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PEOPLE FOR THE ETHICAL TREATMENT OF ANIMALS

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Re: **Endocrine Disruptor Screening Program Tier 1 Screening Order Issuing Announcement (74 FR 54422); EPA–HQ–OPP–2009–0634**

The accompanying comments are being submitted on behalf of the more than two million members and supporters of People for the Ethical Treatment of Animals who are concerned about promoting reliable and relevant toxicity testing strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals. Our comments are submitted in response to issuance of Tier 1 Screening Orders for the Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP) for linuron issued on December 10, 2009, under the request for existing data and "other scientifically relevant information" (OSRI) in which "persons other than recipients" have 90 days to respond.

Introduction

EPA has initiated the EDSP Tier 1 screening for the first group of 67 chemicals by issuing test orders from October 29, 2009, through February 26, 2010. The 67 Phase I chemicals consist of 58 pesticide active and nine High Production Volume (HPV) chemicals used as pesticide inert ingredients (also known as "pesticide inerts"). These chemicals were chosen for testing based on exposure potential considering four exposure pathways for each type of chemical. The four exposure pathways identified for pesticide active ingredients are: food, drinking water, residential use, and occupational exposure. The four exposure pathways identified for HPV/pesticide inert chemicals are: human biological monitoring, ecological biomonitoring, drinking water, and indoor air.[1]

These chemicals are to be tested in five *in vitro* and six *in vivo* assays (**Table 1**). The stated purpose of the Tier 1 battery is to "identify substances that have the potential to interact with the EAT [estrogen/androgen/thyroid] hormonal systems…".[2] The EPA has stated that it intends to use a weight-of-evidence approach to evaluate the results of the Tier 1 studies,[3] and based on this assessment, EPA will determine which, if any, of the Tier 2 tests are necessary. The putative Tier 2 battery consists of developmental and reproductive toxicity tests in several vertebrate species and is designed to identify and establish dose-response relationships for any adverse endocrine-related effects.

These comments address the test orders for linuron, a substituted urea herbicide insecticide that has been extensively tested as part of registration. This testing involves dozens of toxicity tests in vertebrate animals, including reproductive and chronic/lifecycle studies in rodents, fish and birds, as well as metabolism and pharmacokinetics studies.[4] These tests kill thousands of animals

and include many of the same endpoints addressed in the presumptive EDSP Tier 2 tests (**Table 2**).

These comments are a continuation in a series of comments on individual Phase I chemicals from PETA and the Physicians Committee for Responsible Medicine.

References

[1] 74 FR 17579. April 15, 2009; EPA Final List of Initial Pesticide Active Ingredients and Pesticide Inert
Ingredients to be Screened Under the Federal Food, Drug, and Cosmetic Act.

[2] 74 FR54415, October21, 2009. Endocrine Disruptor Screening Program (EDSP); Announcing the
Availability of the Tier 1 Screening Battery and Related Test Guidelines; Notice.

[3] Response to Comments on the Public Review Draft of the Information Collection Request (ECR) entitled "Tier 1 Screening of Certain Chemicals Under the Endocrine Disruptor Screening Program (EDSP)", contained in Docket ID no. EPA-HQ-OPPT-2007-1081, page 16.

[4] 72 FR 60934, October 26, 2007: EPA 40 CFR Parts 9 and 158: Pesticides; Data Requirements for Conventional Chemicals.

Table 1: EDSP Tier 1 Assays

	Species	Mechanism addressed	Endpoints	suggested equivalent information
in vitro				
ER TA: OPPTS 890.1300 OECD TG 455	endogenous human ERα	Estrogen agonists	ERa-dependent transcriptional activation	effect ovary/uterus size, histology, male/female fertility
ER binding OPPTS 890.1250	Rat uterine cytosol	Estrogen agonists, antagonists	ER binding	effect ovary/uterus size, histology, male/female fertility
AR binding: OPPTS 890.1150	rat prostate cytosol	Androgen agonists, antagonists	AR binding	effect on testes size, histology, male/female fertility
Steroidogenesis - H295R OPPTS 890.1550	human	Steroid synthesis (estrogen and testosterone)	testosterone, estrogen hormone levels	effect on estrogen/testosterone levels, sex organs, male/female fertility
Aromatase OPPTS 890.1200	human	Aromatase inhibition, the enzyme responsible for the conversion of androgens to estrogens	$^3H_20$ released during the conversion of androstenedione to estrone	effect on estrogen/testosterone levels, sex organs, male/female fertility
In vivo:				
Uterotrophic OPPTS 890.1600 OECD TG 440	rat, mouse immature: PND 18 - 21 ovarectimized: 6 - 8 weeks	Estrogen agonists, antagonists (in GD, not well developed)	body weight, uterine weight, optional: histolopathology of vagina	evidence of estrogenic activity, uterine or vaginal weight changes, uterine or vaginal histology, effects on fertility reproduction
Hershberger OPPTS 890.1400 OECD TG 441	rat, mouse	Androgen agonists, antagonists, and 5α-reductase inhibitors	ventral prostate (VP), seminal vesicle (SV), levator ani-bulbocavernosus (LABC) muscle, paired Cowper's glands (COW) and the glans penis (GP)	evidence of androgenic activity, male sex organ weights or histology, effects on fertility reproduction

