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In Vitro Endocrine Disruption Screening of 3-nitro-1,2,4-triazol-5-one (NTO)

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14. ABSTRACT

The compound, 5-nitro-1,2,4-triazol-3-one, (NTO) is an explosive that performs similar to RDX but has reduced sensitivity compared to RDX. Routine safety assessments including toxicity testing have been performed with NTO. NTO has been demonstrated to affect testes weights in orally dosed rats. One possible mode of action for adverse testicular effects is hormonal (endocrine) disregulation. A series of in vitro bioassays were performed to assess if NTO disrupts the endocrine system. The assays performed were estrogen receptor binding, androgen receptor binding, estrogen transactivation, aromatase, and steroidogenesis. The endpoints of these assays contribute to a weight of evidence (WoE) strategy for determining NTO endocrine disruption. No NTO effects on the tested in vitro endpoints were observed and using a WoE approach, NTO does not appear to directly affect testosterone or estrogen mediated endocrine regulation.

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Sponsor

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1 Summary

1.1 Overview

The compound, 5-nitro-1,2,4-triazol-3-one, (NTO) has been demonstrated to affect testes weight in rat oral administration 14-day and 90-day studies. Additionally, testicular atrophy and hypospermia were observed in the 90-day study. The following studies were conducted to test the possibility that NTO is an endocrine disrupting compound (EDC). A weight of evidence (WoE) approach described by the USEPA was used as a guideline for evaluating NTO. A WoE strategy improves the sensitivity of and reliability for determining the potential EDC impact on hormonal pathways.

1.2 Purpose

As the next step for assessing NTO testicular toxicity, a series of bioassays that measure endocrine mediated endpoints were performed. Determining if NTO acts as an endocrine disruptor is important because environmentally persistent chemicals that impact reproduction can be highly regulated and receive a great deal of scrutiny from the EPA, which can result in restrictions on the use of these compounds by the U.S. Army.

1.3 Conclusions

NTO was tested in nine endocrine disruptor bioassays. Five of these assays were *in vitro*: estrogen receptor binding, androgen receptor binding, estrogen transactivation, aromatase, and steroidogenesis. No NTO effects on these endpoints were observed. Using a WoE approach, NTO does not appear to directly affect testosterone- or estrogen-mediated regulation.

1.4 Recommendation

The testicular toxicity of NTO in rats is well documented by USAPHC. The results from the Tier 1 *in vitro* screen do not support that NTO disrupts estrogen or androgen (as testosterone) endpoints. Metabolites are not directly tested with these methodologies and, if known, should be included in future EDC Tier 1 assessments. The mode of action for NTO testicular effects should be assessed with timed exposures so that the sequence of toxicity events can be observed using histopathological endpoints.

2 References

See Appendix A for list of references.

3 Authority

Military Interdepartmental Purchase Request (MIPR) 1JDATHR142. This Toxicology Study addresses, in part, the environmental safety and occupational health (ESOH) requirements outlined in Army Regulation (AR) (AR 200-1), AR (AR 40-5), and AR (AR 70-1), Department of Defense Instruction (DoDI) 4715.4, and Army Environmental Requirement and Technology Assessment (AERTA) PP-3-02-04, *Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces*, (AERTA., 2009).

4 Background

The endocrine system produces hormones that control the growth, development, reproduction, and metabolism of the body (Hiller-Sturmhofel and Bartke, 1998). The endocrine glands include the hypothalamus, pituitary, adrenal, ovaries, testes, thyroid, parathyroid, and pancreas. In response to a specific stimulus, hormones are released from these glands and trigger a cascade of reactions that will modify the target cells' function or activity. Hormone production and secretion is tightly controlled by feedback responses so that homeostasis is maintained. Additionally, this highly regulated system allows for proper developmental staging. One example of this type of regulation is sexual maturation. Chemicals that disrupt these processes are called endocrine disrupting chemicals/compounds or EDCs. Chemicals that act as EDCs and specifically target reproductive hormones are of great interest as there is the potential for a negative impact on ecological species at the population level and on human health.

The USEPA was authorized in 1996 to regulate substances that may act like estrogen [21 U.S.C. 346a(p)]. Subsequently, the USEPA adopted a two-tiered screening and testing strategy--endocrine disruption screening program (EDSP)--and expanded the program to include androgen and thyroid hormonal pathways and ecological effects [Dec. 28, 1998 (63 FR 71542)]. A testing battery was developed by the USEPA and peer-reviewed; see Table 1.

5 Statement of the Problem

NTO is a testicular toxicant. One possible mode of action for this type of effect is inhibition or disruption of reproductive hormone function. A series of bioassays suitable for screening estrogen, androgen, and thyroid endpoints has been identified by the USEPA. The USAPHC Toxicology Portfolio has used this test battery as a reference for identifying a series of *in vitro* tests to screen NTO for endocrine effects. The *in vitro* approaches are of relative low cost and provide key information regarding the potential mode of action for NTO toxicity.

6 Methods

6.1 General Approach

The methods that were used for the series of tests reported here are based on the Test Guidelines in Table 1. Modifications or substitutions to these methods were made to accommodate recent scientific data the support the use of alternative approaches. The details for each assay and any substitutions that were made are described below.

6.2 Estrogen and Androgen Receptor Binding

The estrogen and androgen receptor binding assays were performed by Ricerca Biosciences, LLC. Taiwan, R.O.C. The USEPA test guideline uses a radiolabeled endpoint for these assays and it was deemed cost and time efficient to use Ricerca as they are equipped and approved for using radiolabeled reagents.

Table 1. EPA EDC Tier 1 test battery; from (USEPA, 2011).

Screening Assay	Test Guideline	Receptor Binding				Steroidogenesis		HPG Axis	HPT Axis
		E	Anti-E	A	Anti-A	E	A		
In vitro									
ER Binding (Rat uterine cytosol)	OCSPP 890.1250	■	■						
ERα Transcriptional Activation (Human cell line HeLa-9903)	OCSPP 890.1300 OECD 455	■							
AR Binding (Rat prostate cytosol)	OCSPP 890.1150			■	■				
Steroidogenesis (Human Cell Line H295R)	OCSPP 890.1550					■	■		
Aromatase (Human target tissue or cell-line microsomes)	OCSPP 890.1200					■			
In vivo									
Uterotrophic (Rat)	OCSPP 890.1600 OECD 440	■							
Hershberger (Rat)	OCSPP 890.1400 OECD 441			■	■		■[1]		
Pubertal Male (Rat)	OCSPP 890.1500			■	■		■	■	■
Pubertal Female (Rat)	OCSPP 890.1450	■	■			■		■	■
Fish Short-term Reproduction	OCSPP 890.1350 OECD 229	■	■	■	■	■	■	■	
Amphibian Metamorphosis (Frog)	OCSPP 890.1100 OECD 231								■

*Complementary endpoints across assays are indicated (solid black box) within each column.
[1] 5α-reductase inhibition only.

The Ricerca Biosciences screen uses recombinant receptors for the assays. The androgen receptor is of rat origin expressed in bacteria and the estrogen alpha and beta receptors are of human origin expressed in Sf9 cells (Traish et al., 1986; Chang and Liao, 1987; Obourn et al., 1993). The concentration range tested was 3 nM to 30 µM NTO. The experimental conditions for the ER and AR binding assays are provided in the Ricerca report; Appendix B.

6.3 Aromatase Assay

The CYP19/Methoxy-4-trifluoromethyl-coumarin (MFC) High throughput Inhibition Screening Kit (Cat# 459520; Lot # 2177659) was used to screen NTO for potential inhibition of CYP19 catalytic activity (GenTest, BDBiosciences, Woburn MA). The storage conditions and protocol provided with the kit were followed; see Appendix C. The kit included the following reagents: 7-methoxy-4-trifluormethyl coumarin (fluorescent substrate), glucose 6-phosphate dehydrogenase, cofactors, CYP19 enzyme, phosphate buffer- pH 7.4, positive control inhibitor (ketoconazole), and metabolite standard (7-hydroxy-4-trifluormethyl coumarin). Briefly, on the day of the assay, reagents were removed from storage (-80^{O}C) and thawed on ice. Twenty-five mLs of molecular grade H_20 (Cat# SH 30538LS; Hyclone/ThermoFisher, US) and 2 mLs phosphate buffer were warmed to 37^{O}C. The ketoconazole stock was reconstituted in 36 µL acetonitrile (Sigma-Aldrich, US). NADPH-Cofactor mix was prepared per the protocol and 144 µL per well (96-well plate; Costar- black wall) was dispensed. Test compound was added to primary test wells and serially diluted (1:3) across the 96-well plate. The process was repeated for the ketoconazole wells. The plate was then incubated for 10 min at 37^{O}C. During the incubation, the enzyme-substrate mix was prepared and added to the appropriate wells after the 10 minute incubation. The plate was returned to 37^{O}C for an additional 30 minute incubation. At the conclusion of the 30 minute incubation, stop solution (stop reagent plus

acetonitrile) was added. The metabolite standard was prepared and dispensed to the appropriate wells. The test plate was then read using a plate reader (Em 528; Ex 400; Synergy HT, Biotek; Winooski VT). The data were analyzed by calculating the percent decrease in signal of the test and control wells compared to the no inhibitor control wells.

6.4 Estrogen Receptor Transactivation Assay

The BG1Luc4E2 cell line was used for this assay (Rogers and Denison, 2000). The BG1Luc4E2 cell line is of human ovarian cancer origin and is stably transfected with a plasmid containing an estrogen response element pGudLuc7.0. The BG1Luc4E2 assay has been validated by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). The cell line can be used to evaluate both the ER alpha and ER beta ligand mediated responses. The BG1Luc4E2 was substituted for the HeLa Transactivation Assay. The BG1Luc4E2 cells were obtained from Dr. Mike Denison, University of California Davis under a material transfer agreement (UC Davis Control # 2012-21-0476); see Appendix D.

