	*450*	*500*	*700*	*450*	*1000*	*400*	*400*	*450*

					Concentration [LogM]							
Padimate-O (Antag)	blank	10 nM DHT	VC	VC	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0	-3.5	-3.0
A	0	7050	500	25650	17900	20850	28800	22300	23850	23800	16050	18100
B	0	30900	450	19850	21850	23950	28900	30950	34800	18500	22750	18150
C	0	26750	100	27750	19100	20200	30150	30950	30750	33950	17100	20750
D	0	22250	350	22000	21350	31900	27050	33050	29150	21850	17200	18150
E	*100*	*20200*	*28400*	*31050*	*23450*	*27100*	*28350*	*43400*	*51750*	*24350*	*19850*	*26000*
F	*50*	*28100*	*26800*	*28700*	*28850*	*27850*	*38050*	*42000*	*29100*	*28350*	*16650*	*20150*
G	*0*	*30150*	*25650*	*31750*	*31050*	*31200*	*33750*	*33450*	*37500*	*26250*	*25250*	*20900*
H	*0*	*25150*	*34900*	*39350*	*32200*	*31400*	*43800*	*34600*	*48350*	*23850*	*21050*	*24500*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
February 5, 2013

					Concentration [LogM]							
DHT (Ag)	blank	10 nM DHT	VC	VC	-11.5	-11.0	-10.5	-10.0	-9.5	-9.0	-8.5	-8.0
A	0	17500	850	500	450	650	1650	2900	11650	20600	21650	25050
B	50	22550	550	550	700	650	2350	7150	15500	23800	24800	27750
C	0	22450	400	200	750	1300	2300	6750	17200	19250	25600	21550
D	0	18850	400	600	750	600	2750	7300	24550	28250	41800	23150
E	0	34500	450	1000	350	800	1800	6150	23150	25550	25200	30800
F	0	30200	650	700	900	1000	1500	6050	17750	25450	29400	26100
G	*0*	*16900*	*600*	*450*	*400*	*400*	*750*	*550*	*1650*	*1750*	*7550*	*18650*
H	*0*	*16100*	*500*	*600*	*100*	*400*	*600*	*600*	*900*	*2100*	*4800*	*15300*

					Concentration [LogM]							
DHT (Antag)	blank	10 nM DHT	VC	VC	-11.5	-11.0	-10.5	-10.0	-9.5	-9.0	-8.5	-8.0
A	0	4150	350	16250	15100	18400	16700	26500	18300	19500	22900	26900
B	50	24550	500	21750	24850	19650	21850	25950	22700	22050	24950	27200
C	0	23750	350	17350	15900	15400	26800	16850	30300	26800	20950	28900
D	0	30900	500	13050	17250	20850	30600	29800	23300	23550	29300	39350
E	*0*	*20800*	*22500*	*25750*	*23600*	*28550*	*33850*	*21400*	*28450*	*31600*	*35150*	*28600*
F	*100*	*26300*	*16900*	*21750*	*26250*	*25000*	*26550*	*19900*	*26300*	*24550*	*28450*	*26650*
G	*50*	*24050*	*23350*	*20750*	*16050*	*25600*	*26300*	*33100*	*29450*	*34550*	*27150*	*31300*
H	*100*	*21400*	*16350*	*20550*	*19300*	*18950*	*21050*	*25300*	*26750*	*32700*	*23950*	*21700*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 7, 2013

					Concentration [LogM]							
DHT (Ag)	blank	10 nM DHT	VC	VC	-11.5	-11.0	-10.5	-10.0	-9.5	-9.0	-8.5	-8.0
A	0	26500	500	300	500	500	1500	5450	13750	24550	25150	27600
B	50	25750	650	800	750	900	1400	6200	21400	34300	41350	34850
C	50	32750	450	450	850	1050	1900	7200	21000	32350	35550	37350
D	50	30300	800	650	350	950	2400	10000	23150	36000	40050	64500
E	0	37700	200	650	750	900	2150	9500	20850	28200	35650	29500
F	0	32450	600	700	700	850	1750	5900	38400	38150	36900	40100
G	*0*	*14050*	*300*	*400*	*500*	*950*	*600*	*500*	*1050*	*1800*	*3450*	*12050*
H	*0*	*8600*	*750*	*600*	*300*	*250*	*550*	*600*	*800*	*1300*	*3150*	*9400*

					Concentration [LogM]							
DHT (Antag)	blank	10 nM DHT	VC	VC	-11.5	-11.0	-10.5	-10.0	-9.5	-9.0	-8.5	-8.0
A	0	7900	250	22650	16000	18300	17050	25850	20800	19750	22600	30200
B	50	7600	400	18000	17250	17500	28800	25750	20700	25750	28800	35900
C	0	21600	400	24200	19250	20400	29350	23200	26900	27950	23500	39350
D	100	22500	400	15650	19600	21350	25100	22100	29800	27050	23250	26900
E	*50*	*22800*	*26100*	*32100*	*21000*	*23750*	*27500*	*24250*	*29400*	*33100*	*31600*	*31000*
F	*50*	*28600*	*21300*	*27850*	*29550*	*25900*	*30550*	*36050*	*30250*	*34100*	*25950*	*29000*
G	*0*	*23350*	*27750*	*25900*	*25900*	*25350*	*36350*	*30650*	*26950*	*32300*	*26450*	*21400*
H	*0*	*20600*	*10950*	*16150*	*18450*	*16850*	*22650*	*22050*	*39050*	*28150*	*18600*	*12250*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
February 5, 2013

					Concentration [LogM]							
Nilutamide (Ag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	0	18000	350	350	550	550	350	500	750	1100	2050	250
B	50	19200	200	550	300	300	350	850	1000	750	1800	300
C	50	22450	650	350	300	350	600	500	1300	1150	2650	50
D	0	22400	700	900	550	500	850	400	1000	550	2100	100
E	0	25000	450	550	300	700	400	500	1200	950	2200	400
F	0	18100	550	600	350	400	450	500	450	1850	2300	400
G	*50*	*17750*	*600*	*200*	*300*	*500*	*550*	*350*	*1350*	*1500*	*1450*	*150*
H	*0*	*12500*	*800*	*450*	*800*	*950*	*1000*	*550*	*1350*	*1050*	*1500*	*50*

					Concentration [LogM]							
Nilutamide (Antag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	0	17550	200	17100	9500	8550	8300	2350	2850	1400	1350	100
B	50	20350	600	20650	13300	9900	9450	3550	2850	1950	2700	150
C	0	27250	850	17950	15000	10850	7950	3950	2450	2800	1700	250
D	0	21800	350	14650	17100	13350	10450	3550	2200	1900	2700	250
E	*0*	*23800*	*23600*	*19750*	*19400*	*18700*	*14700*	*22500*	*29750*	*23200*	*25150*	*650*
F	*0*	*23900*	*26650*	*20300*	*19700*	*17450*	*24600*	*26650*	*32650*	*20350*	*15450*	*650*
G	*0*	*24850*	*23050*	*28850*	*22150*	*21700*	*21450*	*28600*	*26450*	*29000*	*15350*	*550*
H	*0*	*21650*	*19450*	*21400*	*20950*	*21200*	*19200*	*25050*	*24100*	*22400*	*21700*	*400*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 7, 2013

					Concentration [LogM]							
Nilutamide (Ag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	0	20150	250	350	500	350	250	250	650	750	950	300
B	50	25450	450	550	450	350	100	300	850	950	1600	200
C	0	22300	350	250	350	500	450	500	650	800	1350	50
D	0	20150	400	600	250	200	300	650	1500	1200	1900	150
E	100	26300	400	350	400	200	50	550	1050	1250	1750	100
F	0	40050	500	100	400	550	300	350	750	1350	1250	300
G	*0*	*10900*	*150*	*350*	*450*	*400*	*500*	*400*	*1200*	*1100*	*2050*	*50*
H	*0*	*7700*	*400*	*650*	*500*	*400*	*200*	*700*	*800*	*1450*	*1350*	*150*

					Concentration [LogM]							
Nilutamide (Antag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	50	17700	300	21600	9100	13200	8450	1950	1400	1550	1250	100
B	0	29650	550	15000	17250	19600	8550	3900	2300	1700	1500	100
C	0	36100	500	16000	22800	14400	8200	3650	2550	1400	1600	250
D	0	21000	650	16700	16150	15450	11650	3650	1850	1600	1800	150
E	*50*	*39050*	*20200*	*19050*	*22200*	*20600*	*26050*	*29750*	*26650*	*27650*	*15750*	*550*
F	*50*	*23150*	*24700*	*26750*	*25200*	*21450*	*26350*	*26950*	*30650*	*28650*	*19350*	*250*
G	*0*	*34100*	*36000*	*28050*	*22450*	*28300*	*28300*	*25100*	*27300*	*31600*	*13350*	*250*
H	*0*	*21600*	*23000*	*21150*	*20900*	*37850*	*24850*	*25250*	*38200*	*32950*	*10550*	*100*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 1
February 5, 2013

					Concentration [LogM]							
ppDDE (Ag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	0	16700	350	400	500	550	600	400	900	1150	1250	1500
B	0	21150	600	500	450	750	700	500	1250	1150	1450	1250
C	50	24250	600	400	600	350	550	600	750	1000	1700	2350
D	50	20400	850	250	650	250	750	750	850	1000	1200	2350
E	0	25600	300	500	550	450	750	550	950	850	1350	1400
F	0	19650	600	750	750	400	600	600	1050	1150	1300	2200
G	*0*	*4300*	*300*	*350*	*400*	*400*	*450*	*350*	*450*	*400*	*1100*	*850*
H	*50*	*4500*	*550*	*450*	*300*	*100*	*500*	*400*	*400*	*450*	*500*	*950*

					Concentration [LogM]							
ppDDE (Antag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	50	5050	150	13050	12250	15450	14550	13900	17100	10250	6650	4800
B	0	9400	150	11150	13850	18450	13200	15800	16550	7300	6650	3000
C	0	7950	150	14450	12300	25800	18450	16850	17500	12500	5450	3050
D	0	9800	150	14700	13050	17300	20500	18800	12750	13450	5900	3150
E	*0*	*13500*	*8600*	*9800*	*10900*	*15900*	*16000*	*22000*	*26000*	*30500*	*16800*	*12850*
F	*50*	*8250*	*12650*	*11650*	*11800*	*15700*	*18800*	*23950*	*28050*	*21500*	*15450*	*12450*
G	*0*	*5250*	*5900*	*8400*	*5900*	*8750*	*8600*	*8450*	*11700*	*15050*	*12950*	*7100*
H	*100*	*5000*	*7450*	*8950*	*6100*	*7200*	*8950*	*12400*	*9200*	*13600*	*11400*	*8400*

APPENDIX 1 Raw and Normalized Luminescence Data (Continued)

VALID RUN 2
February 7, 2013

					Concentration [LogM]							
ppDDE (Ag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	0	32100	500	350	450	300	500	900	750	750	1200	1500
B	0	31950	600	750	950	500	850	1000	1550	1600	1400	1800
C	0	33900	450	550	450	750	800	650	1200	1000	2000	3150
D	0	45950	400	800	700	550	1050	750	1050	1300	1900	2450
E	0	53100	500	400	650	500	1400	800	1400	1300	2100	2350
F	0	51400	450	450	800	400	850	800	750	1600	1550	1450
G	*0*	*16750*	*400*	*300*	*500*	*550*	*850*	*900*	*600*	*1300*	*1850*	*1850*
H	*0*	*21400*	*600*	*400*	*500*	*600*	*600*	*500*	*1450*	*1700*	*1550*	*2050*

					Concentration [LogM]							
ppDDE (Antag)	blank	10 nM DHT	VC	VC	-7.5	-7.0	-6.5	-6.0	-5.5	-5.0	-4.5	-4.0
A	0	19050	500	13700	25400	30600	23700	18150	17850	12700	4500	3050
B	100	29900	700	24250	24500	25450	28050	16400	21900	15800	8150	4500
C	0	27500	250	28200	29750	22650	31200	29250	19850	16900	6000	3600
D	0	35450	350	26900	18600	29850	25650	34650	20700	14800	7500	3550
E	*50*	*21900*	*30050*	*20950*	*31350*	*34150*	*35900*	*34800*	*24800*	*41400*	*37800*	*27950*
F	*0*	*20750*	*37600*	*23800*	*24100*	*38250*	*28850*	*24550*	*36150*	*35850*	*36200*	*31600*
G	*0*	*38800*	*30300*	*39400*	*29650*	*22800*	*27250*	*34950*	*39700*	*25950*	*27800*	*22650*
H	*0*	*29800*	*23150*	*28850*	*18850*	*37300*	*44350*	*34300*	*31500*	*34300*	*34800*	*24450*

APPENDIX 1 Raw Propidium Iodide Data

VALID RUN 1
February 5, 2013
READ 1

					Concentration [LogM]							
Ensulizole (Raw Data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	875	883	883	882	884	878	873	880	881	879	875	883
B	891	889	888	887	884	884	883	891	883	889	889	886
C	881	892	888	884	883	881	880	883	884	879	884	885
D	886	883	884	883	878	877	880	878	876	878	878	878
E	886	888	892	884	884	889	878	879	885	883	884	889
F	880	881	882	879	879	880	878	888	874	878	879	889
G	893	1024	1052	1032	1032	1024	1011	1029	1020	1016	1030	1023
H	885	1013	1004	990	1016	996	1012	979	983	980	974	992

READ 2

					Concentration [LogM]							
Ensulizole (Raw Data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	965	1545	1520	1468	1471	1476	1484	1472	1484	1465	1475	1458
B	975	1524	1519	1509	1502	1508	1484	1494	1482	1475	1476	1441
C	969	1521	1531	1530	1516	1520	1484	1479	1520	1471	1487	1451
D	972	1525	1510	1526	1507	1523	1489	1488	1493	1489	1461	1437
E	970	1525	1522	1512	1525	1527	1499	1478	1510	1481	1482	1443
F	961	1513	1497	1502	1491	1511	1452	1473	1471	1474	1469	1445
G	972	1146	1131	1128	1129	1108	1118	1112	1103	1115	1107	1121
H	969	1112	1091	1130	1102	1087	1098	1110	1116	1111	1100	1135

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 7, 2013

READ 1

					Concentration [LogM]							
Ensulizole (Raw Data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	203	227	264	262	260	249	255	273	256	244	371	252
B	201	263	288	246	255	279	276	279	272	264	411	277
C	196	286	272	263	253	249	262	283	260	292	463	260
D	236	279	278	260	229	293	271	267	277	274	460	281
E	234	277	263	269	263	224	260	263	248	243	425	268
F	241	285	254	275	269	279	278	235	282	289	358	281
G	202	417	435	353	369	456	503	462	487	478	1468	415
H	192	303	396	464	455	520	550	416	454	467	442	472