Pubertal female OPPTS 890.1450	rat	Anti-thyroid, estrogenic or anti-estrogenic (including alterations in receptor binding or steroidogenesis), luteinizing hormone, follicle stimulating hormone, prolactin or growth hormone levels or via alterations in hypothalamic function	Growth (daily body weight), Age and body weight at vaginal opening, Organ weights: Uterus, Ovaries, Thyroid, Liver, Kidneys, Pituitary, Adrenals. Histology: Uterus, Ovary, Thyroid, Kidney. Hormones: Serum thyroxine (T4), Serum thyroid stimulating hormone (TSH). Estrous cyclicity: Age at first estrus, length of cycle, percent of animals cycling. Standard blood panel, including creatinine and blood urea nitrogen.	evidence of estrogenic or thyroid activity, uterine or vaginal weight changes, uterine or vaginal histology, effects on fertility reproduction
Pubertal male OPPTS 890.1500	rat	Anti-thyroid, androgenic, or anti-androgenic [androgen receptor (AR) or steroid-enzyme-mediated], alterations in gonadotropins, prolactin, or hypothalamic function	Growth (daily body weight), Age and body weight at preputial separation, Organ weights: Seminal vesicle plus coagulating gs, Ventral prostate, Dorsolateral prostate, Levator ani/bulbocavernosus muscle complex, Epididymides, Testes, Thyroid, Liver, Kidneys, Pituitary, Adrenals. Histology: Epididymis, Testis, Thyroid, Kidney. Hormones: Serum testosterone, Serum thyroxine (T4), Serum thyroid stimulating hormone (TSH). Standard blood panel, including creatinine and blood urea nitrogen.	evidence of androgenic or thyroid activity, male sex organ weights or histology, effects on fertility reproduction
Amphibian metamorphosis OPPTS 890.1100	*Xenopus laevis*	hypothalamic-pituitary-thyroid (HPT) axis, Androgen agonists, antagonists, testosterone synthesis	Day 5: developmental assessment: hind limb and body length, body weight, developmental stage. Day 21 (termination): Developmental stage, SVL, hind limb length and wet body weight, thyroid gland histology.	evidence of androgenic or thyroid activity, male sex organ weights or histology, effects on fertility reproduction
Fish short-term reproductive screen OPPTS 890.1350 OECD 229	fathead minnow	hypothalamus-pituitary-gonadal (HPG) axis	survival, reproductive behavior, secondary sexual characteristics (number and size of nuptial tubercles), gonadal histopathology, gonado-somatic index, plasma concentrations of vitellogenin, 17β-estradiol and testosterone, fecundity (# eggs/female), fertility (%embryos/eggs)	evidence of estrogenic/androgenic activity, effects on fertility of reproduction