The NICEATM protocol was used as a guideline for the BG1Luc4E2 assay. Cells were cultured and maintained using standard tissue culture aseptic practices. Cells were maintained in complete medium (RPMI 1640 (-phenol red) Lot # 17105058, ThermoFisher, US; 0.9 percent Penicillin-streptomycin Cat# SV30010, ThermoFisher, US, 8 percent fetal bovine serum (FBS- Lot # ASA28574; Hyclone, Logan UT); 2 mM L-glutamine (Life Technologies, Carlsbad CA); 37^{O}C +/-1^{O}C, 90 percent +/- 5 percent humidity, and 5 percent +/- 1 percent CO_2/air.). Cells were subcultured when at ~80 percent confluence by decanting the medium, rinsing the adherent cells with 10 mLs of phosphate buffered saline (PBS) without Ca^{+2} or Mg^{+2} (Cat# SH30028LS, Hyclone/ThermoFisher, US), dissociating the cells from the flasks (T-25, T-75, and T-150 plug cap flasks, as appropriate; Corning, ThermoFisher US) with trypsin/EDTA (Cat # 154000-54; Life Technologies, Carlsbad CA) for 3-5 minutes, neutralizing the trypsin with fresh media, and dispensing the cells at a 1:4 ratio into new flasks. Cells were reselected on G418 (Cat# MT30234CR ThermoFisher, US) after receipt from UC Davis and after 5 subcultures. Prior to experimental treatment, the cells were conditioned into estrogen free media (EFM) by replacing the maintenance medium with EFM (Dulbecco's Modification of Eagle's Medium (DMEM) Cat# D1145, Sigma-Aldrich, US; 4.5 percent charcoal/dextran- treated FBS, Cat# SH30068.03, HyClone, Logan UT; 2mM L-Glutamine and 0.9 percent penicillin-streptomycin, Cat# SV30010, ThermoFisher, US) for 48 hrs prior to initiating the experiment.

For the transactivation experiments, BG1Luc4E2 cells were plated at a density of $2x10^4$ cells/well (volume 100 µL; EFM medium) in 96 well plates (Costar- white sided, ThermoFisher, US) and incubated overnight using the standard conditions described above. Dilution series of the positive controls estradiol (E2), 17-alpha estradiol (17-EE), diethylstilbestrol (DES), methoxyclor, and ethylparaben were prepared in dimethyl sulfoxide (DMSO) all from Sigma-Aldrich, US; NTO dilutions were prepared in DMSO; see Table 2 for concentrations. The negative control was vehicle only (0.05 percent DMSO final concentration).

Table 2. Concentration of test and control chemicals for BG1Luc4E2 assay.

Chemical	µg/mL stock	Final µg/mL (DMSO @ 0.05 percent in final)
Estradiol	0.000061, 0.00024, 0.00098, 0.0039, 0.0152, 0.0625, 0.24	$3x10^{-8}$, $1.2x10^{-7}$, $4.9x10^{-7}$,, $1.95x10^{-6}$, $7.6x10^{-6}$, $3.12x10^{-5}$, 0.00012
17-ethenyl estradiol 17-EE	0.0002, 0.002, 0.02, 0.2, 20	$1x10^{-7}$, $1x10^{-6}$, $1x10^{-5}$, 0.0001, 0.001, 0.01
DES	0.002, 0.006, 0.02, 0.06, 0.6, 2	$1x10^{-6}$, $3x10^{-6}$, $1x10^{-5}$, $3x10^{-5}$, 0.0003, 0.001
Ethyl Paraben	0.004, 0.04, 0.4, 2, 10, 50 mg/mL	0.002, 0.02, 0.2, 1, 5, 25
Methoxychlor	1.5, 6.25, 25 mg/mL	0.78, 3.125, 12.5
NTO	0.016, 0.08, 0.4, 10, 50 mg/mL	0.008, 0.04, 0.2, 1, 5, 25

Test and control chemicals were added to duplicate wells in a volume of 100 µL per well so that the final volume per well was 200 µL. The test plates were returned to the incubator for 24 hours. Before measuring the luminescence, cells were observed microscopically for signs of cytotoxicity. The Steady Glo® assay system (Cat# E2510, Promega, Madison WI) was used to develop the luminescence signal. To measure the luminescence, the media was aspirated from the wells and 100 µL RPMI-1640 was added to all test wells on the plate followed by 100 µL of reconstituted Steady-Glo reagent. Cells were incubated for 15 minutes in the dark and then luminescence was detected using plate reader (Synergy HT, Biotek, Winooski VT). Data were analyzed by subtracting the background signal (DMSO control) from the test signal and plotting the results graphically. Two criteria were used to determine a positive signal. First, the standard deviation of the DMSO control x 3 added to the DMSO signal was used as the minimum value for a positive signal. A dose-dependent response where at least two concentrations were above the minimal positive signal was used as the second criteria.

6.5 Steroidogenesis Assay H295R cell line

The H295R cell line (Cat# CRL-2128) was purchased from the American Type Culture Collection (ATCC) Manassas, VA. Cells were cultured according to the OPPTS 890.1550 Steroidogenesis (Human Cell Line H295R) protocol. Briefly, the cells were initiated from the ATCC stock and grown for 5 passages and then frozen in liquid nitrogen. Cells were then initiated from these frozen stocks and cultured for an additional 4 passages before being used for testing. The H295R media is (DMEM/H12 no phenol red Cat# 11039047 Life Technologies, Carlsbad CA; 2.5 percent Nu-Serum (Cat# 51000) + ITS Universal Culture Supplement (Cat# 40351) BD Biosciences, San Jose CA; and 0.9 percent Penicillin-Streptomycin) and the cells were maintained at $37^{O}C$ +/-$1^{O}C$, 90 percent +/- 5 percent humidity, and 5 percent +/- 1 percent CO_2/air. At the time of passage, cells were dissociated from the flasks using the same procedure as for the BG1Luc4E2 cells.

For the steroidogenesis experiment, cells were plated into 24 well plates at a density of $1.3x10^5$ cells/well in a volume of 1 mL per well and incubated for 24 hrs. The media was removed and replaced with fresh media supplemented with 30 µM 22-R hydroxycholesterol (Cat# H9384 Sigma Aldrich, St. Louis MO) which controls for low basal production of estradiol. Dilutions of the known inducer Forskolin (Cat# F3917, Sigma Aldrich, St. Louis MO and inhibitor Prochloraz (Cat# 45631, Sigma Aldrich, St. Louis MO) and NTO were made using DMSO; see Table 3. The negative control for the assay is vehicle only (0.1 percent DMSO). Ten microliters of each diluted stock was added to triplicate wells. An additional triplicate set of negative control wells were prepared for the viability

Table 3. Concentration of test and control chemicals used in the H295R assay.

Chemical	Stock Concentration mM	Final Concentration µM	µg/mL equivalent
Forskolin	1, 10	1, 10	
Prochloraz	0.1, 1	0.1, 1	
NTO	0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30	0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30	0.0013, 0.0039, 0.013, 0.039, 0.13, 0.39, 1.3, 3.9

assay. The treated plates were returned to the incubator for 48 hours. After the 48-hour incubation cells were observed microscopically for morphological indications of cytotoxicity. Then, 30 minutes prior to collecting supernatants for hormone analysis, the supernatant was first removed from the second set of negative control wells and 400 µL of 70 percent methanol (in PBS) was added. The 30 minute incubation in 70 percent methanol kills all the cells in the well and is used as a reference point in the viability assay. After the 30 minute incubation, the supernatant from each well was collected and stored at $-80^{O}C$ until analysis. After supernatant removal, the wells were washed 3 times with 400 µL PBS (Dulbecco's PBS with Ca^{+2} and Mg^{+2}- Cat# SH30264FS, ThermoFisher, Pittsburg PA) and then stained with the Live/Dead ® assay reagents (Cat# L3224, Life Technologies, Carlsbad CA) to measure viability and cell death. The protocol provided with the assay was followed. Briefly, after the 3 PBS washes to remove residual media constituents, 300 µL of PBS was added to each well followed by 300 µL the Live/Dead working solution (10 µL Ethidium homodimer-1 (EthD-1) and 6 µL

Calcein per 10 mL of PBS). Cells were incubated for a minimum of 1 hr and then fluorescence intensity (Ex/Em 494/517 -calcein and 528/617-EthD-1) was measured with the Synergy HT plate reader. The percentage of live cells was calculated from the calcein fluorescence readings and the percentage of dead cells was calculated from the EthD-1 fluorescence readings.

Testosterone levels were measured using a TOSOH Biosciences system (TOSOH Corp. Tokyo Japan) using ST AIA-PACK testosterone test cups (Cat # 025204; Lot 72) and following the manufacturers protocol. The assay is a competitive enzyme immunoassay (EIA) in which the test sample testosterone competes with a enzyme-labeled testosterone for a limited number of binding sites on an immobilized monoclonal antibody. After incubation with a fluorogenic substrate, the resulting intensity of fluorescence is inversely proportional to the amount of test sample testosterone. The amount of testosterone induced or inhibited by the test/control chemicals was calculated by subtracting the treated sample values from the basal (vehicle control) sample values.

6.6 Steroidogenesis Assay BLTK1 cell line

In collaboration with Dr. Tim Zacharewski, (Center for Integrative Toxicology, Michigan State University, East Lansing MI), NTO was tested for steroidogenic activity using a murine Leydig cell line. A 19 mg/mL stock solution of NTO dissolved in DMSO was provided to the collaborator. Using an approach similar to the H295R assay and described in Forgacs et al (Forgacs et al.) BLTK1 cells were exposed to 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 µM NTO. The positive control for this assay was recombinant human chorionic gonadotropin (rhCG) which induces testosterone production. Cytotoxicity of NTO was measured using an MTT assay. Testosterone was detected using a 96-well plate format EIA (Cat#582701 Cayman Chemical, Ann Arbor MI). Data were analyzed by comparing the vehicle control values to the test and positive control values. Test values that generated a 50 percent change compared to the controls were considered significant.

7 Results

7.1 Estrogen and Androgen Receptor Binding

The results of the estrogen and androgen receptor binding assay were negative for all NTO concentrations tested. The detailed data report is provided in Appendix B.

7.2 Aromatase Inhibition Assay

The results of the aromatase inhibition assay were negative for all NTO concentrations tested. The percent inhibition of the positive control inhibitor, ketoconazole and NTO are shown in Figure 1. There was no statistical difference between any of the NTO test values compared to the no inhibitor values (Mann-Whitney Rank Sum, P=0.7; SigmaStat 3.11). Based on the lack of difference between the no inhibitor control and the NTO values, all of the NTO values were averaged together for Figure 1. The data from duplicate experiments performed on separate days were averaged together. The error bars are +/- S.D.

7.3 Estrogen Receptor Transactivation Assay

The BG1Luc4E2 estrogen receptor was not activated by NTO. In contrast, the known estrogen receptor ligands (estradiol, DES, ethylparaben and methoxychlor) did activate the receptor yielding an increased production of the luciferase reporter. The results are presented in Figure 2. The estrogenic controls responded as expected and met the criteria described in the NICEATM protocol. The relative luminescence units (RLU) considered significantly different from baseline is 26.9 x1000 RLU, see Figure 2. The highest concentration of NTO tested, 25 µg/mL or 192 µM, was slightly above this level at 36.2 x1000 RLU.