READ 2

					Concentration [LogM]							
Ensulizole (Raw Data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	473	1634	2036	1695	1760	1654	1847	1676	1705	1707	1745	1811
B	261	1675	2060	1963	2508	1966	1766	1787	2199	2006	1696	2879
C	444	2094	2010	2065	2013	2032	2042	2101	1996	2217	1859	2082
D	467	2141	2087	2147	2193	2218	2085	2201	2149	2276	1895	2167
E	503	2288	2257	2290	2251	2259	2322	2362	2296	2364	2080	2308
F	435	2256	2300	2379	2387	2314	2267	2353	2354	2458	2086	2184
G	432	1354	1579	1546	1597	1612	1520	1524	1578	1127	1137	908
H	451	600	667	685	736	695	671	694	732	724	1276	1025

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
February 5, 2013
READ 1

					Concentration [LogM]							
Avobenzone (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	842	852	855	848	847	853	848	852	858	853	923	932
B	894	887	890	887	887	886	887	894	888	894	947	951
C	881	888	881	885	879	879	879	876	880	883	934	950
D	897	894	887	886	884	886	888	887	887	895	959	960
E	881	887	885	880	882	881	873	873	883	880	937	939
F	891	898	895	889	888	891	887	891	894	899	981	977
G	888	1029	1083	1089	1044	1078	1083	1076	1071	1073	1075	1079
H	897	1032	1039	1005	1024	1024	1021	1007	1001	1034	1047	1048

READ 2

					Concentration [LogM]							
Avobenzone (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	926	1464	1465	1417	1449	1440	1421	1395	1310	1260	1275	1257
B	975	1521	1511	1496	1478	1489	1474	1424	1353	1310	1296	1274
C	966	1517	1519	1494	1499	1503	1476	1438	1359	1319	1267	1269
D	975	1530	1506	1516	1498	1506	1493	1447	1379	1329	1310	1275
E	961	1528	1515	1488	1511	1491	1487	1441	1362	1305	1284	1268
F	976	1536	1513	1505	1520	1494	1474	1450	1366	1321	1294	1293
G	964	1132	1152	1154	1157	1165	1155	1155	1140	1138	1143	1147
H	979	1114	1121	1134	1140	1136	1106	1132	1095	1129	1114	1163

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 7, 2013
READ 1

					Concentration [LogM]							
Avobenzone (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	254	265	275	266	252	288	261	242	236	243	260	483
B	279	259	274	269	292	282	291	282	266	259	342	338
C	249	267	262	261	249	270	237	249	258	262	307	310
D	281	305	277	278	283	260	282	301	282	255	364	389
E	264	255	271	262	269	263	255	255	284	266	318	324
F	278	277	279	241	259	261	271	254	278	261	313	311
G	285	448	399	573	470	513	471	458	537	507	611	580
H	285	390	564	524	608	550	573	494	503	501	597	671

READ 2

					Concentration [LogM]							
Avobenzone (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	489	3174	3929	3427	2671	3414	3498	2992	1922	1489	1251	1712
B	512	2070	2104	1945	2184	1912	2317	1801	1730	1524	1282	1348
C	473	2057	2126	2098	3200	2050	2168	2039	1726	1519	1533	1473
D	473	2197	2250	2455	2430	2318	2168	2112	1770	1671	1523	1475
E	497	2138	2322	2382	2290	2453	2523	2255	1685	1514	1676	1918
F	531	2239	2677	2528	2500	2321	2317	2308	1744	1507	1698	1786
G	480	1599	1091	1377	1392	1556	1104	1138	1468	1077	1211	961
H	486	729	840	765	799	898	726	1025	946	831	806	839

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
February 5, 2013
READ 1

					Concentration [LogM]							
Homosalate (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	880	885	885	882	882	885	882	884	877	880	1047	918
B	889	888	896	885	884	886	889	891	888	886	966	984
C	887	897	892	885	888	886	884	886	885	943	1017	944
D	879	888	893	887	881	883	880	885	890	921	992	1030
E	881	882	886	881	882	877	878	888	879	1027	968	965
F	892	903	895	891	895	896	887	890	896	900	989	901
G	875	1044	1027	1012	1033	1031	1034	1028	1027	1079	1064	1040
H	893	1019	1034	1026	1025	1020	1043	1018	1029	1018	985	1032

READ 2

					Concentration [LogM]							
Homosalate (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	957	1521	1481	1483	1471	1462	1473	1480	1461	1438	1588	1444
B	970	1519	1502	1506	1518	1504	1496	1491	1481	1440	1465	1489
C	970	1532	1484	1489	1476	1477	1490	1462	1461	1462	1367	1371
D	966	1527	1503	1514	1500	1499	1497	1480	1498	1442	1576	1466
E	962	1519	1520	1495	1504	1519	1491	1485	1493	1656	1425	1492
F	977	1545	1524	1539	1516	1525	1509	1495	1524	1461	1474	1455
G	954	1146	1129	1158	1155	1150	1137	1146	1134	1151	1153	1143
H	976	1117	1125	1148	1112	1108	1129	1121	1131	1122	1122	1162

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 7, 2013
READ 1

					Concentration [LogM]							
Homosalate (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	181	207	261	241	241	241	262	238	246	255	622	624
B	240	245	266	259	240	263	275	265	256	384	396	463
C	235	255	279	258	248	278	279	267	280	278	365	303
D	248	324	279	274	253	259	285	291	271	280	349	507
E	224	275	271	254	241	251	261	263	276	331	395	348
F	249	267	280	245	274	274	271	243	259	331	375	418
G	231	268	463	530	499	493	668	443	453	462	529	438
H	204	417	445	416	492	453	477	536	530	476	583	561

READ 2

					Concentration [LogM]							
Homosalate (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	419	1854	1775	1862	1806	1892	1728	2322	1715	1821	3915	2786
B	454	2283	2188	2093	1914	1884	1954	1828	2008	1810	1803	2220
C	468	2246	2033	2203	2165	2127	2157	2160	2139	2006	1441	2176
D	448	2266	2237	2225	2306	2305	2417	2295	2257	2250	1406	2009
E	440	2258	2246	2353	2242	2379	2318	2352	2381	1990	1378	1404
F	466	2188	2312	2404	2321	2333	2298	2348	2714	2017	1768	1573
G	468	606	936	910	881	765	794	774	858	701	718	760
H	427	643	715	684	678	751	753	773	803	888	860	785

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
February 5, 2013
READ 1

					Concentration [LogM]							
Padimate-O (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	863	874	869	868	871	866	863	865	871	864	874	917
B	892	892	895	888	884	886	886	893	891	897	893	933
C	876	877	887	874	875	876	878	880	887	893	880	891
D	898	892	890	895	887	891	883	888	890	891	897	905
E	881	878	884	873	883	879	874	879	880	878	876	908
F	889	891	888	884	887	884	883	885	890	889	892	896
G	891	1035	1041	1060	1022	1063	1058	1046	1045	1064	1065	1046
H	890	988	998	1015	1001	1016	1024	1006	1017	1041	1031	1030

READ 2

					Concentration [LogM]							
Padimate-O (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	945	1504	1466	1514	1524	1506	1516	1492	1497	1472	1490	1463
B	975	1540	1507	1501	1507	1465	1529	1495	1479	1526	1483	1484
C	960	1555	1492	1512	1500	1477	1479	1486	1468	1477	1490	1493
D	983	1552	1519	1508	1500	1502	1499	1496	1480	1488	1503	1485
E	961	1531	1511	1468	1506	1500	1499	1485	1503	1477	1492	1469
F	966	1542	1505	1504	1491	1508	1480	1474	1490	1481	1481	1461
G	973	1117	1126	1136	1121	1136	1117	1142	1127	1120	1129	1122
H	974	1096	1117	1123	1114	1107	1108	1114	1115	1131	1122	1126

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 7, 2013
READ 1

					Concentration [LogM]							
Padimate-O (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	233	274	247	260	248	272	256	251	266	242	242	271
B	261	271	261	275	262	265	277	261	267	293	276	284
C	249	270	284	275	234	291	229	259	272	258	272	288
D	254	304	290	264	295	329	261	272	302	287	312	316
E	255	283	255	273	254	276	281	261	296	308	266	293
F	264	280	295	280	273	298	271	300	262	287	266	303
G	254	483	553	537	468	470	561	620	626	629	596	543
H	261	481	475	577	496	499	595	547	581	619	540	606

READ 2

					Concentration [LogM]							
Padimate-O (Raw Data)	blank	10 nM DHT	VC	VC	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00	-3.50	-3.00
A	484	2022	2258	2014	1786	1613	1513	1613	1513	1688	1566	1761
B	500	1925	2186	2142	2064	2139	1944	1931	1899	1810	1747	1837
C	497	2212	2393	2326	2297	2090	2313	2308	2406	2085	2106	2009
D	502	2113	2461	2419	2461	2139	2336	2278	2373	2207	2181	2097
E	490	2170	2505	2474	2510	2177	2303	2336	2446	2132	2075	2010
F	487	2103	2394	2243	2362	2171	2090	2222	2322	1988	1895	1885
G	479	855	927	1049	1127	978	1062	907	979	868	1120	958
H	498	686	688	711	786	908	742	753	736	764	833	809

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
February 5, 2013
READ 1

					Concentration [LogM]							
DHT (Raw Data)	blank	10 nM DHT	VC	VC	-11.50	-11.00	-10.50	-10.00	-9.50	-9.00	-8.50	-8.00
A	866	882	884	884	884	884	883	891	887	881	879	890
B	885	871	887	882	880	878	882	887	881	878	879	870
C	882	888	879	882	885	879	881	879	877	879	877	869
D	888	897	896	893	892	893	885	892	895	883	887	902
E	881	889	885	883	881	898	878	879	877	885	876	879
F	888	896	893	892	895	895	878	890	892	892	883	888
G	879	1042	1057	1036	1052	1057	1046	1046	1032	1047	1051	1040
H	889	986	1021	1026	1034	1037	1023	1030	1032	1038	1004	1020

READ 2

					Concentration [LogM]							
DHT (Raw Data)	blank	10 nM DHT	VC	VC	-11.50	-11.00	-10.50	-10.00	-9.50	-9.00	-8.50	-8.00
A	955	1525	1530	1502	1540	1506	1488	1523	1548	1528	1524	1490
B	970	1519	1525	1534	1513	1498	1491	1518	1476	1511	1506	1492
C	969	1549	1536	1524	1524	1517	1496	1503	1517	1517	1509	1483
D	979	1563	1542	1540	1546	1535	1522	1513	1543	1519	1536	1520
E	964	1535	1537	1524	1523	1530	1508	1517	1500	1527	1520	1517
F	972	1552	1546	1543	1540	1510	1493	1514	1516	1532	1512	1508
G	966	1132	1141	1125	1148	1155	1146	1125	1122	1124	1128	1153
H	972	1098	1102	1112	1126	1114	1118	1114	1113	1141	1113	1126

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 7, 2013

READ 1

					Concentration [LogM]							
DHT (Raw Data)	blank	10 nM DHT	VC	VC	-11.50	-11.00	-10.50	-10.00	-9.50	-9.00	-8.50	-8.00
A	276	465	284	295	280	290	297	301	286	309	508	281
B	266	505	282	259	297	281	259	278	261	262	512	256
C	260	554	262	268	256	264	287	268	267	268	523	283
D	253	550	288	250	259	265	260	272	272	266	543	279
E	258	549	262	255	265	298	262	263	266	284	515	257
F	262	547	259	279	249	268	253	252	291	275	514	247
G	274	1097	440	427	483	410	523	501	495	457	1114	521
H	261	1103	499	504	586	516	532	538	569	444	1065	518

READ 2

					Concentration [LogM]							
DHT (Raw Data)	blank	10 nM DHT	VC	VC	-11.50	-11.00	-10.50	-10.00	-9.50	-9.00	-8.50	-8.00
A	504	1807	3452	3106	2459	1964	2185	1933	1990	2157	1792	3492
B	514	1779	2165	2192	2136	2112	2023	2000	2038	2153	1784	2050
C	509	1801	2194	2219	2268	2189	2306	2329	2182	2343	1750	2323
D	548	1782	2158	2307	2198	2206	2278	2309	2388	2390	1774	2490
E	523	1743	2318	2269	2351	2349	2407	2493	2408	2421	1830	2411
F	514	1794	2577	2277	2290	2365	2432	2452	2550	2474	1797	2539
G	520	1193	1324	1347	1413	1095	1277	1333	1430	1259	1283	856
H	537	1215	806	968	775	909	792	1025	1164	850	1217	893

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
February 5, 2013

READ 1					Concentration [LogM]							
Nilutamide (Raw Data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	887	899	893	898	895	890	887	888	890	887	885	885
B	885	887	886	888	886	887	877	886	878	879	880	882
C	881	880	881	884	876	877	879	885	882	880	886	880
D	888	888	897	888	882	882	879	885	894	881	883	889
E	876	881	882	870	878	872	875	870	867	876	873	886
F	883	884	887	883	888	886	890	883	888	888	888	887
G	870	1054	1056	1023	1039	1040	1045	1059	1060	1021	1045	1063
H	855	976	966	972	993	977	1000	992	1009	997	1009	1035

READ 2					Concentration [LogM]							
Nilutamide (Raw Data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	982	1508	1487	1459	1440	1453	1454	1449	1450	1414	1373	1285
B	967	1481	1511	1484	1478	1466	1469	1460	1441	1442	1381	1279
C	965	1523	1510	1503	1500	1478	1489	1495	1483	1486	1390	1282
D	966	1526	1501	1512	1495	1502	1503	1477	1505	1480	1412	1279
E	961	1504	1502	1511	1503	1475	1503	1490	1486	1476	1385	1290
F	971	1541	1530	1528	1507	1492	1535	1507	1481	1486	1408	1301
G	945	1132	1116	1124	1127	1124	1118	1128	1129	1101	1107	1169
H	934	1102	1070	1086	1085	1081	1095	1083	1105	1103	1092	1120

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 7, 2013

READ 1					Concentration [LogM]							
Nilutamide (Raw Data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	237	263	269	282	292	311	278	276	292	301	268	249
B	241	279	269	274	273	272	276	283	276	264	271	264
C	233	266	260	265	269	272	282	237	263	257	249	244
D	266	291	288	264	276	275	248	261	274	261	361	275
E	248	284	272	253	282	241	261	249	265	270	338	274
F	252	283	274	247	256	287	265	258	282	278	437	222
G	243	452	538	498	613	568	581	569	523	515	954	591
H	225	478	533	591	537	435	631	575	536	378	1184	547

READ 2					Concentration [LogM]							
Nilutamide (Raw Data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	484	3538	5080	5762	4692	4661	4564	5015	2720	2617	1536	1420
B	513	2085	2241	2144	2191	2321	1859	2303	2196	2365	1795	1499
C	497	2108	2304	2348	2320	2232	2390	2460	2097	2790	1915	1598
D	517	2237	2321	2375	2284	2333	2265	2318	2244	2389	1814	1661
E	514	2219	2628	2467	2378	2419	2448	2508	2252	2415	1890	1609
F	499	2240	2162	2238	2319	2296	2357	2327	2395	2380	1996	1585
G	509	921	914	919	1035	1028	816	829	1146	766	1209	713
H	512	966	684	669	709	681	655	645	661	714	1428	749

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 1
February 5, 2013

READ 1					Concentration [LogM]							
ppDDE (Raw data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	850	860	860	850	855	855	851	852	836	848	859	852
B	882	887	891	887	885	879	881	888	878	888	886	885
C	883	888	888	880	883	889	882	879	876	876	876	889
D	889	891	885	888	882	883	885	880	886	883	883	892
E	864	866	868	867	865	864	862	868	864	865	871	875
F	876	883	880	881	875	879	871	880	877	869	874	889
G	889	1015	1012	1008	1028	1033	1029	1020	1018	1014	1017	1050
H	891	1007	1008	1007	1020	1013	1018	1016	1017	999	1009	1046

READ 2					Concentration [LogM]							
ppDDE (Raw Data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	970	1482	1481	1443	1442	1425	1425	1430	1392	1423	1411	1356
B	964	1510	1490	1457	1488	1487	1471	1452	1462	1438	1412	1352
C	974	1518	1502	1489	1500	1478	1491	1467	1455	1441	1434	1359
D	970	1527	1515	1518	1504	1496	1500	1480	1499	1457	1436	1379
E	947	1503	1503	1488	1511	1502	1487	1480	1477	1455	1433	1343
F	959	1533	1513	1520	1496	1489	1482	1471	1497	1456	1446	1373
G	977	1148	1133	1111	1110	1126	1151	1136	1122	1127	1110	1159
H	975	1111	1106	1097	1106	1109	1099	1106	1110	1131	1125	1135

APPENDIX 1 Raw Propidium Iodide Data (Continued)

VALID RUN 2
February 7, 2013

READ 1

					Concentration [LogM]							
ppDDE (Raw Data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	258	290	256	285	280	274	287	267	275	250	262	280
B	246	234	253	273	272	272	273	273	275	267	293	270
C	242	282	285	256	237	251	251	268	249	242	243	245
D	244	263	278	270	281	257	276	259	268	293	262	232
E	272	279	286	277	249	260	272	280	263	261	276	277
F	261	245	275	272	284	276	267	256	268	264	228	257
G	258	470	535	480	486	499	577	546	530	516	574	661
H	243	413	472	438	467	460	422	447	409	521	551	683

READ 2

					Concentration [LogM]							
ppDDE (Raw Data)	blank	10 nM DHT	VC	VC	-7.50	-7.00	-6.50	-6.00	-5.50	-5.00	-4.50	-4.00
A	490	2066	2346	2099	2036	1858	1842	2068	1851	1863	2144	1643
B	494	1992	2013	2123	2060	1916	2266	2069	2286	2213	2149	1724
C	503	2069	2151	2180	2194	2096	2025	2196	2241	2241	2146	1920
D	500	2012	2148	2148	2160	2171	2200	2234	2259	2387	2173	1924
E	500	2014	2203	2257	2123	2314	2179	2258	2167	2187	2036	1665
F	491	1922	2129	2175	2089	2181	2199	2275	2221	2138	2118	1754
G	523	1086	1118	1111	1017	1089	1001	1037	991	944	976	986
H	471	657	677	688	784	740	734	754	736	748	740	1035

APPENDIX 1 Solubility Data

Valid Run 1 – February 5, 2013

	1	2	3	4	5	6	7	8	9	10	11	12	
A					-11.5	-11	-10.5	-10	-9.5	-9	-8.5	-8	DHT
B					-11.5	-11	-10.5	-10	-9.5	-9	-8.5	-8	
C					-7.5	-7	-6.5	-6	-5.5	-5	-4.5	-4	Nilutamide
D					-7.5	-7	-6.5	-6	-5.5	-5	-4.5	-4	
E					-7.5	-7	-6.5	-6	-5.5	-5	-4.5	-4	ppDDE
F					-7.5	-7	-6.5	-6	-5.5	-5	-4.5	-4	
G													
H													

	1	2	3	4	5	6	7	8	9	10	11	12	
A					109	122	117	107	205	175	103	122	DHT
B					147	187	195	225	236	174	143	132	
C					170	198	180	159	148	180	149	156	Nilutamide
D					215	195	245	138	265	202	173	861	
E					181	174	362	123	227	202	448	5421	ppDDE
F					465	171	554	213	190	321	440	5261	
G													
H													

APPENDIX 1 Solubility Data (Continued)

Valid Run 1 – February 5, 2013

	1	2	3	4	5	6	7	8	9	10	11	12	
A					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	Ensulizole
B					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	
C					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	Avobenzone
D					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	
E					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	Homosalate
F					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	
G					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	Padimate-O
H					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	

	1	2	3	4	5	6	7	8	9	10	11	12	
A					137	143	175	148	172	177	253	415	Ensulizole
B					165	152	157	156	150	195	160	158	
C					133	166	112	125	220	6370	17346	18323	Avobenzone
D					133	113	108	95	251	6140	16898	17912	
E					113	133	117	138	300	742	386	318	Homosalate
F					170	163	179	156	289	582	604	249	
G					124	140	127	136	297	625	382	273	Padimate-O
H					124	120	170	118	302	669	422	249	

APPENDIX 1 Solubility Data (Continued)

Valid Run 2 – February 7, 2013

	1	2	3	4	5	6	7	8	9	10	11	12	
A					-11.5	-11	-10.5	-10	-9.5	-9	-8.5	-8	DHT
B					-11.5	-11	-10.5	-10	-9.5	-9	-8.5	-8	
C					-7.5	-7	-6.5	-6	-5.5	-5	-4.5	-4	Nilutamide
D					-7.5	-7	-6.5	-6	-5.5	-5	-4.5	-4	
E					-7.5	-7	-6.5	-6	-5.5	-5	-4.5	-4	ppDDE
F					-7.5	-7	-6.5	-6	-5.5	-5	-4.5	-4	
G													
H													

	1	2	3	4	5	6	7	8	9	10	11	12	
A					154	149	184	116	166	117	126	120	DHT
B					185	124	124	201	220	132	116	115	
C					126	170	103	121	115	92	104	161	Nilutamide
D					216	188	124	121	159	115	97	107	
E					106	95	83	106	153	112	267	5064	ppDDE
F					186	95	102	103	94	143	300	4709	
G													
H													

APPENDIX 1 Solubility Data (Continued)

Valid Run 2 – February 7, 2013

	1	2	3	4	5	6	7	8	9	10	11	12	
A					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	Ensulizole
B					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	
C					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	Avobenzone
D					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	
E					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	Homosalate
F					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	
G					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	Padimate-O
H					-6.5	-6	-5.5	-5	-4.5	-4	-3.5	-3	

	1	2	3	4	5	6	7	8	9	10	11	12	
A					109	103	138	109	149	350	339	490	Ensulizole
B					139	122	93	122	86	253	543	189	
C					113	112	108	115	220	7039	18642	19685	Avobenzone
D					*14797	106	105	105	234	6882	18736	19518	
E					123	132	106	115	255	1530	882	7237	Homosalate
F					102	109	114	119	256	1492	530	259	
G					105	100	101	130	321	1105	526	258	Padimate-O
H					92	92	135	100	330	1125	872	356	

***bubble**

APPENDIX 2 Certificate of Analysis – Ensulizole

RTI INTERNATIONAL
turning knowledge into practice

NTP Analytical Chemistry Services

3040 Cornwallis Road • PO Box 12194 • Research Triangle Park, NC 27709-2194 • USA
Telephone 919.541.6730 or 919.541.5975 • Fax 919.485.2650 • www.rti.org

Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.080
ChemTask No. CHEM11786
CAS No. 27503-81-7

This pdf is an exact duplicate of the original approved report.
[redacted]
Program Information Coordinator

ENSULIZOLE

CHEMICAL REANALYSIS

September 5, 2012

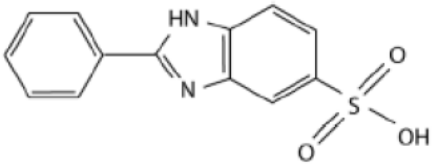
Prepared by:
[redacted]
09-05-12
Date
Task Leader

Approved by:
[redacted]
09/05/12
Date
Reshan Fernando, Ph.D.
Principal Investigator

Submitted to:
[redacted]
National Institute of Environmental Health Sciences
P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

ENSULIZOLE

CAS No.: 27503-81-7	Study Lab: (Investigator): ILS [redacted]
RTI Chemical ID Code: N60	Lot No. (Vendor): 05117JE(Aldrich)
ChemTask No.: CHEM11786	Vendor Purity: 99.9% (by HPLC, Aldrich COA)
RTI Log Nos. (Amt. Received): Analytical: 082010-C-15 (~50 g) Reference: 082010-C-05 (~5 g)	Receipt Date: Aug 20, 2010 (Bulk receipt and reference)
Program Supported: TOX	Receipt Condition: No damage noted
Analysis Dates: May 11, 15 and 24, 2012	Submitter: [redacted] (RTI)
Interim Results Date: May 29, 2012	Shipping Container: NA (in-house transfer)
	Storage Conditions: Bulk: Room temperature Reference: Freezer (~ -20 °C)

STRUCTURE	MOL. WT.	MOL. FORMULA
	274.30	$C_{13}H_{10}N_2O_3S$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, an aliquot of ensulizole was submitted for bulk chemical reanalysis. Chemical purity of the bulk sample was determined relative to a reference standard of the same lot/batch number which had been stored at RTI under freezer conditions. Analytical results obtained by LC chromatographic method indicated that the sample had a percent relative purity of 99.6% when compared to the frozen reference standard. The FTIR spectrum of the bulk sample matched the spectrum of the frozen reference and was consistent with the structure for ensulizole.

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: **Ensulizole**

Task Type: **Chemical Reanalysis**

Chem Task Number: **CHEM11786**

This study/task was audited by the Regulatory and Quality Assurance (RQA) – Quality Assurance Unit and the results of the inspections and audits were reported to the task leader/study director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Sample Preparation Inspection for HPLC Analysis	05/15/12	05/22/12
Data & Report Audit	08/24/12 & 08/28/12	08/28/12

Prepared by:

[redacted] 9-5-12

Quality Assurance Specialist Date

Reviewed by:

[redacted] 9/5/12

Quality Assurance Specialist Date

turning knowledge into practice

TABLE OF CONTENTS

Figures

ENSULIZOLE

1.0 INTRODUCTION

The objective of this work was to determine the purity and verify the identity of ensulizole to the current studies being conducted at RTI International. To accomplish this objective, a bulk chemical reanalysis was performed. The identity of the chemical was confirmed by FTIR and its purity assessed by LC.

2.0 CHEMICAL ANALYSIS

An aliquot of the bulk sample of ensulizole was received at the analytical laboratory on March 27, 2012 for chemical reanalysis (RTI log 082010-C-15). The aliquot was stored at room temperature. A frozen reference (RTI log 082010-C-05) sample was received at the analytical laboratory on May 10, 2012 and was stored at freezer temperature.

3.0 CONFIRMATION OF IDENTITY - INFRARED SPECTROMETRY (IR)

3.1 IR Parameters

System	Thermo Nicolet 6700 FTIR
Software	Omnic, Ver. 7.3
Method	KBr pellet, scan 4000 - 400 cm^{-1}

3.2 Results

Bulk Sample Frequency (1/cm)	Frozen Reference Sample Frequency (1/cm)	Assignment
3367	3372	N-H stretch
3059-2725	3059-2725	O-H, N-H, C-H stretch
1633, 1568	1630, 1567	C=C, C=N stretch
1368	1368	C-N stretch
1176	1176	C-C, SO_2 stretch
1028	1028	N-H bend
780	777	C-H, N-H bend
631	630	S-O stretch

The observed spectrum for the bulk sample matched the spectrum of the frozen reference sample, and is consistent with the structure of ensulizole (as reported in the characterization protocols development task CHEM11291). Figure 1 shows the IR spectra for the bulk and frozen samples.

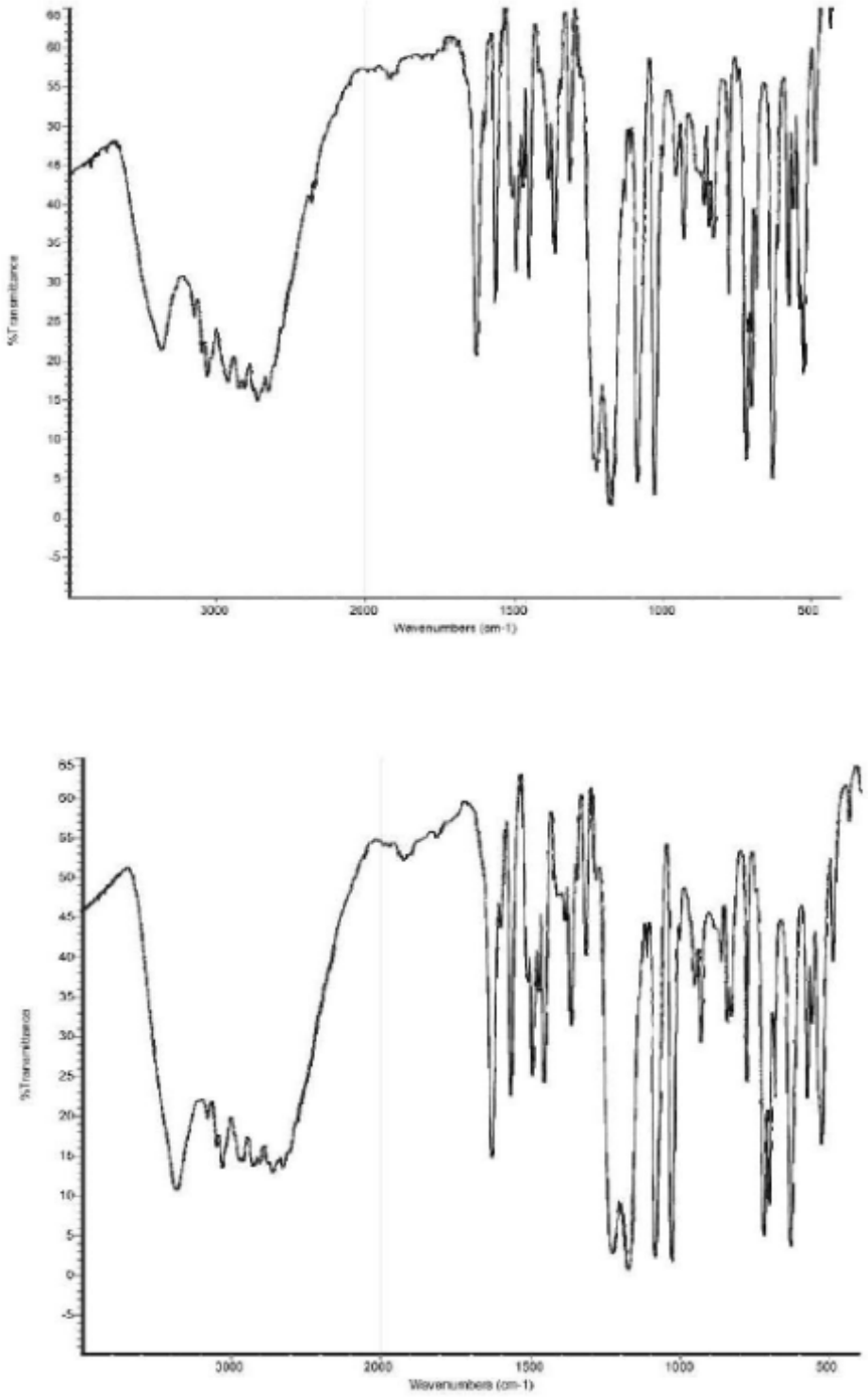


Figure 1: Infrared Spectrum of Ensulizole Frozen Reference (top spectrum) and Bulk Sample (bottom spectrum)

2

4.0 DETERMINATION OF PURITY - LIQUID CHROMATOGRAPHY

This section describes the liquid chromatographic method used to estimate sample purity.