Figure 1. NTO does not inhibit aromatase activity. Error bars = S.D.

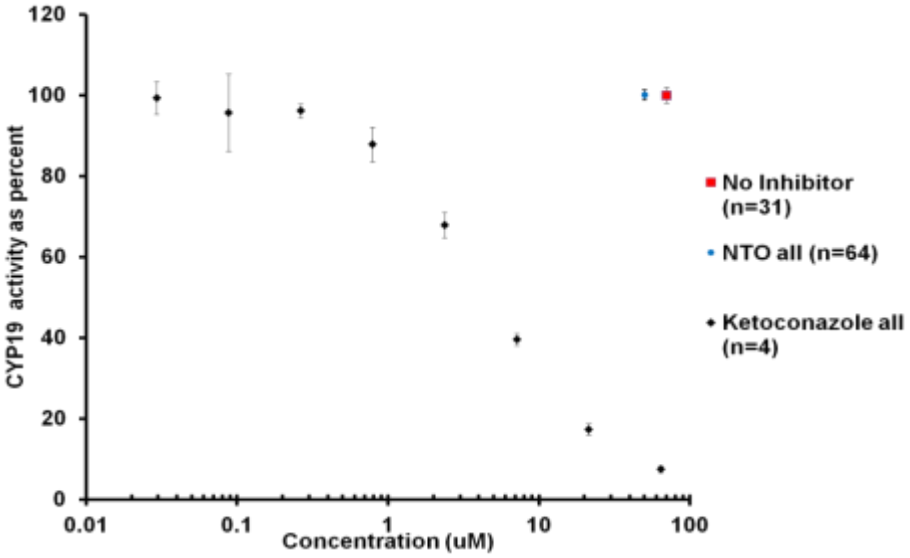
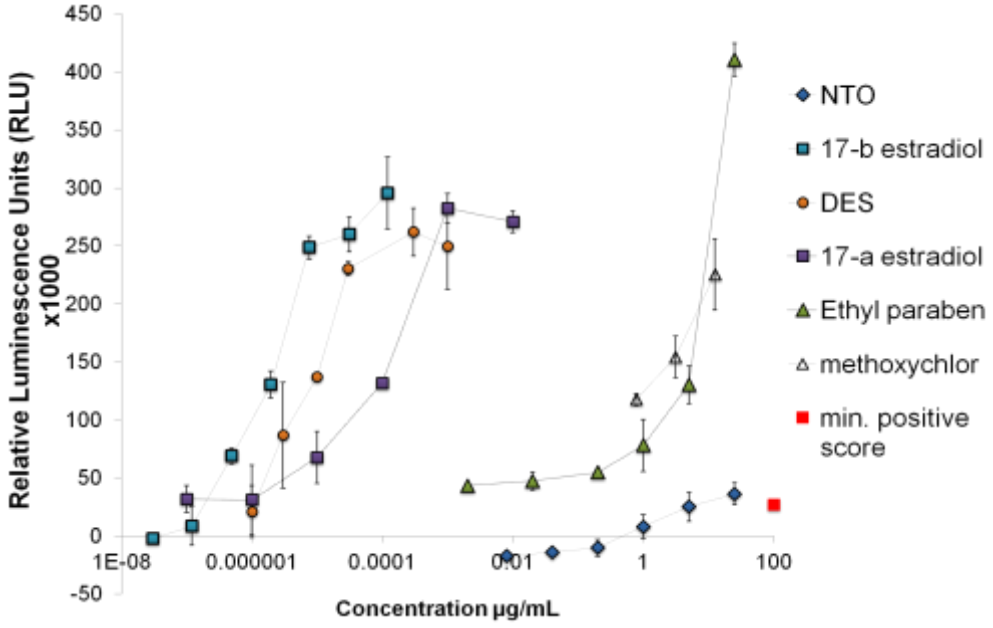


Figure 2. Estrogenic compounds, but not NTO, induce the expression of the BG1Luc4E2 reporter system. Error bars = S.D.



However, the next lowest NTO concentration, 5 µg/mL or 38 µM, did not exceed this minimum positive level. Additionally, both methoxychlor and ethylparaben which are considered weak positive controls exhibit a 10-fold greater response compared to NTO. Based on these observations, NTO is not considered an estrogen receptor ligand in this assay.

7.4 Steroidogenesis Assay H295R cell line

The steroidogenesis assay using the H295R cell line evaluated both cytotoxicity and steroidogenic potential of NTO. Cytotoxicity was measured by comparing fluorescent endpoints for viable and

nonviable cells. At all concentrations tested, NTO was not found to significantly affect the viability of H295R cells; see Figure 3. The ability for NTO to inhibit or induce testosterone was assessed by measuring the level of testosterone produced from H295R cells exposed to NTO. When compared to the level of testosterone produced in cells exposed to either a known inducer (forskolin) or known inhibitor (prochloraz), NTO responses were not different from the vehicle control testosterone levels; see Figure 4.

Figure 3. The viability of H295R cells is not affected by NTO. The 80 percent cut-off for minimum indicator of cytotoxicity is indicated with a dashed line. Error bars = S.D.

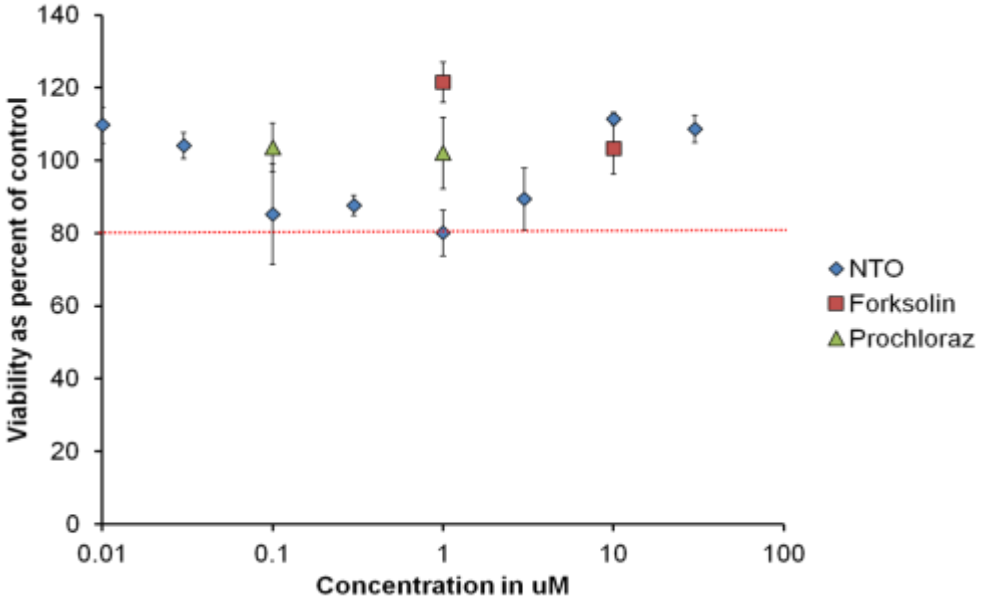
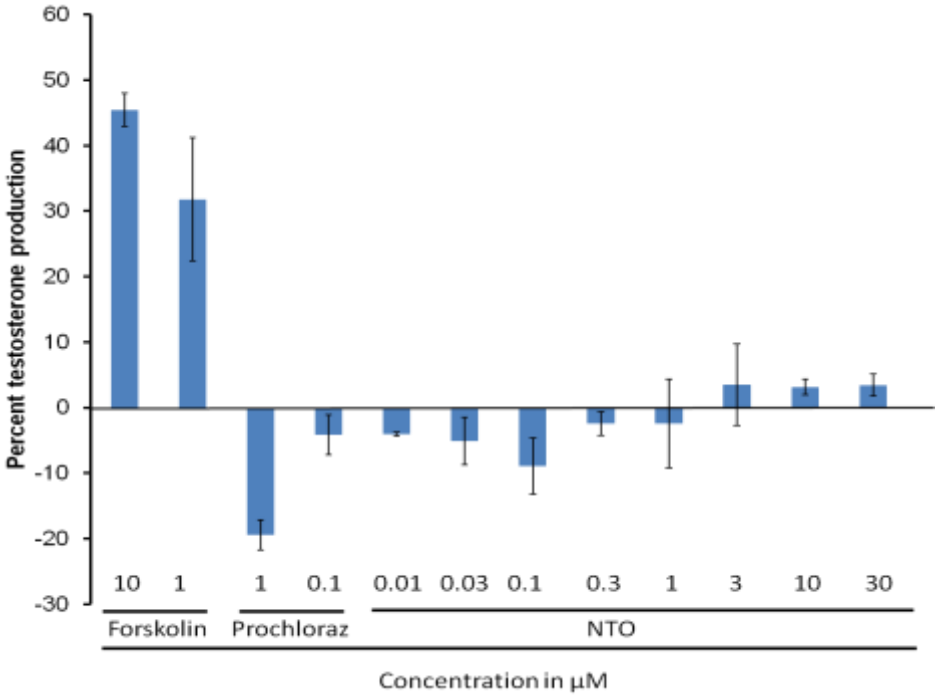


Figure 4. NTO does not significantly induce or inhibit testosterone in H295R cells compared to 10 µM forskolin and 1 µM prochloraz. Error bars = S.D.



7.5 Steroidogenesis Assay BLTK1 cell line

The BLTK1 cell line assay evaluated both the cytotoxicity and steroidogenic potential of NTO. Cytotoxicity was measured using the MTT assay and NTO was found to not be cytotoxic to BLTK1 cells, either in the presence or absence of rhCG; see Figure 5. The induction and inhibition of testosterone production was measured by treating cells with NTO in the presence or absence of rhCG. In the absence of rhCG, NTO did not stimulate the production of testosterone. In the presence of rhCG, NTO did not reduce the production of testosterone; see Figure 6. Combined, the results indicate that NTO does not impact the steroidogenesis pathway in BLTK1 cells.

Figure 5. NTO is not cytotoxic to BLTK1 cells. BLTK1 cells were treated with 0.03-100 µM NTO (left panel), or 0.03-100 µM NTO co-treated with 3 ng/ml rhCG (right panel) for 24 hrs. Cytotoxicity was evaluated by MTT assay revealing that NTO does not have a significant effect on cell viability; figure and analysis provided by Dr. Tim Zacharewski and Agnes Forgacs. Error bars = S.D.