4.1 Preparation of Internal Standard (IS) Solution

A stock solution of IS was prepared by weighing 500 mg of padimate O and transferring it into a 10-mL volumetric flask. The IS was diluted to volume with mobile phase B (methanol with 0.1% formic acid). The flask was mixed by inversion. A working IS solution (WIS) was prepared as a 1 mL to 1 L dilution with mobile phase B and mixing by inversion, yielding 0.050 mg/mL working IS.

4.2 Bulk Sample and Frozen Reference Standard Solution Preparation

Triplicate solutions of the reference standard and bulk samples were prepared by transferring approximately 25 mg of compound to individual 100-mL volumetric flasks and diluting to volume with WIS and mixing by inversion. All samples were transferred to autosampler vials and analyzed by liquid chromatography.

4.3 Analysis

LC Parameters

System	Waters Alliance 2695
Software	Empower 2; Build 2154
Column	Waters XBridge C18 3.5 µm, 100 x 2.1 mm, guard column, 5 µm 2.1 x 10 mm
Column Temp	40 °C
Mobile Phases	A: 0.1% formic acid in water B: 0.1% formic acid in methanol
Flow Rate	0.25 mL/min
Gradient	Hold 90 % A for 0.67 min., 90% A to 90% B in 10 min., hold 90% B for 10 min., 90% B to 90% A in 5 min., hold 90% A for 5 min.
Injection Volume - Solvent	2 µL – Mobile Phase B
Retention Time (min)	Ensulizole – 5.73 min Padimate O (IS) – 16.59 min
Detector	Waters 2996 PDA, 312 nm

The suitability of the system was evaluated, and the results are shown below.

Parameter	Result	Criteria	Pass/Fail
Capacity Factor, k	2.8	$2 \geq k \leq 12$	Pass
Tailing Factor, T	1.2	$0.5 \geq T \leq 2.0$	Pass
Column Efficiency, N	29,000	$N \geq 6{,}000$ plates	Pass

4.4 Results

Calculations based on a major peak comparison technique gave the results shown in the following table.

RTI Log No.	Chemical	RRF[a]	Mean RRF[a] (%RSD)	Percent Relative Purity[b]
082010-C-15	Analytical Replicate #1	3.072	3.046 (0.82)	99.6
	Analytical Replicate #2	3.022		
	Analytical Replicate #3	3.045		
082010-C-05	Reference Replicate #1	3.034	3.057 (0.81)	--
	Reference Replicate #2	3.083		
	Reference Replicate #3	3.054		

[a] RRF = Relative Response Factor; normalized to sample concentration.

[b] Relative Purity = (Mean RRF, bulk/Mean RRF, ref.) x 100.

Based on the chromatographic results, the bulk sample had not significantly changed as compared to the frozen reference, and no significant impurities were observed. Typical chromatograms are shown in Figure 2.

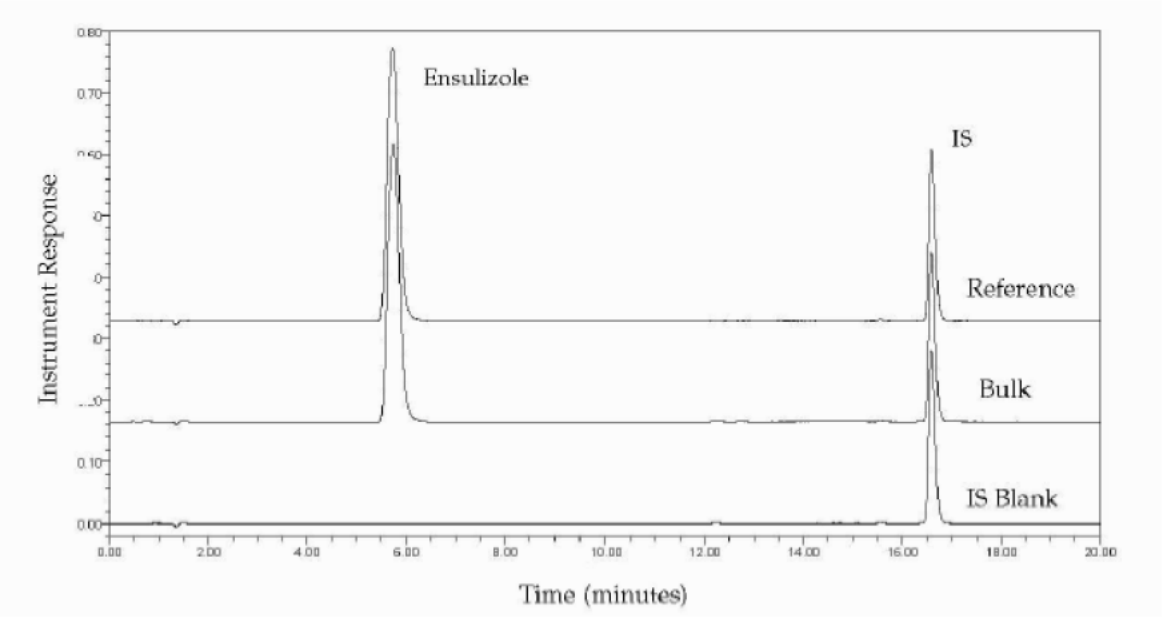


Figure 2: Example Liquid Chromatograms of Ensulizole Reference and Bulk Sample, and a Blank

5.0 REFERENCE

RTI International report "Ensulizole, Characterization Protocols Development, (CHEM11291), January 9, 2012.

6.0 ACKNOWLEDGMENTS

Personnel contributing to this task: [redacted]

APPENDIX 2 Certificate of Analysis – Avobenzone

This PDF File is an Exact Copy of the Report
Signature: [redacted]
Date: 2-16-12

Analytical Chemistry Services for the NTP
NIEHS Contract No. HHSN273201100001C
MRI Project No.: 110730
NTP ChemTask No.: CHEM10985

Chemical Comprehensive Analysis Final Report

Avobenzone

Chemical Comprehensive Analysis of Avobenzone

MRI Assignment No.: 2003

February 16, 2012

Prepared by:

[redacted]
Study Director

Approved by:

[redacted]
Joseph W. Algaier, Ph.D.
Principal Investigator

Reviewed by:

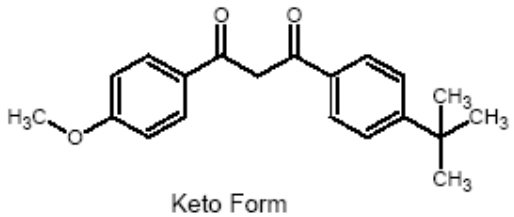
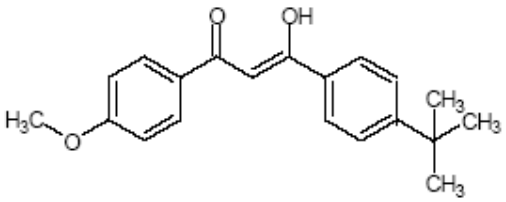
[redacted]
Group Leader

Submitted to:

[redacted]
National Institute of Environmental Health Sciences
111 T. W. Alexander Drive, MD K2-07
P.O. Box 12233
Research Triangle Park, NC 27709-2233

Chemical Comprehensive Analysis of Avobenzone

Chemical Information

CAS No.: 70356-09-1 MRI Assignment No.: 2003 ChemTask No. CHEM10985 Program Supported: TOX Analysis Dates: 2/11/11 to 12/14/11 Interim Result Date(s): 2/25/11, 4/7/11, 5/17/11	Lot No.: L802809 MRI Assigned Batch No.: 01 Amount Received: 20 Kg Sample Receipt Date: 1/5/11 Appearance: Off white to yellowish crystalline powder per CoA; confirmed by visual observation Supplier: Universal Preserv-A-Chem Inc. Supplier Purity: 98.30% per CoA Storage conditions (at Analytical Lab): Ambient, protected from light
 Keto Form  Enol Form (predominant)	Mol. Wt.: 310.39 Mol. Formula: $C_{20}H_{22}O_3$

Executive Summary

The purpose of this assignment was to perform a chemical comprehensive analysis for avobenzone, Lot No. L802809, received from Universal Preserv-A-Chem Inc. Based on the results, the identity of the test article was confirmed to be avobenzone, with a purity of approximately 98.5%. Evaluation by gas chromatography with flame ionization detection of samples stored at various temperatures indicated avobenzone is stable when stored for 2 weeks, protected from light, at temperatures up to approximately 60°C. Nuclear magnetic resonance spectroscopic analysis of these samples, as well as samples exposed to light for 1 week, detected some conversion of enol to keto form under elevated temperature and light exposure.

The chemical comprehensive analysis included identity confirmation using infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy, residual solvent analysis for volatile content using gas chromatography (GC)/headspace analysis, ultraviolet/visible (UV/Vis) spectroscopy, water content using Karl Fischer titration, elemental analysis, determination of melting point, and log P, differential scanning calorimetry (DSC), and chromatographic profiling using gas chromatography (GC) with flame ionization detection (FID). Additionally, gas chromatography/mass spectrometry (GC/MS) was performed to confirm identity of the test article.

Spectra obtained for the test article using IR and NMR spectroscopy techniques were consistent with reference spectra and the proposed structure for the enol form of the test article. One absorbance maximum was observed using ultraviolet/visible spectroscopy: 358 nm, ϵ_{max} = 36241 ± 186(s). Analysis using GC/MS with electron capture ionization provided confirmation of identity based on the molecular ion (310 Da) observed, as well as comparison to a reference spectrum.

Water content determined by Karl Fischer was 0.223 ± 0.008(s) %. Elemental analysis determined 77.36% carbon, 7.39% hydrogen, and 0.02% nitrogen compared to expected values of 77.39 carbon, 7.15% hydrogen, and no nitrogen. The observed melting point range was 83.0° to 85.5°C (literature values of 83.5°C and 81° to 86°C). The determined log P was 3.10.

Differential scanning calorimetry was performed, and the observed melting point range was consistent with the melting point range from the MSDS. The results indicated a purity of 98.8 ± 0.5(d) %. Chromatographic profiling, using GC with a DB-5 column and FID, indicated 98.7% purity, with seven reportable impurities totaling 1.26% relative to the total peak area. GC/headspace analysis indicated residual solvent peak responses for methanol and cis-1,2-dichloroethene, but they were not present at levels greater than the Class 2 Mixture A Standard. There were no other Class 1 or Class 2 solvents observed to be present in the test article.

Accelerated stability was performed using GC with FID to evaluate possible degradation of the test article. The test variability limit (TVL), which is statistically determined, established that in order to be statistically significant at the 95% confidence level, the loss or gain under ambient, refrigerated, or elevated storage conditions must be greater than 3.8% relative to the sample under the frozen storage condition. The maximum variance from the frozen storage condition was +0.7%, observed for the sample stored at approximately 60°C. Using the TVL criteria,

avobenzone is stable when stored for 2 weeks as the bulk chemical, protected from light, at temperatures up to approximately 60°C. An additional evaluation using ^{1}H-NMR spectroscopy of the accelerated stability samples and stability samples exposed to light exhibited decreased enol/keto ratios of the –OH and –CH_2 functional groups for the samples stored at 60°C, as well as samples exposed to fluorescent or mercury/xenon lighting. This indicates some conversion of the enol to the keto form.

Quality Assurance Statement

Chemical Comprehensive Analysis of Avobenzone

ChemTask No. CHEM10985
MRI Project No. 110730
MRI Assignment No. 2003

This study was inspected by the Quality Assurance Unit of MRI (QAU) and the findings reported to the Study Director and Management as follows:

Phase inspected	Date inspected	Date reported
Protocol Audit	3/1/11	3/1/11
In-life Audit; Stability analysis	3/1/11	3/1/11
Protocol Amendment No. 1 Audit	2/8/12	2/10/12
Protocol Amendment No. 2 Audit	2/8/12	2/10/12
Protocol Amendment No. 3 Audit	2/8/12	2/10/12
Data Audit	2/9/12	2/10/12
Draft Final Report Audit	2/9/12	2/10/12

In addition to the study-specific audits/inspections cited above, inspection of applicable facilities and equipment was performed by the QAU and reports were submitted to management as follows:

Facility/equipment	Inspection date	Management submitted date
285N laboratory complex	7/13/11	7/14/11
GC facility	7/14/11	7/15/11

MIDWEST RESEARCH INSTITUTE

[redacted]

Senior Quality Assurance Officer

Approved:

[redacted]

Director, Quality and Regulatory Systems

February 16, 2012

Good Laboratory Practice Compliance Statement

Chemical Comprehensive Analysis of Avobenzone

ChemTask No. CHEM10985
MRI Project No. 110730
MRI Assignment No. 2003

All work performed at Midwest Research Institute for this assignment was conducted in compliance with the Good Laboratory Practice regulations of the U.S. Food and Drug Administration (21 *CFR* Part 58). Elemental analysis was performed by ICON Developmental Solutions, LLC, in compliance with FDA current Good Laboratory Practices (21 *CFR* Part 58).

The raw data and report will be stored in the MRI Archives.

[redacted]
Study Director

2/16/12
Date:

APPENDIX 2 Certificate of Analysis – Homosalate

RTI INTERNATIONAL
turning knowledge into practice

NTP Analytical Chemistry Services
3040 Cornwallis Road • PO Box 12194 • Research Triangle Park, NC 27709-2194 • USA
Telephone 919.541.6730 or 919.541.5975 • Fax 919.485.2650 • www.rti.org

Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.082
ChemTask No. CHEM11788
CAS No. 118-56-9

This pdf is an exact duplicate of the original approved report.
[redacted]
Program Information Coordinator

HOMOSALATE

CHEMICAL REANALYSIS

September 5, 2012

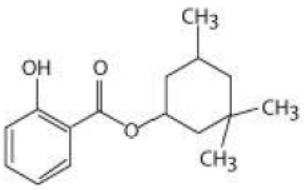
Prepared by:
[redacted]
09-05-12
Date
Task Leader

Approved by:
[redacted]
09/05/12
Date
Reshan Fernando, Ph.D.
Principal Investigator

Submitted to:
[redacted]
National Institute of Environmental Health Sciences
P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

HOMOSALATE

CAS No.: 118-56-9	Study Lab: (Investigator): ILS ([redacted]
RTI Chemical ID Code: N67	Lot No. (Vendor): YT0976 (Spectrum)
ChemTask No.: CHEM11788	Vendor Purity: 99.88% (Spectrum COA)
RTI Log Nos. (Amt. Received): Analytical: 091410-A-14 (~50 g) Reference: 091410-A-05 (~5 g)	Receipt Date: Sep 14, 2010 (Bulk) Receipt Condition: No damage noted
Program Supported: TOX	Submitter: [redacted] RTI)
Analysis Date: May 11, 21-23, 2012	Shipping Container: NA (in-house transfer)
Interim Results Date: May 29, 2012	Storage Conditions: Bulk: Room temperature Reference: Freezer (~ -20 °C)

STRUCTURE	MOL. WT.	MOL. FORMULA
	262.34	$C_{16}H_{22}O_3$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, an aliquot of homosalate was submitted for bulk chemical reanalysis. Chemical purity of the bulk sample was determined relative to a reference standard of the same lot/batch number which had been stored at RTI under freezer conditions. Analytical results obtained by a GC/FID chromatographic method indicated that the sample had a percent relative purity of 99.3% when compared to the frozen reference standard. The FTIR spectrum of the bulk sample matched the spectrum of the frozen reference and was consistent with an identity of homosalate.

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: **Homosalate**

Task Type: **Chemical Reanalysis**

Chem Task Number: **CHEM11788**

This study/task was audited by the Regulatory and Quality Assurance (RQA) – Quality Assurance Unit and the results of the inspections and audits were reported to the task leader/study director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Sample Preparation Inspection	05/21/12	05/21/12
Data & Report Audit	08/16/12	08/16/12

Prepared by:

[redacted]
Quality Assurance Specialist

9/5/12
Date

Reviewed by:

[redacted]
Quality Assurance Specialist

9/5/12
Date

turning knowledge into practice

TABLE OF CONTENTS

Figures

HOMOSALATE

1.0 INTRODUCTION

The objective of this work was to determine the purity and verify the identity of homosalate in support of studies being conducted at ILS. To accomplish this objective, a chemical reanalysis was performed. The identity of the chemical was confirmed by FTIR and its purity assessed by GC.

2.0 CHEMICAL ANALYSIS

An aliquot of the bulk sample of homosalate was received on March 27, 2012 for chemical reanalysis (RTI log 091410-A-14). The aliquot was stored at room temperature. A frozen reference (RTI log 091410-A-05) sample was received May 10, 2012 and was stored at freezer temperature.

3.0 CONFIRMATION OF IDENTITY - INFRARED SPECTROMETRY (IR)

3.1 IR Parameters

System	Thermo Nicolet 6700 FTIR
Software	Omnic, Ver. 7.3
Method	NaCl disks, scan 4000 - 400 cm^{-1}

3.2 Results

Bulk Sample Frequency (1/cm)	Frozen Reference Sample Frequency (1/cm)	Assignment
3150	3150	O-H stretch
2953-2869	2953-2869	C-H stretch
1672	1672	C=C, C=0 stretch
1614	1614	C=C stretch
1585	1585	C=C stretch
1089	1089	C-C, C-O stretch
757	757	C-H bend

The observed spectrum for the bulk sample matched the spectrum of the frozen reference sample, and is consistent with the structure of homosalate (as reported in the bulk chemical comprehensive task CHEM11090). Figure 1 shows the bulk and frozen reference IR spectra.

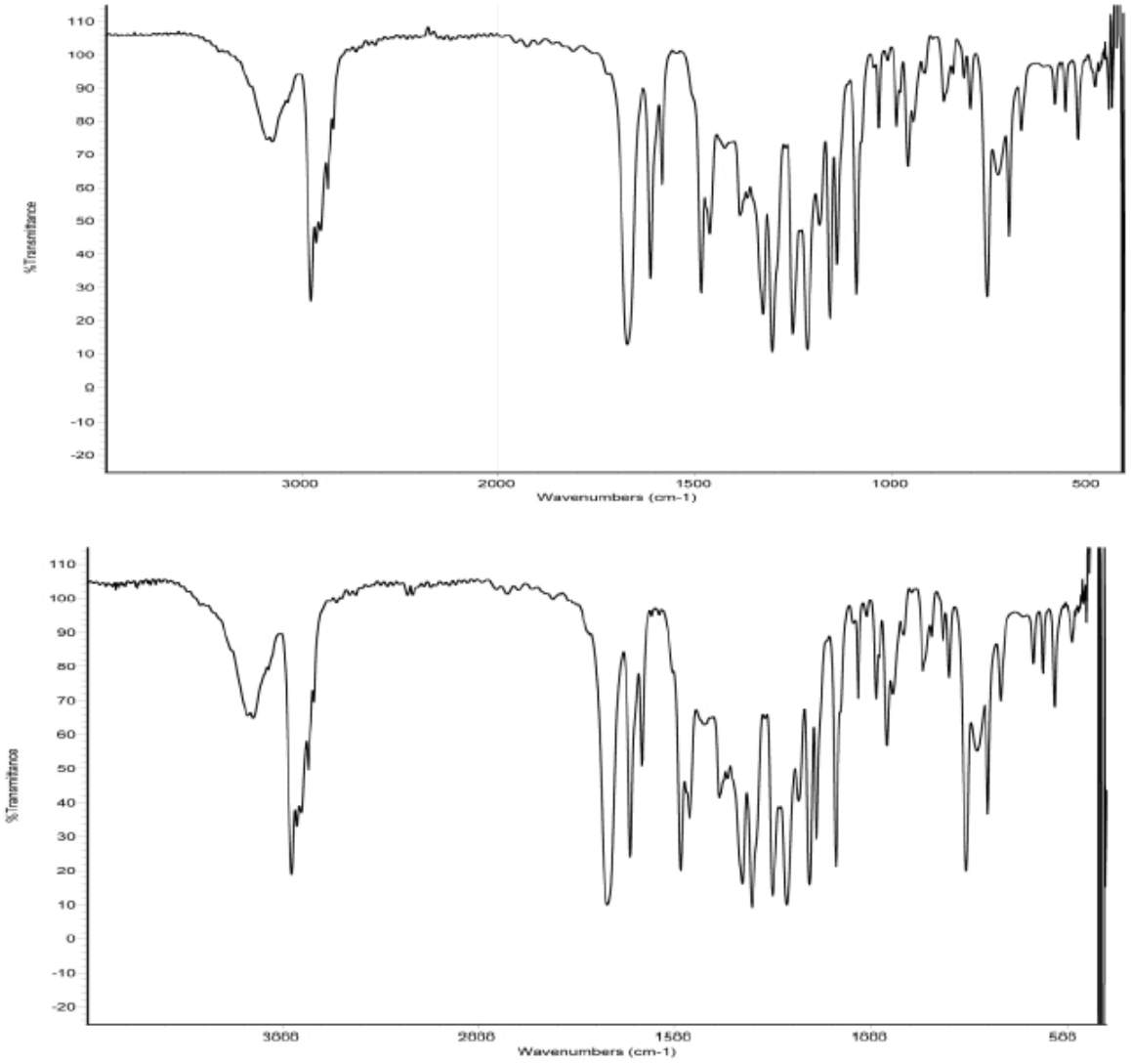


Figure 1: Infrared Spectrum of Homosalate Bulk (top spectrum)
and Frozen Reference (bottom spectrum)

4.0 DETERMINATION OF PURITY - GAS CHROMATOGRAPHY

This section describes the gas chromatographic method used to estimate sample purity.

4.1 Preparation of Internal Standard (IS) Solution

A solution of IS was prepared by weighing 115.49 mg of octanophenone and transferring it into a 200-mL volumetric flask. The IS was diluted to volume with dichloromethane. The flask was mixed by inversion. The IS solution had a concentration of 0.577 mg/mL.

4.2 Bulk Sample and Frozen Reference Standard Solution Preparation

Triplicate solutions of the reference standard and bulk samples were prepared by transferring approximately 25 mg of compound to individual 25-mL volumetric flasks and diluting to volume with IS solution and mixing by inversion. An aliquot of the bulk and reference solutions were transferred to GC vials for analysis. The samples were analyzed by gas chromatography.

4.3 Analysis

GC Parameters

Instrument	Agilent 6890N GC
Data System	Empower 2; Build 2154
Column	Phenomenex ZB-5MS (30 m x 0.25 mm ID, 0.5 µm film) with 5 m pre-guard
Carrier Gas	Helium
Flow Rate	1.5 mL/min
Oven Temperature	70 °C for 1 min., ramp to 270 °C at 20 °C/min with a 7 min hold
Retention Times	Homosalate: ~11.1 min. and 11.2 min (two peaks – cis/trans isomers) Octanophenone (IS): ~9.9 min.
Injector Type and Volume	Split (20:1), 1 µL
Injector Temperature	250 °C
Detector-Temperature	FID at 290 °C

The suitability of the system was evaluated, and the results are shown below.

Parameter	Criteria	Result	Pass/Fail
Tailing Factor, T	$0.5 \geq T \leq 2.0$	1.0	Pass
Column Efficiency, N	$\geq$ 250,000 plates	2,460,486	Pass
Precision (%RSD)	$\leq$ 5% (n=6)	0.2	Pass
Resolution	$\geq$ 40	41	Pass

4.4 Results

Calculations based on a major peak comparison technique gave the results shown in the following table. Typical chromatograms are shown in Figure 2.

RTI Log No.	Chemical	RRF[a]	Mean RRF[a] (%RSD)	Percent Relative Purity[b]
091410-A-14	Analytical Replicate #1	1.443	1.414 (2.0)	99.3
	Analytical Replicate #2	1.412		
	Analytical Replicate #3	1.388		
091410-A-05	Reference Replicate #1	1.430	1.424 (0.69)	--
	Reference Replicate #2	1.430		
	Reference Replicate #3	1.413		

[a] RRF = Relative Response Factor; normalized to sample concentration.
[b] Relative Purity = (Mean RRF, bulk/Mean RRF, ref.) x 100.

Based on the chromatographic results, the bulk sample had not significantly changed as compared to the frozen reference, and no significant impurities were observed.

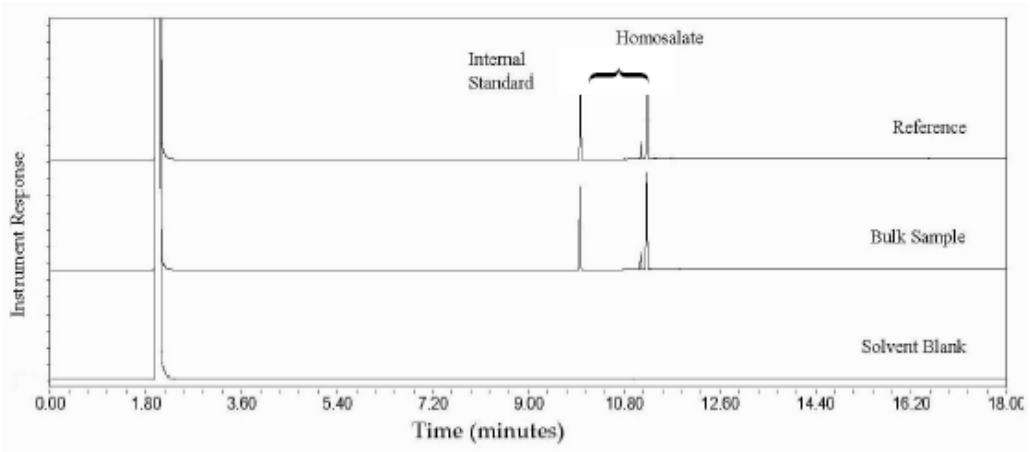


Figure 2: Example Gas Chromatograms of Homosalate Reference and Bulk Sample, and a Blank

5.0 REFERENCE

RTI International report "Homosalate, Characterization Protocols Development, (CHEM11293), January 6, 2012.

6.0 ACKNOWLEDGMENTS

Personnel contributing to this task: [redacted]

APPENDIX 2 Certificate of Analysis – Padimate O

RTI INTERNATIONAL
turning knowledge into practice

NTP Analytical Chemistry Services

3040 Cornwallis Road • PO Box 12194 • Research Triangle Park, NC 27709-2194 • USA
Telephone 919.541.6730 or 919.541.5975 • Fax 919.485.2650 • www.rti.org

Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.081
ChemTask No. CHEM11787
CAS No. 21245-02-3

This pdf is an exact duplicate of the original approved report.

[redacted]
Program Information Coordinator

2-ETHYLHEXYL-P-DIMETHYL-AMINOBENZOATE (PADIMATE O)

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:

[redacted] 09-05-12
Task Leader Date

Approved by:

[redacted] 09/05/12
Reshan Fernando, Ph.D. Date
Principal Investigator

Submitted to:

[redacted]
National Institute of Environmental Health Sciences
P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

2-ETHYLHEXYL-P-DIMETHYL-AMINOBENZOATE (PADIMATE O)

CAS No.: 21245-02-3

RTI Chemical ID Code: L98

ChemTask No.: CHEM11787

RTI Log Nos. (Amt. Received):
Bulk Analytical: 082010-B-14 (~50 g)
Reference: 082010-B-05 (~5 g)

Program Supported: TOX

Analysis Dates: May 21-22, 24, 2012

Interim Results Date: May 30, 2012

Study Lab: (Investigator): ILS [redacted]

Lot No. (Vendor): MKBF0590V (Aldrich)

Vendor Purity: 98.3% (Aldrich COA)

Receipt Date: Aug 20, 2010 (Bulk)

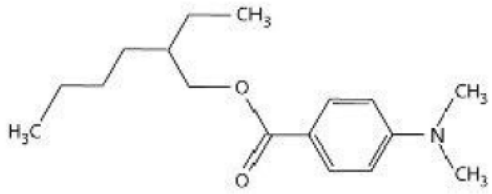
Bulk Receipt Condition: Good, room temperature

Submitter: [redacted] RTI)

Shipping Container: NA (in-house transfer)

Storage Conditions:
Bulk: Room temperature
Reference: Freezer (~ -20 °C)

STRUCTURE



MOL. WT.
277.40

MOL. FORMULA
$C_{17}H_{27}NO_2$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, an aliquot of padimate O was submitted for bulk chemical reanalysis. Chemical purity of the bulk sample was determined relative to a reference standard of the same lot/batch number which had been stored at RTI under freezer conditions. Analytical results obtained by a GC/FID chromatographic method indicated that the sample had a percent relative purity of 98.1% when compared to the frozen reference standard. The FTIR spectrum of the bulk sample matched the spectrum of the frozen reference and was consistent with an identity of padimate O.