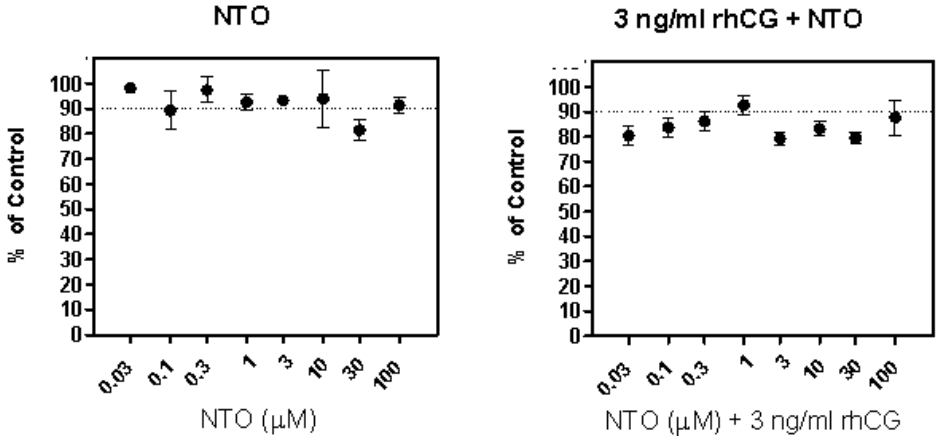
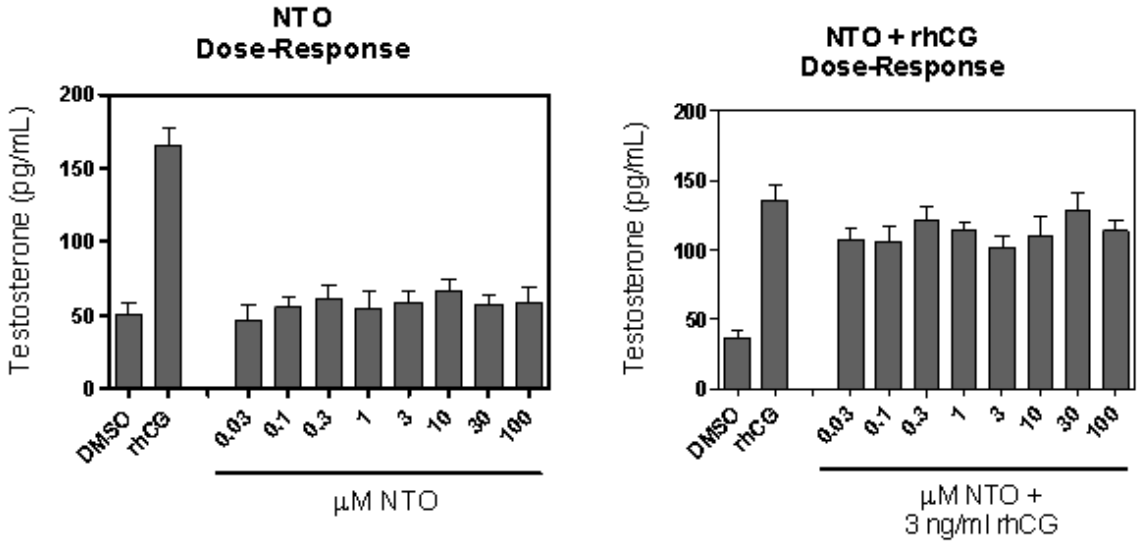


Figure 6. NTO does not induce or inhibit testosterone production in BLTK1 cells. BLTK1 cells were treated with 0.03-100 µM NTO (left panel), or 0.03-100 µM NTO co-treated with 3 ng/ml rhCG (right panel) for 24 hrs. Testosterone levels were determined by EIA revealing that NTO does not significantly alter basal or rhCG-induction of testosterone levels. Figure and analysis provided by Dr. Tim Zacharewski and Agnes Forgacs. Error bars = S.D.



8 Discussion

8.1 General

The tiered approach to screening a chemical of concern permits increased focus on specific toxic responses. The testicular toxicity of NTO observed in subacute and subchronic oral toxicity rat studies warranted further exploration into the mode of action for the observed findings. One mode of testicular toxicity is through endocrine signaling, specifically via inhibition of testosterone production or function. The *in vitro* assays used here screened NTO for interaction with the predominant gonadal hormones: estrogen and androgen.

The results from the estrogen receptor binding, estrogen receptor activation, androgen receptor binding, aromatase inhibition, and steroidogenesis were consistently negative. No interactions between NTO and these assays were identified; see Table 4.

8.2 Areas of Uncertainty

The *in vitro* assays used to screen for EDC activity gave consistent and robust results. However, the *in vitro* assays are not able to capture toxic effects as a result of metabolism of the parent compound. Additionally, the *in vivo* steroidogenic pathway is quite complex and each *in vitro* endpoint tested offers only a snapshot of likely interactions at the molecular level. The use of both the H295R and BLTK1 cell lines for the steroidogenesis assay improves the strength of the test as there are subtle differences between these two cell lines. Indeed, the BLTK1 cell line may be a better testes model as the cell of origin is a Leydig cell, the steroidogenic cell population in the testes (Forgacs et al.). The *in vitro* assays do not evaluate endpoints within the larger Hypothalmo-Pituitary-Gonadal (HPG) axis such as follicular stimulating hormone (FSH) or luteinizing hormone (LH).

Table 4. Summary of findings for NTO *in vitro* EDC test battery screen.

Assay	NTO concentration	Results
Estrogen Receptor Binding ER α (recombinant-human) ER β (recombinant-human)	0.003-30 uM 0.0004-3.9 ug/mL	Negative- no significant interaction
Androgen Receptor Binding (recombinant-rat)	0.003-30 uM 0.0004-3.9 ug/mL	Negative- no significant interaction
Estrogen receptor α/β transcriptional assay (BG1 cells ovarian-human)	0.02-200 uM 0.0025-25 ug/mL	Negative- no significant interaction
Steroidogenesis Two cell lines- H295R (adrenal-human) BLTK1 (Leydig-mouse)	0.03-100 uM 0.004-13 ug/mL	Negative- no significant interaction (Testosterone) Follow up with estradiol only if testosterone affected
Aromatase inhibition (cell line microsomes)	0.0001-100 uM $1.3x10^{-5}$-13 ug/mL	Negative- no significant inhibition

9 Recommendations

The *in vitro* portions are complete for the EDC tier 1 evaluation of NTO. If testicular toxicity of NTO is a priority concern, the *in vitro* assays could be used to screen metabolites of NTO. Thus far, urazole has been identified as metabolite in isolated rat hepatocytes (Le Campion et al., 1997; Le Campion et al., 1998). Urazole is commercially available and it is recommended that it be screened with the EDC *in vitro* assays.

To ascertain the affected cell populations *in vivo*, short duration timed NTO exposures where testes are harvested at 24 hr intervals are recommended. Histopathology of these tissues would characterize the order of cells impacted by NTO and provide a likely mode of action for testicular toxicity of NTO.

10 Point of Contact

The Point of Contact for this report is Dr. Valerie H. Adams. She may be reached at 410-436-3980 or DSN 584-3980; e-mail:usaphctoxinfo@amedd.army.mil.

APPENDIX A

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APPENDIX B

RICERCA REPORT FOR TESTOSTERONE AND ESTROGEN RECEPTOR BINDING

Individual Tests Data Report

US Army Center for Health Promotion & Preventative Medicine

Study Completed: July 26, 2011

Report Printed: July 26, 2011

Ricerca PT#: 1151889

Alt. Code 1: NTO

Alt. Code 2:

Alt. Code 3:

Sample(s): UAL-6

M.W.: 130.07

Objectives:

To evaluate, in Radioligand Binding assays, the activity of test compound NTO (PT# 1151889).

ricerca

Taking you from IP to IND

Ricerca Biosciences, LLC • Tel: 425-487-8217 • Fax: 425-487-8211 • e-mail: pharmacology@ricerca.com http://www.ricerca.com

Ricerca Biosciences, LLC Pharmacology Data Report

On Compound UAL-6, NTO For US Army Center for Health Promotion & Preventative Medicine

Work Order Number:	1-1043298-0	**Services Being Reported:**	Individual Tests
Study Number:	AB05533	**Alternative Work Order No:**	
Quote No:	25881-1	**Purchase Order Number:**	
Compound Information:		**Total # of Assays:**	3
Compound Code:	UAL-6		
Alternative Code 1:	NTO		
Alternative Code 2:			
Alternative Code 3:			
Ricerca Internal #:	1151889		
Molecular Weight:	130.07		

Sponsor:
US Army Center for Health Promotion & Preventative Medicine
5158 Blackhawk Rd ATTN MCHB-TS-THE
Health Effects Research Program, Directorate of Toxicology
Bldg E2100, 1st Floor, Room 1401
Aberdeen Proving Ground, MD 21010-5403
United States

Undertaken at:
Ricerca Biosciences, LLC
Pharmacology Laboratories
158 Li-Teh Road, Peitou
Taipei, Taiwan 112
Taiwan R.O.C.

Date of Study: July 19, 2011 - July 26, 2011

Study Directors: Kun-Yuan Lin, Ricerca Biosciences, LLC
Kuo-Hsin Chen, Ricerca Biosciences, LLC

Distribution: US Army Center for Health Promotion & Preventative Medicine

"This study was conducted according to the procedures described in this report. All data presented are authentic, accurate and correct to the best of our knowledge."

Kun-Yuan Lin
Study Director for Animal Assays

Kuo-Hsin Chen
Study Director for Biochemical Assays

Peter Chiu, Ph.D
Technical Director

TABLE OF CONTENTS

SUMMARY

STUDY OBJECTIVE

To evaluate, in Radioligand Binding assays, the activity of compound NTO (UAL-6, PT# 1151889).

METHODS

Methods employed in this study have been adapted from the scientific literature to maximize reliability and reproducibility. Reference standards were run as an integral part of each assay to ensure the validity of the results obtained. Assays were performed under conditions described in the accompanying "Methods" section of this report. The literature reference(s) for each assay are in the "Literature References" section. If either of these sections were not originally requested with the accompanying report, please contact us at the number below for a printout of either of these report sections.

Where presented, IC_{50} values were determined by a non-linear, least squares regression analysis using MathIQ™ (ID Business Solutions Ltd., UK). Where inhibition constants (K_i) are presented, the K_i values were calculated using the equation of Cheng and Prusoff (Cheng, Y., Prusoff, W.H., Biochem. Pharmacol. 22:3099-3108, 1973) using the observed IC_{50} of the tested compound, the concentration of radioligand employed in the assay, and the historical values for the K_D of the ligand (obtained experimentally at **Ricerca Biosciences, LLC**). Where presented, the Hill coefficient (n_H), defining the slope of the competitive binding curve, was calculated using MathIQ™. Hill coefficients significantly different than 1.0, may suggest that the binding displacement does not follow the laws of mass action with a single binding site. Where IC_{50}, K_i, and/or n_H data are presented without Standard Error of the Mean (SEM), data are insufficient to be quantitative, and the values presented (K_i, IC_{50}, n_H) should be interpreted with caution.

RESULTS

A summary of results meeting the significance criteria is presented in the following sections. Complete results are presented under the section labeled "Experimental Results". Individual responses, if requested, are presented in the appendix to this report.

SUMMARY/CONCLUSION

Significant results are displayed in the following table(s) in rank order of potency for estimated IC_{50} and/or K_i values.

SUMMARY OF SIGNIFICANT PRIMARY RESULTS

Biochemical assay results are presented as the percent inhibition of specific binding or activity throughout the report. All other results are expressed in terms of that assay's quantitation method (see Methods section).

- *For primary assays, only the lowest concentration with a significant response judged by the assays' criteria, is shown in this summary.*
- *Where applicable, either the secondary assay results with the lowest dose/concentration meeting the significance criteria or, if inactive, the highest dose/concentration that did not meet the significance criteria is shown.*
- *Unless otherwise requested, primary screening in duplicate with quantitative data (e.g., IC50 ± SEM, Ki ± SEM and nH) are shown where applicable for individual requested assays. In screening packages, primary screening in duplicate with semi-quantitative data (e.g., estimated IC50, Ki and nH) are shown where applicable (concentration range of 4 log units); available secondary functional assays are carried out (30 μM) and MEC or MIC determined only if active in primary assays >50% at 1 log unit below initial test concentration.*
- *Please see Experimental Results section for details of all responses.*

Significant responses (≥ 50% inhibition or stimulation for Biochemical assays) were noted in the primary assays listed below:

PRIMARY TESTS

No significant responses noted.

PT#: 1151889
CODE: UAL-6, NTO

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EXPERIMENTAL RESULTS - BIOCHEMICAL ASSAYS

Cat. #	TARGET	BATCH*	SPP.	n=	CONC.	†% INHIBITION	IC_{50}	K_i	n_H	R
						% -100 -50 0 50 100				
285010	Androgen (Testosterone) AR	298542	rat	2	30 µM	4	>30 µM			
				2	3 µM	6				
				2	0.3 µM	3				
				2	0.03 µM	-2				
				2	3 nM	0				
226010	Estrogen ERα	298540	hum	2	30 µM	0	>30 µM			
				2	3 µM	-2				
				2	0.3 µM	10				
				2	0.03 µM	4				
				2	3 nM	6				
226050	Estrogen ERβ	298541	hum	2	30 µM	-1	>30 µM			
				2	3 µM	0				
				2	0.3 µM	4				
				2	0.03 µM	6				
				2	3 nM	5				

* Batch: Represents compounds tested concurrently in the same assay(s). ‡ Partially soluble in *in vitro* test solvent.
♦ Denotes item meeting criteria for significance
† Results with ≥ 50% stimulation or inhibition are highlighted.
R=Additional Comments
hum=human

METHODS - RADIOLIGAND BINDING ASSAYS

■ 285010 Androgen (Testosterone) AR

Source:	Rat recombinant E. coli
Ligand:	1.5 nM [^{3}H] Mibolerone
Vehicle:	1% DMSO
Incubation Time/Temp:	4 hours @ 4°C
Incubation Buffer:	50 mM Tris-HCl, pH 7.4, 0.8 M NaCl, 10% Glycerol, 2 mM Dithiothreitol, 0.1% BSA, 2% EtOH
Non-Specific Ligand:	10 µM Mibolerone
K_D:	3 nM *
B_{MAX}:	930 pmole/mg Protein *
Specific Binding:	90% *
Quantitation Method:	Radioligand Binding
Significance Criteria:	≥ 50% of max stimulation or inhibition

■ 226010 Estrogen ERα

Source:	Human recombinant insect Sf9 cells
Ligand:	0.5 nM [^{3}H] Estradiol
Vehicle:	1% DMSO
Incubation Time/Temp:	2 hours @ 25°C
Incubation Buffer:	10 mM Tris-HCl, pH 7.4, 0.1% BSA, 10% Glycerol, 1 mM DTT
Non-Specific Ligand:	1 µM Diethylstilbestrol
K_D:	0.2 nM *
B_{MAX}:	1400 pmole/mg Protein *
Specific Binding:	85% *
Quantitation Method:	Radioligand Binding
Significance Criteria:	≥ 50% of max stimulation or inhibition

■ 226050 Estrogen ERβ

Source:	Human recombinant insect Sf9 cells
Ligand:	0.5 nM [^{3}H] Estradiol
Vehicle:	1% DMSO
Incubation Time/Temp:	2 hours @ 25°C
Incubation Buffer:	10 mM Tris-HCl, pH 7.4, 10% Glycerol, 1 mM DTT, 1 mg/ml BSA
Non-Specific Ligand:	1 µM Diethylstilbestrol
K_D:	0.13 nM *
B_{MAX}:	3000 pmole/mg Protein *
Specific Binding:	90% *
Quantitation Method:	Radioligand Binding
Significance Criteria:	≥ 50% of max stimulation or inhibition

* Historical Values

PT #: 1151889
CODE: UAL-6, NTO

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REFERENCE COMPOUND DATA - BIOCHEMICAL ASSAYS

CAT. #	ASSAY NAME	REFERENCE COMPOUND	HISTORICAL IC_{50}	HISTORICAL K_i	n_H	CONCURRENT BATCH *	CONCURRENT IC_{50}
285010	Androgen (Testosterone) AR	Testosterone	6.5 nM	4.3 nM	1	298542	4.11 nM
226010	Estrogen ERα	Diethylstilbestrol	0.77 nM	0.22 nM	1	298540	1.02 nM
226050	Estrogen ERβ	Diethylstilbestrol	0.61 nM	0.13 nM	1.2	298541	1.22 nM

* Batch: Represents compounds tested concurrently in the same assay(s). ‡ Partially soluble in *in vitro* test solvent.

LITERATURE REFERENCES

CAT. #	REFERENCE
226010	**Obourn JD, Koszewski NJ and Notides AC (1993)** Hormone- and DNA-binding mechanisms of the recombinant human estrogen receptor. Biochemistry 32(24): 6229 - 6236.
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APPENDIX C

GENTEST CYP19/MFC PROTOCOL

BD Biosciences – Discovery Labware
BD Gentest™ Products and Services
6 Henshaw Street
Woburn, MA 01801
Tel: 781.935.5115
Fax: 781.938.8644
bdbiosciences.com

Info_gentest@bd.com

CYP19/MFC High Throughput Inhibitor Screening Kit
Data Sheet

New Catalog Number.....459520 **Lot Number**.....32930
Date Released: 2012 January

Storage Conditions .....**STORE AT -80°C** (Individual components can be stored as instructed below.)
QC Assay for HTS Kit: IC_{50} Determination for Ketoconazole...3.43 uM (*see note below)

Kit Components

1. **CYP19 + P450 Reductase** Catalog Number: 459760
   Storage Condition: Store at -80°C Lot Number: 19703
   Package Contents: 1.5 nmol P450 in 1.5 mL — 150 λ per plate
   Buffer Composition: 100 mM potassium phosphate (pH 7.4)
   Protein Content: 3.9 mg/mL
   Aromatase Activity: 6.4 pmol product/(min x pmol P450)
2. **Control Insect Cell Membrane Protein** Catalog Number: 04-80701
   Volume: 1.0 mL Lot Number: 15404
   Storage Condition: Store at -80°C
   Protein Content: 15 mg/mL in 100 mM Potassium Phosphate (pH 7.4)
3. **Buffer Solution: 0.5 M Potassium Phosphate (pH 7.4),** filter sterilized.
   Volume: 18 mL
   Storage Condition: Buffer solution can be stored at room temperature or 4°C.
4. **Stop Solution: 0.5 M Tris Base,** filter sterilized.
   Volume: 18 mL
   Reconstitution: Add 72 mL of acetonitrile prior to use.
   Storage Condition: Store at 4°C or room temperature prior to addition of acetonitrile.
   Stop solution should be stored at room temperature after addition of acetonitrile.
5. **Cofactors:** 1.3 mM NADP+, 66 mM $MgCl_2$, and 66 mM Glucose 6-Phosphate.
   Volume: 1.7 mL
   Storage Condition: Store at -20°C
6. **Glucose 6-Phosphate Dehydrogenase:** 40 Units/mL in 5 mM Sodium Citrate Buffer (pH 7.5).
   Volume: 1.3 mL
   Storage Condition: Store at -20°C
7. **MFC (7-Methoxy-4-trifluoromethylcoumarin):** Fluorescence substrate, 1.21 mg. 20 λ per plate
   → Reconstitution: Add 200 ul of acetonitrile prior to use. Concentration = 25 mM
   Storage Condition: Store at -20°C before and after reconstitution.
8. **KTZ (Ketoconazole):** CYP19 selective positive control inhibitor, 0.032 mg
   → Reconstitution: Add 30 ul of acetonitrile prior to use. Concentration = 2mM = 1.67 mM – 12 λ per plate
   Storage Condition: Store at -20°C before and after reconstitution. Resuspend in 316 λ instead of 30 so that 2 full runs can be performed
9. **HFC (7-Hydroxytrifluoromethylcoumarin):** Metabolite standard (0.25 mM in 0.1 M Tris-pH 9.0) — 24 λ per plate
   Volume: 0.04 mL
   Storage Condition: Store at -80°C

Note

- Freeze thaw stability: This CYP19 inhibitor screening kit was subjected to 6 freeze thaw cycles without a change in IC_{50} value for ketoconazole or a significant loss of signal to noise.
- *QC assay (IC_{50} determination for ketoconazole) was performed using all the components contained in the current lot kit.

THIS PRODUCT IS SUPPLIED FOR LABORATORY RESEARCH USE ONLY.