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: 2-Ethylhexyl-p-dimethyl-aminobenzoate (Padimate O)

Task Type: Chemical Reanalysis

RTI Task Number: 0212839.200.003.081

Chem Task Number: CHEM11787

This study/task was audited by the Regulatory and Quality Assurance (RQA) – Quality Assurance Unit and the results of the inspections and audits were reported to the task leader/study director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Sample Analysis Inspection	05/15/12	05/22/12
Data & Report Audit	08/20/12	08/20/12

Prepared by:

[redacted]
Quality Assurance Specialist

9/5/12
Date

Reviewed by:

[redacted]
Quality Assurance Specialist

9/5/12
Date

turning knowledge into practice

TABLE OF CONTENTS

Figures

2-ETHYLHEXYL-P-DIMETHYL-AMINOBENZOATE (PADIMATE O)

1.0 INTRODUCTION

The objective of this work was to determine the purity and verify the identity of 2-Ethylhexyl-p-dimethyl-aminobenzoate (padimate O) in support of studies being conducted at ILS. To accomplish this objective, a chemical reanalysis was performed. The identity of the chemical was confirmed by FTIR and its purity assessed by GC.

2.0 CHEMICAL ANALYSIS

An aliquot of the bulk sample of padimate O was received on March 27, 2012 for chemical reanalysis (RTI log 082010-B-14). The aliquot was stored at room temperature. A frozen reference (RTI log 082010-B-05) sample was received May 10, 2012 and was stored at freezer temperature.

3.0 CONFIRMATION OF IDENTITY - INFRARED SPECTROMETRY (IR)

3.1 IR Parameters

System	Thermo Nicolet 6700 FTIR
Software	Omnic, Ver. 7.3
Method	NaCl disks, scan 4000 - 400 cm^{-1}

3.2 Results

Bulk Sample Frequency (1/cm)	Frozen Reference Sample Frequency (1/cm)	Assignment
2958-2860	2958-2860	C-H Stretch
2819	2820	N-CH_3 stretch
1703	1703	C = O stretch
1609, 1527	1609, 1527	C=C Stretch
1317	1317	C-N (tertiary amine stretch)
1183	1184	C = O Stretch
1107	1107	C-O-C Stretch

The observed spectrum for the bulk sample matched the spectrum of the frozen reference sample, and is consistent with the structure of padimate O (as reported in the bulk chemical comprehensive task CHEM11089). Figure 1 shows the bulk and frozen reference IR spectra.

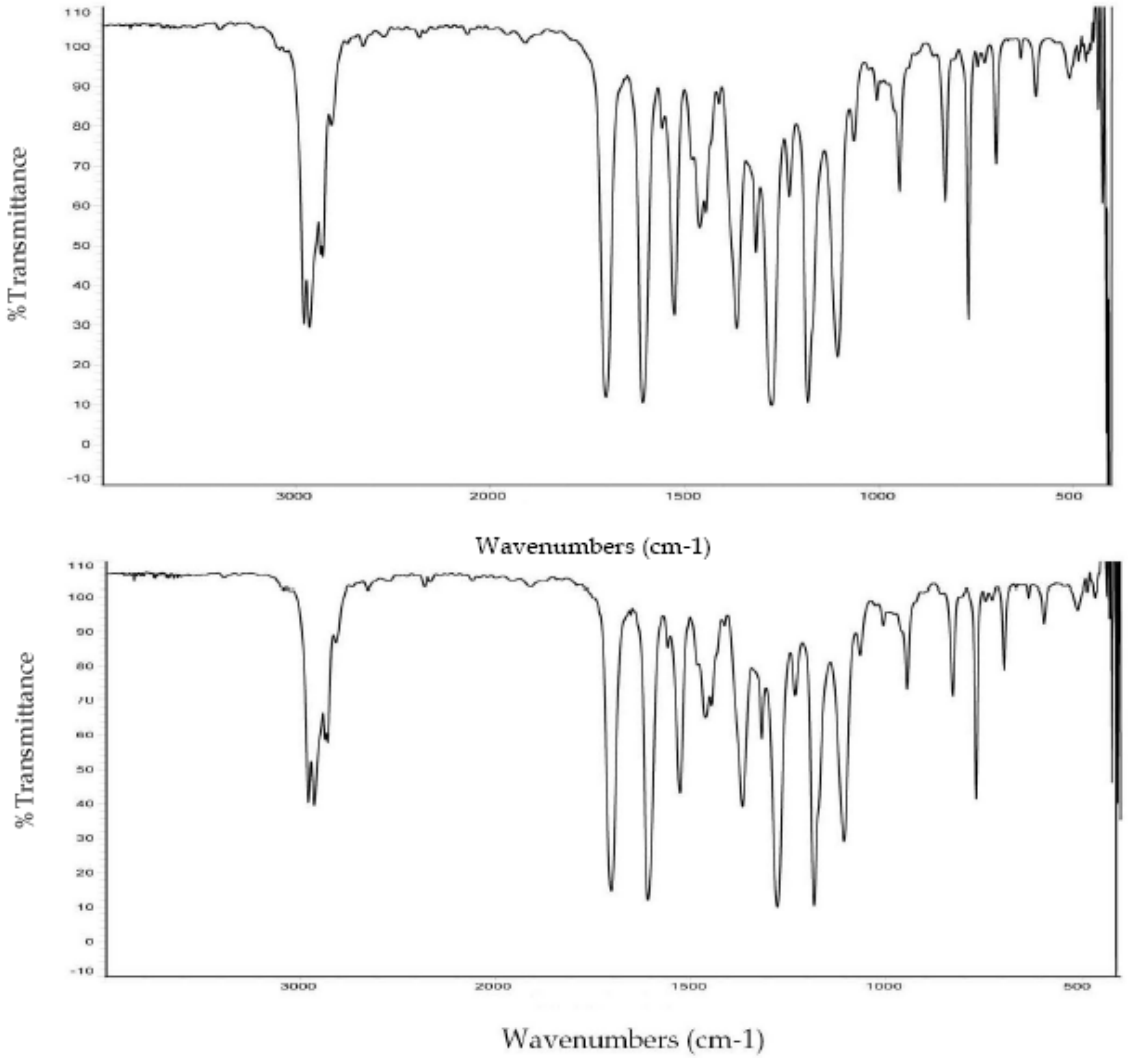


Figure 1: Infrared Spectrum of Padimate O Bulk (top spectrum)
and Frozen Reference (bottom spectrum)

4.0 DETERMINATION OF PURITY - GAS CHROMATOGRAPHY

This section describes the gas chromatographic method used to estimate sample purity.

4.1 Preparation of Internal Standard (IS) Solution

A solution of IS was prepared by weighing 103.4 mg of octanophenone and transferring it into a 200-mL volumetric flask. The IS was diluted to volume with dichloromethane. The flask was mixed by inversion. The IS solution had a concentration of 0.517 mg/mL.

4.2 Bulk Sample and Frozen Reference Standard Solution Preparation

Triplicate solutions of the reference standard and bulk samples were prepared by transferring approximately 25 mg of compound to individual 25-mL volumetric flasks and diluting to volume with IS solution and mixing by inversion. An aliquot of the bulk and reference solutions were transferred to GC vials for analysis. The samples and an IS blank was analyzed by gas chromatography.

4.3 Analysis

GC Parameters

Instrument	Agilent 6890N GC
Data System	Empower 2; Build 2154
Column	Phenomenex ZB-5MS (30 m x 0.25 mm ID, 0.5 µm film) with 5 m pre-guard
Carrier Gas	Helium
Flow Rate	1.5 mL/min
Oven Temperature	70 °C for 1 min., ramp to 270°C at 20 °C/min with a 7 min hold;
Retention Times	Padimate O: ~13.6 min. ; Octanophenone (IS): ~9.9 min.
Injector Type (ratio)	Split (20:1); 1 µL
Injector Temperature	250 °C
Detector-Temperature	FID at 290 °C

The suitability of the system was evaluated, and the results are shown below.

Parameter	Criteria	Result	Pass/Fail
Tailing Factor, T	$0.5 \leq T \leq 2.0$	0.79	Pass
Column Efficiency, N	$\geq$ 250,000 plates	1,070,819	Pass
Precision (%RSD)	$\leq$ 5% (n=6)	0.6%	Pass
Resolution	$\geq$ 40	91.5	Pass

4.4 Results

Calculations based on a major peak comparison technique gave the results shown in the following table. Typical chromatograms are shown in Figure 2.

RTI Log No.	Chemical	RRF[a]	Mean RRF[a] (%RSD)	Percent Relative Purity[b]
082010-B-14	Analytical Replicate #1	1.637	1.640 (0.4)	98.1
	Analytical Replicate #2	1.647		
	Analytical Replicate #3	1.637		
082010-B-05	Reference Replicate #1	1.661	1.672 (2.1)	--
	Reference Replicate #2	1.645		
	Reference Replicate #3	1.711		

[a] RRF = Relative Response Factor; normalized to sample concentration.
[b] Relative Purity = (Mean RRF, bulk/Mean RRF, ref.) x 100.

Based on the chromatographic results, the bulk sample had not significantly changed as compared to the frozen reference, and no significant impurities were observed.

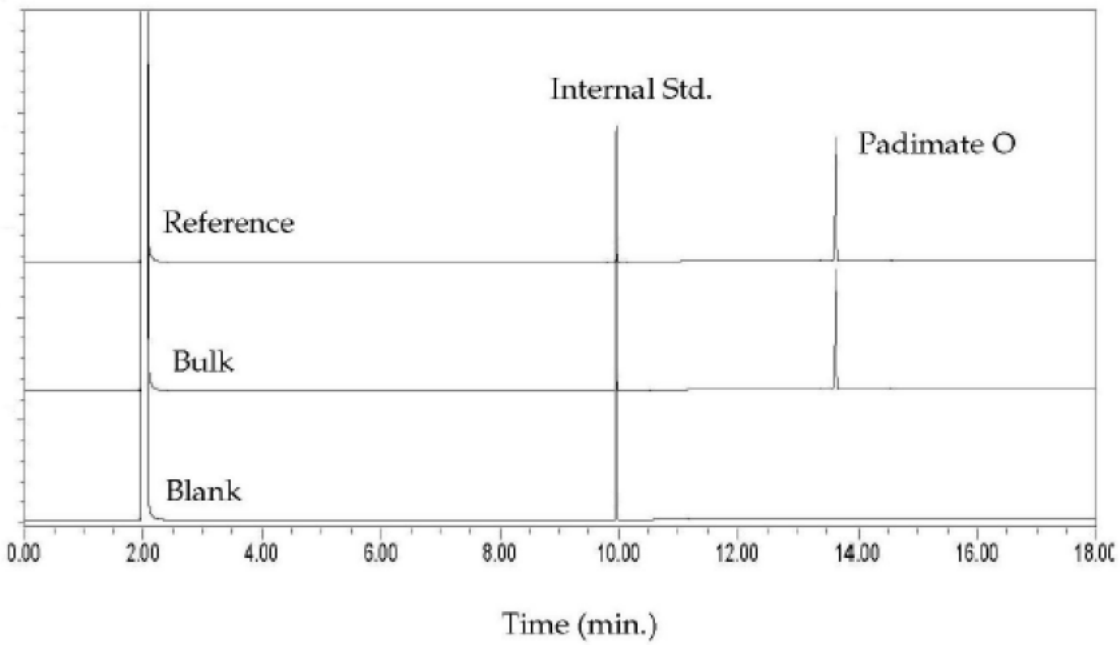


Figure 2: Example Gas Chromatograms of Padimate O Reference and Bulk Sample, and an IS Blank

5.0 REFERENCE

RTI International report "2-Ethylhexyl-p-dimethyl-aminobenzoate (Padimate O), Characterization Protocols Development, (CHEM11292), January 6, 2012.

6.0 ACKNOWLEDGMENTS

Personnel contributing to this task: [redacted]

APPENDIX 2 Certificate of Analysis - DHT

SIGMA-ALDRICH®

SIGMA Life Science

Industriestrasse 25, CH-9471 Buchs (SG), Switzerland
Tel: +41 81 755 2511 Fax: +41 81 756 5449

Certificate of Analysis

Product Name:	5α-ANDROSTAN-17β-OL-3-ONE >= 97.5 %
Product Number:	A8380
Product Brand:	Sigma
Molecular Formula:	$C_{19}H_{30}O_2$
Molecular Mass:	290.44
CAS Number:	521-18-6

TEST	SPECIFICATION	LOT BCBF1698V RESULTS
APPEARANCE (COLOR)	WHITE TO OFF-WHITE	WHITE
APPEARANCE (FORM)	POWDER	POWDER
PURITY (TLC AREA %)	≥ 97.5 %	100.0 %
SPECIFIC ROTATION (20/D)	+31.8 TO +33.5 DEGREES	32.7 DEGREES
CONCENTRATION	C=1 IN ETHANOL AT 20C	C=1 IN ETOH AT 20C
SOLUBILITY (COLOR)	COLORLESS TO VERY FAINT YELLOW	COLORLESS
SOLUBILITY (TURBIDITY)	CLEAR TO SLIGHTLY HAZY	CLEAR (<3.5 NTU)
SOLUBILITY (METHOD)	50 MG/ML IN ETHANOL	50 MG/ML IN ETHANOL
CARBON CONTENT	77.0 TO 80.2 %	78.60 %
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

QC RELEASE DATE	16/FEB/11
RECOMMENDED RETEST DATE	JAN/15

[redacted]Manager
Quality Control
Buchs, Switzerland

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Nilutamide, solid
Product Number	N8534
Product Brand	SIGMA
CAS Number	63612-50-0
Molecular Formula	$C_{12}H_{10}F_3N_3O_4$
Molecular Weight	317.22

TEST	**LOT 057K1157V RESULTS**
APPEARANCE	WHITE POWDER
SOLUBILITY	CLEAR FAINT YELLOW SOLUTION IN METHANOL:ACETONE (1:1)
PROTON NMR SPECTRUM	CONSISTENT WITH STRUCTURE
CARBON	45.5%
NITROGEN	13.2%
PURITY BY THIN LAYER CHROMATOGRAPHY	100%
QC RELEASE DATE	MAY 2007

[redacted]

[redacted] Manager
Quality Control
St. Louis, Missouri USA

Certificate of Analysis

SIGMA-ALDRICH

Product Name	1,1-Dichloro-2,2-bis(4-chlorophenyl)ethene, 99%
Product Number	123897
Product Brand	ALDRICH
CAS Number	72-55-9
Molecular Formula	$(ClC_6H_4)_2C=CCl_2$
Molecular Weight	318.03

TEST	SPECIFICATION	LOT 11923EOV RESULTS
APPEARANCE	WHITE POWDER, CRYSTALS, CRYSTALLINE POWDER	WHITE CRYSTALS
INFRARED SPECTRUM	CONFORMS TO STRUCTURE.	CONFORMS TO STRUCTURE.
GAS LIQUID CHROMATOGRAPHY	98.5% (MINIMUM)	98.6%
QUALITY CONTROL ACCEPTANCE DATE	REVISED JUNE 12, 2007 RJM	JUNE 2001 REANALYZED DECEMBER 2006

[redacted] Supervisor
Quality Control
Milwaukee, Wisconsin USA

APPENDIX 3 Certificate of Analysis – MDA-kb2

ATCC

Certificate of Analysis

ATCC® Number: CRL-2713™
Lot Number: 3984776

Name: MDA-kb2
Description: Breast Carcinoma
Species: Human (*Homo sapiens*)
Expiration Date: Not applicable

Test	Specifications	Results
Total cells/mL	Report results	5.7×10^6
Ampule passage number	Report results	37
Post-freeze viability	≥ 50.0%	89.8%
Growth properties	Adherent	Adherent
Morphology	Epithelial-like* and/or rounded	Epithelial-like and rounded
Test for mycoplasma contamination Hoechst DNA stain (indirect) Agar culture (direct)	 None detected None detected	 None detected None detected
Species determination: Isoenzyme assay (interspecies)	Human B (G6PD variant)	Human B (G6PD variant)
Species determination: STR analysis (intraspecies)	Human (Unique DNA Profile) D5S818: 11 D13S317: 12 D7S820: 10 D16S539: 9 vWA: 17, 18 THO1: 6 Amelogenin: X TPOX: 10 CSF1PO: 10, 12	Human (Unique DNA Profile) D5S818: 11 D13S317: 12 D7S820: 10 D16S539: 9 vWA: 17, 18 THO1: 6 Amelogenin: X TPOX: 10 CSF1PO: 10, 12
Sterility test (BacT/ALERT 3D) iAST bottle (aerobic) at 32°C iNST bottle (anaerobic) at 32°C	 No growth No growth	 No growth No growth

* Epithelial-like: Any adherent cells of a polygonal shape with clear, sharp boundaries between them.