Patent Pending

A BD Biosciences Company
GENTEST

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CYP19/MFC High Throughput Inhibitor Screening Kit

Instruction Manual

This CYP19 high throughput inhibition assay kit is designed to rapidly screen for potential inhibitors of CYP19 catalytic activity. The instructions are written to perform the assay with 8, 96-well plates at one time. Using these instructions one can measure 30 IC_{50} values, in duplicate (30 test compounds), 1 positive control and 1 standard curve. Volumes can be scaled to perform fewer than 8 plates at a time; however, the total number of plates that can be performed will depend on the dead volume of your liquid handling system. For single plate IC_{50} determinations refer to the *Supplemental Instructions* (Number 1) included with the kit.

The use of this kit assumes the test compounds are dissolved in acetonitrile. Alternative solvents such as methanol may be used, following the instructions below. Ethanol has been shown to inhibit CYP19 activity at concentrations of 1.0% and above. A set of instructions for using the kit with ethanol as an alternative solvent is included in the *Supplemental Instructions* (Number 2). DMSO has been shown to inhibit CYP19 activity at very low concentration (0.16%), and should not be used. Additional information on the inhibition of CYP19 by organic solvents can be obtained on BD-Gentest website.

Kit Components/Storage Conditions

Name	Description	Quantity	Storage Temp.
Buffer	Phosphate Buffer (0.5 M, pH 7.4)	18 mL	4°C or RT
G6PDH	Glucose 6-Phosphate Dehydrogenase Solution	1.3 mL	-20°C
Cofactors	Cofactors	1.7 mL	-20°C
Control Protein	Control Protein (15 mg/mL)	1.0 mL	-80°C
HTS-760	CYP19 (1 uM P450 content)	1.5 mL	-80°C
MFC	(7-Methoxy-4-trifluoromethyl coumarin (fluorescent substrate)	1.21 mg	-20°C
KTZ	Ketoconazole (positive control inhibitor)	0.032 mg	-20°C
HFC	(7-Hydroxy-4-trifluoromethyl coumarin) (0.25 mM)	0.04 mL	-80°C
Stop Reagent	Tris Base (0.5 M)	18 mL	4°C or RT

Disposables Not Supplied in Kit

1. 96-well black microtiter plates (We recommend BD Falcon™ Assay Plates (Catalog No. 353241) and lids (Catalog No. 353958)).
2. Mixing trough for use with multichannel pipette (approximate dead volume-2mL)
3. Secondary mixing containers (e.g. 100 and 200 mL beakers)
4. Lids for 96-well microtiter plates
5. Deionized water
6. Acetonitrile

Other Equipment

1. Multichannel pipetter
2. Incubator (37°C)
3. Fluorescence plate scanner

Getting Started

1. Add 72 mL of acetonitrile to the Stop Reagent. Store at room temperature.
2. Reconstitute the MFC in 200 uL of acetonitrile (25 mM final concentration). Store at –20°C.
3. Reconstitute the KTZ in 30 uL of acetonitrile (2 mM final concentration). Store at –20°C.
4. Dissolve test compound at desired concentration in acetonitrile. The test compound should be prepared at 50 times the highest final concentration desired in the IC_{50} assay.

Performing the IC_{50} Assay

8 Plate Procedure

- Eight 96-well plates can accommodate IC_{50} determinations for 30 test compounds, one positive control inhibitor (KTZ), and a standard curve.
- Plates 1-7: 4 test compounds per plate in duplicate (2 rows per compound).
- Plate 8: 2 test compounds in duplicate; 1 positive control in duplicate; 1 standard curve in duplicate.

Section I. Serial Dilution of Test Compounds and Positive Control (refer to "sample 96-well plate" on page 2 of the instructions)

1. Pre-warm approximately 200 mL of deionized water to 37°C.
2. Thaw all kit components, and place on ice. Warm the container of Buffer to 37°C.
3. In a suitable container, prepare the NADPH-Cofactor Mix; add 1.13 mL of Cofactors, 0.9 mL of G6PDH, and 0.6 mL of Control Protein (standardization of final protein concentration) to 87.4 mL of 37°C water. Mix well. If control protein is not desired, substitute 0.6 mL of water.
4. For each row of test compound and KTZ (positive control reagent), add 144 uL of NADPH-Cofactor Mix to each well of Column 1.
5. In a suitable container, add 3.2 mL of acetonitrile to 77 mL of NADPH-Cofactor Mix (Cofactor/acetonitrile mix). Mix well.
6. For the remaining Columns 2 through 12, add 100 uL of Cofactor/acetonitrile mix to each well.
7. Add 6 uL of test compound to Column 1 of each row of test compound IC_{50}. Mix by pipetting 3 to 5 times in each well.
8. Add 6 uL of 2 mM KTZ to Column 1 of each of the two rows of positive control IC_{50}. Mix as in step 7.
9. For each row, serial dilute 50 uL from Column 1 through Column 8. Mix as in step 7. Changing of tips during the serial dilution is recommended. Discard the extra 50 uL from Column 8.
10. Cover the plate and pre-incubate at 37°C for 10 minutes.

Section II. Preparation of Enzyme Substrate Mix/Reaction Initiation and Termination

1. Prepare the Enzyme/Substrate Mix; to the container of pre-warmed Buffer, add 70.5 mL of 37°C water, 1.35 ml of HTS-760, and 180 uL of 25 mM MFC. Mix well.
2. After the 10 minute pre-incubation of the plate (Part I, Step 10), remove the plate from the incubator, and add 100 uL of Enzyme/Substrate Mix to Columns 1 through 10 of all rows of test compounds and positive control. Dispense the liquid in a stream, not dropwise. Mixing of the components in the wells is dependent upon mixing during dispensing.
3. Cover the plate and incubate at 37°C for 30 minutes.
4. After 30 minutes, remove the plate from the incubator, and add 75 uL of Stop Reagent to all wells 1 through 12 of each row of IC_{50}. Dispense the liquid in a stream, not dropwise.
5. Add 100 uL of Enzyme/Substrate Mix to Columns 11 and 12 of each row of IC_{50}. These wells are blanks.

Section III. Preparing the Standard Curve

1. Thaw the 0.25 mM HFC standard solution.
2. Add 138 uL of NADPH-Cofactor Mix (without acetonitrile) to well 1 of each of the two rows.
3. Add 12 uL of HFC standard to well 1 of each of the two rows. Mix by pipetting 3 to 5 times in each well.
4. Add 100 uL of NADPH-Cofactor Mix (without acetonitrile) to wells 2 through 12.
5. Serially dilute 50 uL from well 1 through well 8. Mix as in Step 3. Discard 50 uL from well 8.
6. Add 75 uL of Stop Reagent to all wells 1 through 12. Mix as in step 3.
7. Add 100 uL of Enzyme/Substrate Mix to all wells 1 through 12. Mix as in step 3.

Note: Using this procedure, well 1 will contain 2000 pmol of standard. Remaining wells 2 through 8 are one-third serial dilutions (i.e. 666.6, 222.2, 74, 24.7, 8.22, 2.74 and 0.91 pmol). Wells 9 through 12 are blanks.

Section IV. Reading the Results

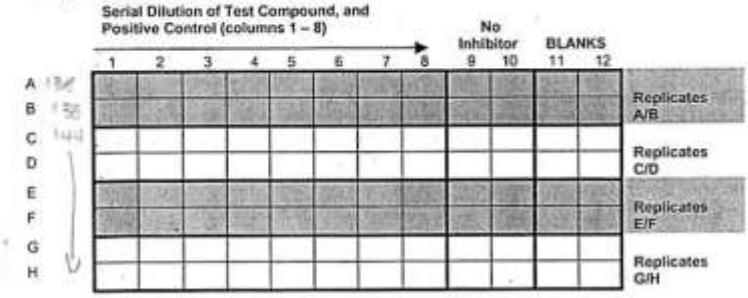
1. Read all plates at an excitation and emission wavelength suitable to detect the HFC metabolite (e.g. 409 nm excitation and 530 nm emission).

Section V. Analysis of Results/IC_{50} Calculation

1. Blank values should be subtracted from the sample wells to obtain the net fluorescence signal.
2. The number of pmol product formed per well can be calculated by comparison to the standard curve. Alternatively, the same IC_{50} value can be determined from the net fluorescent signal directly, without the use of a standard curve.
3. Calculate percent inhibition of each inhibitor concentration relative to the wells without inhibitor (average of wells 9 and 10).
4. Determine the concentrations of test compound that bracket 50% inhibition (High Conc. and Low Conc.). The bracketing concentrations and corresponding percent inhibition are used to calculate the IC_{50} via linear interpolation as described below:

$$IC_{50} = \frac{(50\% - \text{Low \%Inhibition})}{(\text{High \%Inhibition} - \text{Low \%Inhibition})} \times (\text{High Con.} - \text{Low Con.}) + \text{Low Con.}$$

Sample 96-Well Plate



Note: The Gentest Web Site (**www.gentest.com**) has many useful tips on performing the high throughput fluorescent assay (e.g. solvent effects, fluorometer evaluation, FAQ, posters and other literature on fluorescent assays).

A BD Biosciences Company
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BD

CYP19/MFC High Throughput Inhibitor Screening Kit
Supplemental Instructions

Number 1: Performing the IC_{50} assay using a single 96-well plate

One 96-well plate can accommodate IC_{50} determinations for 2 test compounds, in duplicate, one positive control, and one standard curve. The following instructions are written assuming test compounds and ketoconazole (positive control) are dissolved in acetonitrile or methanol. The instructions are essentially identical to the 8-plate method with volumes scaled down to accommodate one 96-well plate. When following the "single plate instructions" the user is referred to the "8-plate instruction manual" for several procedures. It is strongly advised that the 8-plate procedure be thoroughly reviewed before using the single plate method.

Performing the IC_{50} Assay

Section I. Serial Dilution of Test Compounds and Positive Control (refer to "sample 96-well plate" on page 2 of 10 plate instructions)

1. Pre-warm approximately 25 mL of deionized water to 37°C.
2. Thaw all kit components, and place on ice. Warm the required amount of Buffer (2 mL-see Section II) to 37°C.
3. In a suitable container, prepare the NADPH-Cofactor Mix; add 187.5 uL of Cofactors, 150 uL of G6PDH, and 100 uL of Control Protein to 14.56 mL of 37°C water. Mix well. If control protein is not desired, substitute 100 uL of water.
4. For each row of test compound and KTZ (positive control reagent), add 144 uL of NADPH-Cofactor Mix to each well of Column 1.
5. In a suitable container, add 400 uL of acetonitrile to 9.6 mL of NADPH-Cofactor Mix (Cofactor/acetonitrile mix). Mix well.
6. For the remaining Columns 2 through 12, add 100 uL of Cofactor/acetonitrile mix to each well.
7. Add 6 uL of test compound to Column 1 of each row of test compound IC_{50}. Mix by pipetting 3 to 5 times in each well.
8. Add 6 uL of 2 mM KTZ to Column 1 of each of the two rows of positive control IC_{50}. Mix as in step 7.
9. For each row, serial dilute 50 uL from Column 1 through Column 8. Mix as in step 7. Changing of tips during the serial dilution is recommended. Discard the extra 50 uL from Column 8.
10. Cover the plate and pre-incubate at 37°C for 10 minutes.

144λ –

Section II. Preparation of Enzyme Substrate Mix/Reaction Initiation and Termination

1. In a suitable container, prepare the Enzyme/Substrate Mix; add 7.83 mL of 37°C water, 150 uL of HTS-760, and 20 uL of 25 mM MFC to 2 mL of pre-warmed Buffer. Mix well.
2. After the 10 minute pre-incubation of the plate (Part I, Step 10), remove the plate from the incubator, and add 100 uL of Enzyme/Substrate Mix to Columns 1 through 10 of all rows of test compounds and positive control. Dispense the liquid in a stream, not dropwise. Mixing of the components in the wells is dependent upon mixing during dispensing.
3. Cover the plate and incubate at 37°C for 30 minutes.
4. After 30 minutes, remove the plate from the incubator, and add 75 uL of Stop Reagent to all wells 1 through 12 of each row of IC_{50}. Dispense the liquid in a stream, not dropwise.
5. Add 100 uL of Enzyme/Substrate Mix to Columns 11 and 12 of each row of IC_{50}. These wells are blanks.

For instructions on the following procedures the user should refer to the Instruction Manual (for 8 plates).

A. Preparing the Standard Curve(Section III)
B. Reading the Results (Section IV)
C. Analysis of Results/IC_{50} Calculation (Section V)

for ketoconazole
6 uL x 2 = 12λ/assay
alternate? use 5λ instead of 6
6:150
5:150 = 30x dilution
25x dilution
2 mM / 25 = 80 uM
27
88
2.9
0.99 → 0.3 → 0.1 → 0.037
66.6
22.2
7.4
2.46
0.82
0.27

APPENDIX D

EXECUTED MATERIAL TRANSFER AGREEMENT BETWEEN USAPHC AND UC DAVIS

SUMMARY OF ACTION

1. DATE: 24 January 2012

2. SUBJECT: University of California, Davis Material Transfer Agreement

3. OFFICE SYMBOL: MCHB-IP-THE

4. SUSPENSE DATE:

5. PURPOSE OF ACTION:

Coordination of Material Transfer Agreement

6. SUMMARY OF ACTION: *(Briefly provide background information, pertinent facts and possible impacts on resources and/or operations)*

Dr. Adams requested a cell line from the University of California to bring in vitro technologies and assays in house to augment our current toxicology approaches. Focusing on (endocrine disrupting chemical) EDC assays and the HeLa9903 assay (preferred by USEPA) in particular. In review of the available data, protocols, etc. questions arose about the existing assay and the improved BGI strategy. The fact that the BGI line expresses both (estrogen receptor) ER-a and ER-b is a great advantage over the HeLa cells. Although a tiered approach to testing (starting with acute/occupational exposure scenarios) is typically followed, HERP has the capacity to explore the environmental contaminant concerns as well (e.g. potential contamination on military installations). The tiered EDC testing strategy fits well with our approach and while part of our effort is to meet regulatory criteria we can expand our efforts to validate and test comparable methods/approaches that have been supported by ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). The BGI cell line was developed using public dollars and is available for free to Federal agencies.
A Material Transfer Agreement (MTA) is required to facilitate the transfer of the above-referenced material. The legal department is required to review the agreement prior to approval. If the MTA is acceptable, please sign and date the MTA, and have an authorized official of your organization sign the MTA, and send a copy of the MTA to me by e-mail.

7. RECOMMENDATION:

Coordination and approval.

8. COORDINATIONS:

OFFICE	NAME	INITIALS	DATE
MCHB-SJA	Mr. Friedman		30 Jan 12
MCHB-CS-RE	Ms. Owens		
MCHB-CG	Valhmt		2 Apr 12

9. REVIEWS/APPROVALS

OPERATIONS	INITIALS	DATE
PRG MGR/DIV CHIEF	xxx	
PF EXEC OFCR	xxx	
PF DIRECTOR		2/20/12
G-STAFF DIRECTORS	xxx	
REGIONAL CDR	xxx	
SGS	xxx	
AIPH EXEC OFCR	xxx	
DIR AIPH/DEP TO THE CDR		3/14/12
CHIEF OF STAFF		
DEPUTY CDR VET SVCS	xxx	
CSM	xxx	
COMMANDER	xxx	

10. ACTION OFFICER *(Name, Grade, Office Symbol, & Phone Number)*

Valerie H. Adams, GS-12, MCHB-IP-THE, 436-5063

11. SIGNATURE:

USAPHC FORM 384-RE, (MCHB-CG) JAN 12

UNIVERSITY OF CALIFORNIA, DAVIS
MATERIAL TRANSFER AGREEMENT

This Agreement is made this 3rd day of April, by and between THE REGENTS OF THE UNIVERSITY OF CALIFORNIA, as represented by its Davis campus ("UC DAVIS"), having an address at the UC Davis Innovation Access, Technology Transfer Services, University of California, Davis, 1850 Research Park Drive, Suite 100, Davis, California 95618-6134, and THE ARMY INSTITUTE OF PUBLIC HEALTH ("RECIPIENT"), having its principal place of business at Health Effects Research, 5158 Blackhawk Road, Aberdeen Proving Ground, Maryland 21010-5403 (collectively "the PARTIES").

The RECIPIENT has requested from UC DAVIS the MATERIAL as set out in UC Case No. 2011-003 and defined in Section 1.B. below for the RESEARCH USE defined in Section 1.F. below by the RECIPIENT INVESTIGATOR(S) defined in Section 1.G. below. In consideration of the supply of the MATERIAL from UC DAVIS to the RECIPIENT, the PARTIES agree as follows:

1. Definitions

A. "ORIGINAL TRANSFERRED MATERIAL": The physical material actually delivered to the RECIPIENT by UC DAVIS, as identified in Appendix A attached hereto. The ORIGINAL TRANSFERRED MATERIAL contains material covered by Promega's Limited Use Label License in Appendix B attached hereto.

B. "MATERIAL": ORIGINAL TRANSFERRED MATERIAL, PROGENY, and UNMODIFIED DERIVATIVES.

C. "PROGENY": Unmodified descendant from the MATERIAL. Examples include but are not limited to: virus from virus; cell from cell; and organism from organism.

D. "UNMODIFIED DERIVATIVES": Substances created by the RECIPIENT that constitute an unmodified functional sub-unit or an expression product of the ORIGINAL TRANSFERRED MATERIAL. Examples include but are not limited to: subclones of unmodified cell lines; purified or fractionated sub-sets of the ORIGINAL TRANSFERRED MATERIAL; transcription and translation products (e.g., RNA and protein derived from provided DNA); reverse transcription and reverse translation products (e.g., DNA synthesized on a template using provided RNA); monoclonal antibodies secreted by a hybridoma cell line; and chemically-synthesized copies.

E. "MODIFICATIONS": Substances, exclusive of PROGENY and UNMODIFIED DERIVATIVES, created by the RECIPIENT that either contain or incorporate the MATERIAL or were otherwise created through the use of the MATERIAL.

F. "RESEARCH USE": The scientific RESEARCH USE specified in Appendix A.

G. "RECIPIENT INVESTIGATOR(S)": The RECIPIENT's scientific investigator(s) named in Appendix A.

H. "CONFIDENTIAL INFORMATION": Information, data or material in written or other tangible form related to the MATERIAL that is identified as confidential at the time of disclosure. CONFIDENTIAL INFORMATION does not include information which:

(i) the RECEIVING PARTY can demonstrate by written records was previously known to it;
(ii) at the time of disclosure is, or subsequently becomes, public knowledge other than through acts or omissions of the RECEIVING PARTY;
(iii) is lawfully obtained by the RECEIVING PARTY from sources independent of the DISCLOSING PARTY;
(iv) the RECEIVING PARTY is required to disclose under the California Public Records Act; or
(v) is otherwise required to be disclosed by the RECEIVING PARTY due to law or judicial action.

U.C. AGREEMENT CONTROL NUMBER
2012-21-0476

UNIVERSITY OF CALIFORNIA, DAVIS
MATERIAL TRANSFER AGREEMENT

2. Terms and Conditions

A. Use

i. The RECIPIENT shall use the MATERIAL or MODIFICATIONS solely for the RESEARCH USE, and in accordance with the restrictions required by Promega, as specified in Appendices A and B (with respect to the Promega product). Any other use of the MATERIAL or MODIFICATIONS by the RECIPIENT is expressly prohibited without the prior written consent of UC DAVIS. In addition, the RECIPIENT shall use the MATERIAL or MODIFICATIONS in compliance with all applicable statutes and regulations, including, but not limited to, those related to research involving the use of animals or recombinant DNA. The MATERIAL or MODIFICATIONS may not be used on any human subjects or for commercial purposes or any other use other than the RESEARCH USE.

ii. The RECIPIENT shall not analyze the MATERIAL for chemical composition or physical structure or have or allow any component of the MATERIAL to be analyzed or make any use of any such analysis. The RECIPIENT shall not alter the chemical structure of the MATERIAL in any way.

iii. The ORIGINAL TRANSFERRED MATERIAL contains material covered by Promega's Limited Use Label License in Appendix B attached hereto. RECIPIENT hereby agrees to comply with all terms set forth therein.

B. Tangible Property Ownership: UC DAVIS retains ownership of the MATERIAL, including any MATERIAL contained or incorporated in MODIFICATIONS.

C. Confidentiality: Any CONFIDENTIAL INFORMATION disclosed by the disclosing party to the receiving party shall be treated as confidential and maintained in confidence by the receiving party. The receiving party shall not disclose any CONFIDENTIAL INFORMATION of the disclosing party, except to its own personnel who have a need to know. Without limiting the foregoing, the receiving party shall take at least the same steps and use the same methods to prevent the unauthorized use or disclosure of CONFIDENTIAL INFORMATION of the disclosing party as it takes to protect its own CONFIDENTIAL INFORMATION or proprietary information. The confidentiality obligations of each party under this Agreement shall remain in effect for five (5) years from the effective date hereof.