[redacted]

13 April 2009
Date

Quality Control Manager; Quality, Compliance and Biosafety

ATCC hereby represents and warrants that the material provided under this certificate has been subjected to the tests and procedures specified and that the results described, along with any other data provided in this certificate, are true and correct to the best of the company's knowledge and belief.

This product is intended to be used for laboratory research use only. It is not intended for use in humans, animals, or for diagnostics.

ATCC products may not be resold, modified for resale, used to provide commercial services, or to manufacture commercial products without prior written agreement from ATCC.

ATCC (American Type Culture Collection)
P.O. Box 1549
Manassas, VA 20108 USA
www.atcc.org

800-638-6597 or 703-365-2700
Fax: 703-365-2750
E-mail: tech@atcc.org
or contact your local distributor

APPENDIX 4 **Mycoplasma Free**

bionique Testing Laboratories **CELLshipper®**

Mycoplasma Testing Services
156 Fay Brook Drive • Saranac Lake NY 12983
Phone: 518-891-2356 • Fax: 518-891-5753

Please enclose this completed form with each slide to avoid delays in processing.

Date Sent: 15MAR2012
Name: [redacted]
(Bionique will submit results only to the person named above)
Company/University CeeTox Inc
Complete Mailing Address: (Results are mailed 1st class USPS)
CeeTox Inc [redacted]
4717 Campus Drive
Kalamazoo, MI 49008 [redacted]
Optional: FAX #: _____
(one Fax # only)

Sample Designation or #: MDA-kb2 p46
LOT: 3984776
Cell Type: X *Adherent* ____ *Nonadherent*
Normal ____ *Transfect* ____ *Monoclonal* ____ *Tumor* ____
Flask 150 *Roller Bottle* ____ *≤ 2 liter suspension*
Bioreactor ____ *Other* _____

For Research Use Only

www.bionique.com 110364

Date received at Bionique Testing Labs: 3/16/12 Code #: 48659

M-100 CELLSHIPPER DNA FLUOROCHROME ASSAY RESULTS:

X **NEGATIVE:** A reaction with staining limited to the nuclear region, which indicates no mycoplasmal contamination.

____ **POSITIVE:** A significant amount of extranuclear staining which strongly suggests mycoplasmal contamination.

____ **INCONCLUSIVE:**

____ A significant amount of extranuclear staining consistent with low - level mycoplasmal contamination or nuclear degeneration.

____ A significant amount of extranuclear staining consistent with bacterial, fungal or other microbial contaminant or viral CPE. Morphology not consistent for mycoplasmal contamination.

COMMENTS: _____

Date Processed: 3/19/12 **By:** [redacted]

Thank you for allowing us to assist you, and for using the CELLshipper. **(dc: 3003 att # 2; 10/9/2003)**

APPENDIX 5 Protocol and Protocol Amendments

4717 Campus Drive, Kalamazoo, MI 49008 (269) 353-5555 (office) www.ceetox.com

CeeTox®
In vitro models to predict toxicity

PROTOCOL

Androgenic Receptor Transactivation Activity in MDA-kb2

Study Number:
9070-100794ARTA

Sponsor:
NIEHS
National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle Park, NC 27709 USA

Test Facility:
CeeTox
4717 Campus Drive
Kalamazoo, MI 49008

APPENDIX 5 Protocol and Protocol Amendments

TEST PROTOCOL

TO BE COMPLETED BY THE STUDY SPONSOR:
Study Sponsor: NIEHS/NTP [redacted] Chief Toxicology Branch)
Address: P.O. Box 12233
Research Triangle Park, NC 27709 **Phone:** [redacted]
Study Monitor: [redacted] **E-mail:** [redacted] **Phone:** [redacted]
CoStudy Monitor: N/A **Phone:** N/A
Sponsor Protocol/Project No: N/A
Test Substance Name(s): 2-Phenyl-5-benzimidazolesulfonic Acid (Ensulizole)
Purity: 99.6%
Batch or Lot#: 05117JE
Test Substance Name(s): Butyl-methoxydibenzoylmethane (Avobenzone)
Purity: 98.5%
Batch or Lot#: L802809
Test Substance Name(s): 3, 3, 5-Trimethlycyclohexyl Salicylate (Homosalate)
Purity: 99.3%
Batch or Lot#: YT0976
Test Substance Name(s): 2-Ethylhexyl-P-Dimethyl-Aminobenzoate (Padimate-O)
Purity: 98.1%
Batch or Lot#: MKBF0590V
Proposed Experimental Start Date: January 16, 2013 (date subject to change; actual experimental start date to be provided in final report)
Proposed Experimental Termination Date: February 15, 2013 (date subject to change; actual experimental termination date to be provided in final report)

APPENDIX 5 Protocol and Protocol Amendments

Sponsor
National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle Park, NC 27709

[redacted]
Contract Office Technical Representative
National Toxicology Program, National Institutes of Environmental Health

[redacted]
National Toxicology Program (NTP) Investigator
Telephone No.: [redacted]
Facsimile No.:
E-mail: [redacted]

Study Monitor
[redacted]
Integrated Laboratory Systems, Inc.
Telephone No.: [redacted]
Facsimile No.:
E-mail: [redacted]

Project Identification
ILS Project No.: N135
Study No.: 007
Human and Health Science Number: HHSN273200900005C
NIEHS contract number: N01ES00005

APPENDIX 5 Protocol and Protocol Amendments

Table of Contents

APPENDIX 5 Protocol and Protocol Amendments

CeeTox, Inc. Study No. 9070-100794 ARTA

Signatures

[redacted]

Chief, Toxicology Branch
National Toxicology Program, NIEHS

1/15/13
Date

[redacted]

Contract Office Technical Representative
National Toxicology Program, NIEHS

~~1/15/13~~ 1/15/13 (1)
Date

[redacted]

Integrated Laboratory Systems, Inc.
Study Monitor

15 Jan 2013
Date

[redacted]

Study Director

16 Jan 2013
Date

(1) REWRITTEN FOR CLARITY [redacted] 1/16/13

APPENDIX 5 Protocol and Protocol Amendments

1. **Title of Study**

   Androgen Receptor Transactivation Activity in MDA-kb2

2. **Purpose of Study**

   The purpose of this study is to analyze four test substances for androgenic transactivation activity using the MDA-kb2 reporter cell line. The MDA-kb2 cell line is derived from a human breast cancer line transfected with an androgen receptor promoter linked to a luciferase gene. Consequently, the MDA-kb2 cell line can measure the ability of a test substance to induce or antagonize Androgen Receptor mediated transactivation via luciferase gene expression.

3. **Compliance Statement**

   This study will be conducted in compliance with the U.S. Environmental Protection Agency Good Laboratory Practice regulations Title 40, Part 160 with the exception of section 160.113. Dose concentrations of test substance and control substances will not be verified using analytical methods.

4. **Quality Assurance**

   This study will be subjected to periodic inspections. The data and the draft final report will be reviewed by the Quality Assurance Unit of CeeTox in accordance with CeeTox standard operating procedures (SOPs).

5. **Regulatory Citations / Guidelines**

   Currently this assay is not mandated or validated by the Environmental Protection Agency Endocrine Disruptor Screening Program (EDSP) Tier 1 testing program.

6. **Test Facility**

   CeeTox, Inc.
   4717 Campus Drive
   Kalamazoo, MI 49008 USA

7. **Experimental Design**

   The androgen receptor transactivation assay will be used to identify the ability of test substances to bind to and activate androgen receptors (AR) present in the MDA-kb2 cell line (agonism and antagonism). In this assay, the test substances and the controls are exposed to the MDA-kb2 cells at eight concentrations for approximately 24 hours. At the end of the exposure period, the medium is removed from the cells, cell viability is monitored using a propidium iodide (PI) uptake assay, and effects on androgen receptor transactivation are determined using a firefly luciferase reporter construct. The positive control for AR agonism is Dihydrotestosterone (DHT). The negative control is Nilutamide. The positive control for AR Antagonism is 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene. These controls are assessed every time the activation assay is performed.

APPENDIX 5 Protocol and Protocol Amendments

8. Justification of the Test System

The human cell line MDA-kb2 (ATCC CRL-2713) will be used for the assay. The MDA-kb2 cell line was derived from the breast cancer cell line, MDA-MB-453 by stable transfection with a mouse mammary tumor virus (MMTV) luciferase-neo reporter gene construct (Wilson et al., 2002). The MDA-MB-453 parent cell line has been shown to express high levels of functional, endogenous androgen receptor. Estrogen receptor alpha and progesterone receptor however are not detectable at the mRNA level and estrogen receptor beta is expressed only at very low levels. This cell line does contain glucocorticoid receptor (GR). The MDA-kb2 cell line can measure the ability of a test substance to induce or antagonize AR-mediated transactivation via luciferase gene expression.

9. Identification of the Test System

The cells used for the androgen receptor transcriptional activation assay will be the MDA-kb2 cell line (ATCC CRL-2713).

The cells used in the study will be appropriately labeled and will be identified by cell type and passage number. The passage number used in the assay will be provided in the report. Bias is not a factor in this test system.

10. Test & Control Substance(s)

10.1 Preparation of Test Substance

Test substances will be prepared as a stock in DMSO (Dimethylsulfoxide), or appropriate vehicle and serially diluted in the same solvent to prepare solutions for dilutions with media (to a final concentration of ≤ 0.5% (v/v) DMSO). Fresh dilutions of the stock solutions will be prepared on the day of use in the assay. Dose concentrations of test and control substances will not be verified using analytical methods.

Test Substance:	2-Ethylhexyl-p-dimethyl-aminobenzoate (Padimate O)
CAS No.	21245-02-3
Source:	Sigma-Aldrich
Lot/Batch No.:	MKBF0590V
Formula:	$(CH_3)_2NC_6H_4CO_2CH_2CH(C_2H_5)(CH_2)_3CH_3$
Description:	Colorless liquid
Purity:	98.1%
Test Substance:	2-Phenyl-5-benzimidazolesulfonic acid (Ensulizole)

APPENDIX 5 Protocol and Protocol Amendments

CAS No.	27503-81-7
Source:	Sigma-Aldrich
Lot/Batch No.:	05117JE
Formula:	$C_{13}H_{10}N_2O_3S$
Description:	White powder
Purity:	99.6%
Test Substance:	3, 3, 5-Trimethlycyclohexyl Salicylate (Homosalate)
CAS No.	118-56-9
Source:	Spectrum Chemical Mfg. Corp
Lot/Batch No.:	YT0976
Formula:	$C_{16}H_{22}O_3$
Description:	Colorless to light yellow liquid
Purity:	99.3%
Test Substance:	Butyl-methoxydibenzoylmethane (Avobenzone)
CAS No.	70356-09-1
Source:	Universal Preserv-A-Chem Inc.
Lot/Batch No.:	L802809
Formula:	$C_{20}H_{22}O_3$
Description:	Off White to Yellowish Crystalline Powder
Purity:	~98.5%

Note: A certificate of analysis for the test substance will be provided by the sponsor and will be stored in the study data and appended to the study report. Confirmation of the identity of the test substance, characterization and stability

APPENDIX 5 Protocol and Protocol Amendments

will be verified by the sponsor or sponsor's designee. Test substance will be either returned to the Sponsor or destroyed following finalization of the study report.

Certificates of analysis for the positive and negative reference substances will be provided by the vendor, stored in the study data and appended to the study report.

10.2 Control and Reference Substances

Positive control for AR Agonism: DHT (Dihydrotestosterone): CAS No: 521-18-6

Negative control: Nilutamide: CAS No: 63612-50-0

Positive control for AR Antagonism: p,p'-DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene): CAS No: 82413-20-5

Positive control for cytotoxicity Digitonin: CAS No: 11024-24-1 (one concentration, as a positive control in the cytotoxicity assay)

Vehicle control DMSO: CAS No: 67-68-5 (one concentration)

Each control substance will be prepared as a stock in DMSO (final concentration DMSO in media of ≤0.5% (v/v)) and serially diluted in the same solvent to prepare solutions for dilutions with media.

11. Test System

11.1 Source

The human cell line MDA-kb2 (ATCC CRL-2713) will be used for the assay. The MDA-kb2 cell line was derived from the breast cancer cell line, MDA-MB-453 by stable transfection with a mouse mammary tumor virus (MMTV) luciferase-neo reporter gene construct (Wilson *et al.*, 2002). The MDA-MB-453 parent cell line has been shown to express high levels of functional, endogenous androgen receptor. However estrogen receptor alpha, and progesterone receptor are not detectable at the mRNA level and estrogen receptor beta is expressed only at very low levels. This cell line does contain glucocorticoid receptor (GR). The cells used in the study will be appropriately labeled and will be identified by cell type and passage number (3 to 20). Only MDA-kb2 cells that test negative for mycoplasma will be used and certification will be included in the final report as an appendix. Bias is not a factor in this test system.

11.2 Stability of the Cell Line

The stability of the cell line will be monitored by reference substances p,p'-DDE (positive control for antagonism), DHT (positive control for agonism) and Nilutamide (negative control). Reference substance values should fall within CeeTox historical data. A complete concentration response curve for each reference substance will be run each time the assay is performed.

APPENDIX 5 Protocol and Protocol Amendments

11.3 Cell Culture and Plating Conditions

Cells will be maintained in Lebovitz's L-15 medium containing 10% fetal bovine serum, without CO_2 incubator at ~37°C. When the cells reach confluency, they will be subcultured. The cells will be suspended with Lebovitz's L-15 containing 10% fetal bovine serum and plated into wells of a 96 well plate at a density of ~1 X 10^4 cells/100 µL/well. The cells will then be placed into an incubator without CO_2 at ~37°C for at least 3 hours prior to substance exposure.

12. Methods

12.1 Substance Exposure and Assay Plate Organization

The procedure for substance dilutions (steps 1 and 2) and exposure to cells (step 3) will be conducted as follows:

Step 1: Each test/reference substance will be diluted in DMSO (or appropriate solvent), serially diluted and added to wells of a 96-well microtiter plate to achieve final serial concentrations. All concentrations will be tested in replicates of at least 3. The final eight concentrations used will be noted in the final report, but are typically in the range of 1 X 10^{-13} to 1 X 10^{-3}. Control groups will be included as follows: vehicle control, agonist maximal response control (DHT), antagonist control (Nilutamide), cytotoxic control (Digitonin). Note: cytotoxicity plates will include the control Digitonin in place of Nilutamide.

Step 2: Test substance will be diluted in media (Lebovitz L-15) as appropriate (if DMSO is used the final concentration of DMSO in media will not exceed 0.5%).

Step 3: Seventy-five microliters of test substance dilution (2X) in media (prepared in step 2) will be added to wells by pipet containing ~1 X 10^4 cells/75 µL/well for a final volume of 150 µL/well.

APPENDIX 5 Protocol and Protocol Amendments

Table 1. Agonism Assay Plate Example

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank*	DHT (10 nM)	VC**	VC	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Conc. 6	Conc. 7	Conc. 8
B	↓***	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	----------As above + antagonist (10 µM nilutamide)----------											
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

DHT = dihydrotestosterone
*Blank wells contain media only (no cells)
**Vehicle control (VC) wells contain cells and media + 0.5% (v/v) DMSO
***↓ Indicates the composition of the well is identical to the well directly above it

Table 2. Antagonism Assay Plate Example

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank*	DHT (10 nM)	VC**	Induced Control	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Conc. 6	Conc. 7	Conc. 8
B	↓***	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	----------As above + (1000 nM DHT instead of 1 nM DHT)----------											
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

*Blank wells contain media only (no cells)
**Vehicle control (VC) wells contain cells and media + 0.5% (v/v) DMSO
***↓ Indicates the composition of the well is identical to the well directly above it
Rows A-D are low agonist (1 nM DHT)
Rows E-H are high agonist (1000 nM DHT)

APPENDIX 5 Protocol and Protocol Amendments

Table 3. Cytotoxicity Assay Plate Example

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank*	DHT (10 nM)	VC**	VC	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Conc. 6	Conc. 7	Conc. 8
B	↓***	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	----------As above + 250µM Digitonin----------											
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

DHT = dihydrotestosterone
*Blank wells contain media only (no cells)
**Vehicle control (VC) wells contain cells and media + 0.5% (v/v) DMSO
***↓ Indicates the composition of the well is identical to the well directly above it

After adding the test/reference substances, the plates will be incubated in a ~37°C incubator without CO_2 for ~24 hours to allow for induction of the reporter gene products. If test substances are thought to be volatile, plate sealers (breathe easy) will be used and noted in the final report. Two definitive test runs as described in section 14 Data Interpretation will be performed.

12.2 Luciferase Assay

A luciferase assay as described in CeeTox SOP 2041 (proprietary) will be performed. Luciferase assay reagent will be prepared as described in CeeTox SOP 2041. Briefly, cells will be lysed and the cell lysate will be combined with CeeTox proprietary Luciferase reagent and read using a Packard alphaHT Fusion or equivalent plate reader.

12.3 Cytotoxicity Assay

Cell viability will be monitored by propidium iodide (PI) uptake. PI is a dye that cannot cross the plasma membrane of intact and viable cells. Cells that are dead or dying have weakened plasma membranes which allows PI to enter the cytosol of the damaged cells. Once inside the cell, it intercalates into DNA/RNA and yields a fluorescent signal. PI is a light sensitive compound; therefore all procedures will be conducted under low light conditions.

Cells for the cytotoxicity assay will be seeded into a 96-well black sided culture plate at the same time cells are seeded for the AR transactivation assays described above. The PI working solution will be prepared by adding PI powder to either the cell culture media used or phosphate buffered saline (PBS) in an amount sufficient to yield a final concentration of 44 µM. Following an ~24 hour incubation with the test substance, the growth medium will be removed from the plate designated for cytotoxicity and 50 µL of the PI working solution will be added. Background fluorescence will be evaluated by reading fluorescence on

a fluorescent plate reader at an excitation wavelength of 544 nm and an emission wavelength of 612 nm. Following this determination 50 µL of a 2% triton X-100 solution prepared in water will be added and the plate incubated at room temperature for a minimum of 15 minutes and read at the same wavelengths. The total amount of fluorescence or cells present on the plate will be determined by subtracting the first read from the second read. The change in cell viability will be determined by comparing treated wells to the untreated or control wells. A 20% drop below vehicle treated controls will be considered cytotoxic.

12.4 Solubility/Precipitation Assay

The limit of test substance solubility will be determined by laser based light scattering. The test substance will be prepared in the MDA-kb2 culture media at the final exposure concentrations and added to wells of a 96-well plate. The samples will be assessed using a NEPHELOstar nephelometer (BMG LabTech, Ortenberg, Germany).

13. Analysis of Data

13.1 Agonism Assay Plate Data Analysis

All cytotoxic concentrations of test substance (as defined in section 12.3) will be excluded from data analysis.

In order to determine the relative transcriptional activity as compared to the positive control (PC), 10 nM DHT, the luminescence data from each plate will be analyzed according to the steps outlined below. Wells incorporating nilutamide will be analyzed in an identical fashion to wells not incorporating nilutamide, except that the data will be normalized by subtracting the mean value for the nilutamide-containing vehicle control (VC) wells. Data will be analyzed using Microsoft Excel.

Step 1: The mean value for the VC wells will be calculated.

Step 2: The mean value for the VC wells will be subtracted from each well to normalize the data.

Step 3: The mean value for the normalized PC wells will be calculated.

Step 4: The normalized value for each well will be divided by the mean value of the normalized PC wells (with the normalized mean of the PC wells being defined as 100% relative transcriptional activity). The final value for each well will be the relative transcriptional activity for that well compared to the mean normalized PC response.

13.2 Antagonism Assay Plate Data Analysis

All cytotoxic concentrations of test substance (as defined in section 12.3) will be excluded from data analysis.

APPENDIX 5 Protocol and Protocol Amendments

In order to determine the relative transcriptional activity as compared to the positive control (PC), 10 nM DHT, the luminescence data from each plate will be analyzed according to the steps outlined below. Wells incorporating 1 nM DHT will be analyzed in an identical fashion to wells incorporating 1000 nM DHT, except that the data will be normalized to the induced control with 1 nM DHT or 1000 nM DHT, respectively.

Step 1: The mean value for the VC wells will be calculated.

Step 2: The mean value for the VC wells will be subtracted from each well to normalize the data.

Step 3: The mean value for the induced control with 1 nM DHT will be calculated.

Step 4: The mean value for the induced control with 1000 nM DHT will be calculated.

Step 5: The wells dosed with test or control substance and 1 nM DHT will be normalized to the mean value for the induced control with 1 nM DHT.

Step 6: The wells dosed with test or control substance and 1000 nM DHT will be normalized to the mean value for the induced control with 1000 nM DHT.

Step 7: Averages of antagonist % maximal induction control will be calculated (test or control substance a with 1 nM DHT).

Step 8: Averages of high agonist control % maximal induction control will be calculated (test or control substance a with 1000 nM DHT).

Step 9: Differentials will be calculated (averages of high agonist control % maximal induction control minus averages of antagonist % maximal induction control).

13.3 Proposed Statistical Methods

For data generated at CeeTox, basic statistical analysis will be performed on the data, which will include means of replicates, standard error of the mean, standard deviations and %CV. Data will be analyzed using Microsoft Excel.

13.4 Considerations for Induction of EC_{50}, PC_{50} and PC_{10}

The full concentration response curve is required for the calculation of the EC_{50}. Calculating an EC_{50} might not always be achievable or practical due to the limitations of the test concentration range (for example due to cytoxicity and/or solubility issues). However, as the EC_{50} and maximum induction level (corresponding to the top value of the Hill-equation) are informative parameters, these parameters will be reported where possible. XLfit will be used for the calculation of EC_{50} and maximum induction level.

If the Hill's logistic equation is applicable to the concentration response data, the EC_{50} will be calculated by using the following equation:

APPENDIX 5 Protocol and Protocol Amendments

$Y = Bottom + (Top-Bottom)/ (1+10 EXP ((log EC_{50} – X) x Hill slope))$

Where:

X is the logarithm of concentration; and,

Y is the response and Y starts at the Bottom and goes to the Top in a sigmoid curve.

Bottom is fixed at zero in the Hill's logistic equation

For each test substance the following data will be provided when possible:

- RPC_{Max} which is the maximum level of response induced by a test substance, expressed as a percentage of the response induced by 10 nM of DHT on the same plate, as well as the PC_{Max} (concentration associated with the RPC_{Max}).
- For positive substances, the concentrations that induce the PC_{10} and, if appropriate, the PC_{50} will be determined.

14. Data Interpretation

14.1 Agonism Assay Interpretation

- Where appropriate, LogPC50, LogPC10, LogEC50 and Hill slope values will be calculated.
- For the test substance, the maximum response relative to the positive control (RPCMax) will be determined. In each individual run of the transcriptional activation assay, if RPCmax is less than 20%, the test substance will be considered to have given a negative response for AR agonism.
- For each individual run of the transcriptional activation assay, the acceptability of the data will be evaluated using the following criteria:
  - The mean normalized luciferase signal of the PC (10 nM DHT) should be at least 4-fold that of the mean VC on each plate.
  - The results of the reference compounds, nilutamide and DHT, should be within the acceptable ranges.
- If the acceptability criteria outlined above are met, that run of the transcriptional activation assay will be considered to be definitive
- The test substance will be considered negative if RPCMax is <20% in at least 2 definitive runs of the transcriptional activation assay. The test substance will be considered positive if RPCMax is ≥20% in at least 2 definitive runs of the transcriptional activation assay.

14.2 Antagonism Assay Interpretation

- Where appropriate, RICMax, Differential IC50, Differential IC30, LogEC50 and Hill slope values will be calculated.
- If the differential between the high antagonism and the low antagonism is greater than 50% and has a dose response (more than one data point) in two runs, than the test substance will be considered positive.

- If the differential between the high antagonism and the low antagonism is less than 50% and does not have a dose response (more than one data point) in two runs, than the test substance will be considered negative.
- For each individual run of the transcriptional activation assay, the acceptability of the data will be evaluated using the following criteria:
  - The mean normalized luciferase signal of the PC (10 nM DHT) should have been at least 4-fold that of the negative control on each plate.
- If the acceptability criteria outlined above is met, that run of the transcriptional activation assay will be considered to be definitive.

15. Final Study Reports

The data to be reported in the draft report and final report will be determined per Standard Operating Procedure (SOP) and will include (but will not be limited to) the following information: assay date and run number, laboratory personnel involved in the study, chemical/test substance information (including but not limited to substance name, code, molecular weight, concentrations tested, notes regarding solubility, data interpretation).

The draft report will be submitted to the Sponsor in electronic form. The final report will be submitted as one hard copy and one electronic copy.

16. Alterations of the Study Design

Alterations of this protocol may be made as the study progresses. No changes in the protocol will be made without the specific written request or consent of the Sponsor. In the event that the Sponsor authorizes a protocol change verbally, CeeTox will honor such a change. However, written authorization will be obtained to document this verbal request. All protocol amendments with justifications will be documented, signed and dated by the Study Director and Sponsor's Representative. A copy of the protocol and all amendments will be issued to the Sponsor and the originals will be placed into the study binder.

17. Data Retention and Archiving

All original data [including the original signed study protocol and all amendments (if any), test substance information, observations, etc.] and the original final report will be transferred to the National Toxicology Program Archives following finalization of the study report to the address below:

NTP Archives
[redacted]
615 Davis Drive, Suite 300
Durham, NC 27713

APPENDIX 5 Protocol and Protocol Amendments

18. Test Substance Disposition

Test substance will be either returned to the sponsor or destroyed following finalization of the study report.

CeeTox

Protocol Amendment #1

Study Number: 9070-100794ARTA

Title of Study to be Amended: Androgenic Receptor Transactivation Activity in MDA-kb2

Reason for Amendment to Protocol: Section 12.1 page 11 of the protocol: the volume of the test substance to be added to each well is not consistent with CeeTox Standard Operating Procedure SOP-2076 and will be updated to correspond with the SOP.

Change:

Section 12.1 Substance Exposure and Assay Plate Organization Step 3 stated:
Seventy-five microliters of test substance dilution (2X) in media (prepared in step 2) will be added to wells by pipet containing ~1 X 10^4 cells/75 µL/well for a final volume of 150 µL/well.

Section 12.1 Substance Exposure and Assay Plate Organization Step 3 will now state:
Fifty microliters of test substance dilution (2X) in media (prepared in step 2) will be added to wells by pipet containing ~1 X 10^4 cells/50 µL/well for a final volume of 100 µL/well.

Signature

[redacted]

Chief, Toxicology Branch
National Toxicology Program, NIEHS

1/22/13
Date

[redacted]

Contract Office Technical Representative
National Toxicology Program, NIEHS

1/23/13
Date

[redacted]

Integrated Laboratory Systems, Inc
Study Monitor

22 Jan 2013
Date

[redacted]

Study Director

31 Jan 2013
Date

APPENDIX 5 Protocol and Protocol Amendments

CeeTox

Protocol Amendment #2

Study Number: 9070-100794ARTA

Title of Study to be Amended: Androgenic Receptor Transactivation Activity in MDA-kb2

Reason for Amendment to Protocol: A typographical error was found in the protocol

Change:
Section 10.2 Control and Reference Substances Positive control for AR Antagonism stated:
p,p'-DDE (1,1-dichloro-2,2-bis(pchlorophenyl)ethylene): CAS No: 82413-20-5

Section 10.2 Control and Reference Substances Positive control for AR Antagonism will now state:
p,p'-DDE (1,1-dichloro-2,2-bis(pchlorophenyl)ethylene): CAS No: 72-55-9

Signature

[redacted]
2/5/13
Date

Chief, Toxicology Branch
National Toxicology Program, NIEHS

[redacted]
2/7/13
Date

Contract Office Technical Representative
National Toxicology Program, NIEHS

[redacted]
05 Feb 2013
Date

Integrated Laboratory Systems, Inc
Study Monitor

[redacted]
7 Feb 2013
Date

Study Director