D. Distribution: The RECIPIENT shall not transfer the MATERIAL or MODIFICATIONS to anyone other than to one who works under the direct supervision of the RECIPIENT INVESTIGATOR within the RESEARCH USE without the prior written consent of UC DAVIS.

E. Disclosure, Inventorship, and Intellectual Property Rights

i. Disclosure: The RECIPIENT shall promptly notify UC DAVIS of any potentially patentable discoveries or inventions made through the use of the MATERIAL, whether or not made within the specified limits of the approved RESEARCH USE. The RECIPIENT shall promptly supply UC DAVIS with a copy of the invention disclosure.

ii. Inventorship: Inventorship shall be determined according to United States patent law.

iii. Intellectual Property Rights: Collaborative efforts of UC DAVIS and the RECIPIENT may create inventorship rights under United States patent law as well as under the law of any applicable jurisdiction in which a party or the PARTIES may elect to file patent application(s). Each party shall own its undivided interest in joint inventions. The PARTIES shall cooperate in discussing and securing intellectual property rights to protect potentially patentable inventions.

iv. No Implied Rights: The RECIPIENT acknowledges that the MATERIAL is or may be the subject of a patent application. Except as provided in this Agreement, no express or implied license or other rights are provided to the RECIPIENT under any patents, patent applications, trade secrets or other proprietary rights of UC DAVIS, including any altered forms of the MATERIAL made by UC DAVIS. In particular, no express or implied licenses or other rights are provided to use the MATERIAL, MODIFICATIONS or any related patents of UC DAVIS for commercial use or any other use other than the RESEARCH USE.

F. Warranty and Licenses

i. Any MATERIAL delivered pursuant to this Agreement is understood to be experimental in nature and may have hazardous properties. UC DAVIS MAKES NO REPRESENTATIONS AND EXTENDS NO WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED. THERE ARE NO EXPRESS OR IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, OR THAT THE USE OF THE MATERIAL SHALL NOT INFRINGE ANY PATENT, COPYRIGHT, TRADEMARK, OR OTHER PROPRIETARY RIGHTS.

ii. Commercial Use (as defined in Appendix B) of MATERIAL and MODIFICATIONS is expressly prohibited under this Agreement.

G. Liability: The RECIPIENT assumes all liability for damages that may arise from its use, storage or disposal of the MATERIAL or MODIFICATIONS. UC DAVIS shall not be liable to the RECIPIENT for any loss, claim or demand made by the RECIPIENT, or made against the RECIPIENT by any other party, due to or arising from the use, storage or disposal of the MATERIAL or MODIFICATIONS by the RECIPIENT. The RECIPIENT shall indemnify, hold harmless and defend UC DAVIS against any claims, costs or other liabilities which may arise as a result of the RECIPIENT'S use, storage or disposal of the MATERIAL or MODIFICATIONS.

H. Publication of Research Results: The RECIPIENT may publish or present results of research relating to the MATERIAL, provided the RECIPIENT provides UC DAVIS with a copy of any proposed manuscript, abstract, poster session or presentation at least thirty (30) days prior to such publication or presentation. UC DAVIS shall review such publication or presentation for CONFIDENTIAL INFORMATION or patentable material and may request a delay of the proposed publication or presentation for up to an additional thirty (30) days to allow for the removal of CONFIDENTIAL INFORMATION or the filing of patent application(s). Unless UC DAVIS directs otherwise, any publication or presentation reporting the research carried out with the MATERIAL shall contain proper referencing in academic journal format, acknowledging UC DAVIS as the source of the MATERIAL.

I. Termination

i. Date: This Agreement shall terminate on the earliest of the following dates:
(a) on completion of the RECIPIENT'S current RESEARCH USE with the MATERIAL;
(b) on thirty (30) days' written notice by one party to the other; or
(c) one (1) year from the date of execution of this Agreement by UC DAVIS.

ii. Surviving Obligations: Obligations with respect to Tangible Property Ownership (2.B.), Confidentiality (2.C.), Distribution (2.D.), Disclosure, Inventorship, and Intellectual Property Rights (2.E.), Warranty and Licenses (2.F.), Liability (2.G.), Publication of Research Results (2.H.), and this Section (2.I.ii) shall survive termination.

iii. Return of MATERIAL: As directed by UC DAVIS, the RECIPIENT shall stop using the MATERIAL and shall return or destroy any remaining MATERIAL on the termination of this Agreement.

UNIVERSITY OF CALIFORNIA, DAVIS
MATERIAL TRANSFER AGREEMENT

J. Applicable Law: The validity and interpretation of this Agreement and legal relations of the PARTIES in the performance of this Agreement shall be governed by the laws of the State of California without regard to conflicts of law provisions.

K. Notice: Any notice required under this Agreement shall be considered properly given and effective on the date of the postmark if mailed by prepaid postage first-class certified mail; on the date of delivery if delivered in person; or on the date of receipt if mailed by any global express carrier service that requires the recipient to sign the documents demonstrating the delivery of such notice. Notice shall be given to the designated authorized official at the address provided below:

FOR THE REGENTS OF THE UNIVERSITY OF CALIFORNIA:

Authorized Official:	Executive Director
Address:	UC Davis Innovation Access Technology Transfer Services University of California, Davis 1850 Research Park Drive, Suite 100
City, State, Zip:	Davis, CA 95618-6134
Country:	USA
Telephone:	(530) 754-8649
Fax:	(530) 754-7620

FOR RECIPIENT:

Authorized Official:	Director
Recipient Institution:	Army Institute of Public Health
Address:	Health Effects Research 5158 Blackhawk Road
City/State/Zip:	Aberdeen Proving Ground, MD 21010-5403
Country:	USA
Telephone:	(410) 436-8717
Fax:	(410) 436-8258

[Remainder of this page intentionally left blank.]

UNIVERSITY OF CALIFORNIA, DAVIS
MATERIAL TRANSFER AGREEMENT

3. **Complete Agreement**

This Agreement constitutes all the agreements between the PARTIES, both written and oral with respect to the subject matter hereof. All prior agreements respecting the subject matter hereof, either written or oral, expressed or implied, between the PARTIES are hereby canceled. This Agreement may be executed in any number of counterparts, including facsimile or scanned PDF documents. Each such counterpart, facsimile or scanned PDF document shall be deemed an original instrument, and all of such counterparts, together, shall constitute one and the same executed Agreement.

THE REGENTS OF THE UNIVERSITY OF CALIFORNIA

Jan D. Carmikle
Senior Intellectual Property Officer
Technology Transfer Services

Date: 4/3/2012

ARMY INSTITUTE OF PUBLIC HEALTH

John Resta
Director
Army Institute of Public Health

Date: 4/2/12

UC DAVIS INVESTIGATOR and RECIPIENT INVESTIGATOR acknowledge reading and understanding this Agreement and shall abide by the terms and conditions thereof.

UC DAVIS INVESTIGATOR

Name: Michael Denison, Ph.D.
Title: Professor

Date: April 3, 2012

RECIPIENT INVESTIGATOR

Name: Valerie Adams, Ph.D.
Title: Biologist

Date: 2012 MARCH 21

UNIVERSITY OF CALIFORNIA, DAVIS
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APPENDIX B

pGL3 Luciferase Reporter Vectors

Patents/Disclaimers
[a]LIMITED USE LICENSE
For research use only. The terms of the limited license conveyed with the purchase of this product are as follows: Researchers may use this product in their own research and they may transfer derivatives to others for such research use provided that at the time of transfer a copy of this label license is given to the recipients and the recipients agree to be bound by the conditions of this label license. Researchers shall have no right to modify or otherwise create variations of the nucleotide sequence of the luciferase gene except that Researchers may: (1) clone heterologous DNA sequences at either or both ends of said luciferase gene so as to create fused gene sequences provided that the coding sequence of the resulting luciferase gene has no more than four deoxynucleotides missing at the affected terminus when compared to the intact luciferase gene sequence, and (2) insert and remove nucleic acid sequences in furtherance of splicing research predicated on the inactivation or reconstitution of the luminescent activity of the encoded luciferase. In addition, Researchers must do one of the following: (1) use luminescent assay reagents purchased from Promega Corporation for all determinations of luminescence activity resulting from the research use of this product and its derivatives; or (2) contact Promega to obtain a license for the use of the product and its derivatives. No other use or transfer of this product or its derivatives is authorized without the express written consent of Promega including, without limitation, Commercial Use. Commercial Use means any and all uses of this product and derivatives by a party for monetary or other consideration and may include but is not limited to use in: (1) product manufacture; and (2) to provide a service, information or data; and/or resale of the product or its derivatives, whether or not such product or derivatives are resold for use in research. With respect to such Commercial Use, or any diagnostic, therapeutic or prophylactic uses, please contact Promega for supply and licensing information. If the purchaser is not willing to accept the conditions of this limited use statement, Promega is willing to accept the return of the unopened product and provide the purchaser with a full refund. However, in the event the product is opened, then the purchaser agrees to be bound by the conditions of this limited use statement. The above license relates to Promega patents and/or patent applications on improvements to the luciferase gene.
[b]U.S. Pat. No. 5,670,356.
[c]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

UNIVERSITY OF CALIFORNIA, DAVIS
MATERIAL TRANSFER AGREEMENT

APPENDIX A

1. ORIGINAL TRANSFERRED MATERIAL:

Recombinant BG1Luc4E2 human ovarian carcinoma cells, which are BG-1 cells that have been stably transfected with the estrogen receptor-responsive firefly luciferase reporter gene plasmid, pGudLuc7.0ere (which contains estrogen-responsive elements). Please refer to Rogers and Denison (In Vitro and Molecular Toxicology 13, 67-82 (2000)) for details. The pGL3 Luciferase Reporter Vector ("pGL3") was purchased from Promega and is for non-clinical and non-commercial research uses only. pGL3 is covered under Promega's Limited Use Label License in Appendix B attached hereto.

2. RESEARCH USE:

The ORIGINAL TRANSFERRED MATERIAL is provided to the RECIPIENT for the detection of estrogenic and antiestrogenic chemicals for non-clinical and non-commercial research purposes only.

3. RECIPIENT INVESTIGATOR (name):

Valerie Adams, Ph.D.