Table 2: Pesticide Data requirements related to EDC

Toxicological data requirements			**Use**	
OPPT guideline		**Relevant endpoints**	**food**	**non-food**
870.4100	Chronic oral: rodent	12 months exposure: gross necropsy plus histopathology of liver, kidneys, adrenals, testes, epididymides, ovaries, uterus, thyroid (with parathyroid), spleen, brain	R	CR
870.6200	90-day neurotoxicity	FOB: autonomic function (lacrimation, salvation, etc), convulsions, tremors, abnormal motor movements, reactivity to general stimuli (no reaction to hyperreactivity), general level of activity (unresponsive to hyperactive), posture and gait abnormalities, forelimb and hindlimb grip strength, foot splay, sensorimotor responses, body weight, neuropathology.	R	R
870.4200	Carcinogenicity	24 month exposure: clinical observations, blood smears, gross necropsy, possible histopathology of salivary glands, esophagus, stomach, intestine, liver, pancreas, gallbladder, brain, pituitary, peripheral nerve , spinal cord , eyes, adrenals, parathyroid, thyroid, trachea, lungs, pharynx, larynx, nose. aorta, heart, bone marrow, lymph nodes, spleen, kidneys, urinary bladder, prostate, testes, epididymides, seminal vesicle(s), uterus, ovaries, female mammary gland, all gross lesions and masses, skin.	R	CR
870.3700	Prenatal developmental toxicity, rat and rabbit	Exposure througout gestation: fetal deaths, resoption, sex and weight of each fetus, skeletal and soft-tissue abnormalities of fetuses	R	R
870.3800	Reproduction and fertility	Standard 2-gen: integrity and performance of the male and female reproductive systems, including gonadal function, the estrous cycle, mating behavior, conception, gestation, parturition, lactation, and weaning, and on the growth and development of the offspring. **P animals:** Cycling in females, sperm count, morphology, motility in males. Organ weights: uterus , ovaries, testes, epididymides, seminal vesicles, prostate, brain, pituitary, liver, kidneys, adrenal glands, spleen. Hisotpathology of vagina, uterus with oviducts, cervix, and ovaries, testis, epididymis, seminal vesicles, prostate, coagulating gland, pituitary and adrenal glands. **F1:** weight and gross abnormalities throughout developement, age of vaginal opening and preputial separation, anogenital distance, same organ weights as P, same histopath as P. **F2 weanlings:** histopathological examination of treatment-related abnormalities.	R	R
870.6300*	Developmental neurotoxicity	Perinatal exposure. Pup weight during growth, gross developmental abnormalities, motor activity, learning and memory, neuropathology (brain)	R	CR
870.7800*	Immunotoxicity	Functional tests: either antibody plaque-forming cell (PFC) assay or ELISA-based antibody reaction, NK cell activity. Cell counts of splenic or peripheral blood total B cells, total T cells, and T cell subpopulations.	R	R

Terrestrial and aquatic non-target organism data requirements			Use				
			terrestrial	aquatic	forestry	residential	Greenhouse/ indoor
850.2300	Avian reproduction	Eggs laid, percent fertilized, eggs not cracked, shell thickness, hatching, chick survival	R	R	R	R	NR
850.1400 (OECD TG 210)	Fish early life stage (freshwater)	Exposure of eggs until hatching: cumulative mortality, numbers of healthy fish at end of test, time to start of hatching and end of hatching, numbers of larvae hatching each day, length and weight of surviving animals, numbers of deformed larvae, numbers of fish exhibiting abnormal behavior.	R	R	R	R	NR
850.1500	Fish life cycle	Locomotion, behavioral, physiological, and pathological effects, spawning, egg numbers, fertility, and fecundity.	CR	CR	CR	CR	NR

*new in 2007

Linuron CAS 330-55-2
Test Order Numbers: EDSP -035506-122 through 123
Test Order Date: December 10, 2009

Introduction:

Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] is a substituted urea herbicide registered for use on asparagus, carrots, celery, corn , cottonseed , parsley, parsnips, potatoes, sorghum, soybeans, and wheat. linuron was initially registered as a pesticide in 1966 (RED 1995).

Linuron is of relatively low acute toxicity (oral LD50 for rats is 2600 mg/kg). It is slightly toxic by the oral, dermal and inhalation routes, and has been placed in Toxicity Category III (the second-to-lowest of four categories) for these effects. It causes slight eye irritation in rabbits (Toxicity Category III), and is not a skin irritant (Toxicity Category IV) or sensitizer (RED factsheet 1995).

Anti-androgenic activity is well documented for linuron, both *in vivo* and *in vitro*. Linuron has a duel mechanism for androgen disruption: androgen receptor antagonist and decreasing fetal testosterone production (Wilson 2008). Linuron was included in validation studies of the Hershberger assay through the Organization of Economic Cooperation and Development (OECD) and in both versions of the method (weanling and castrate), linuron demonstrated activity as an androgen antagonist.

Reproductive and Developmental activity:

In a ***3-Generation Reproduction*** study, the no observed effect level (NOEL) for adult rats was 1.25 mg/kg/day and the lowest effect level (LEL) for adult rats was 6.25 mg/kg/day for general toxicity endpoints. For reproductive toxicity endpoints, NOEL was 1.25mg/kg/day and LEL was 6.25mg/kg/day however, reduced pup weights were more consistently seen at 31.25mg/kg/day (E.I. du Pont de Nemours and Co., Inc., 1984).

In a ***teratology*** study, maternal rat NOEL was 2.50 mg/kg/day and maternal LEL was 6.25 mg/kg/day. Maternal toxicity manifested as decreased food consumption and decreased body weight gain. Fetal NOEL was 6.25 mg/kg/day and fetal LEL was 31.25 mg/kg/day, manifested as an increased number of resorption sites. Teratogenic NOEL was 31.25mg/kg (E.I. du Pont de Nemours and Co., Inc., 1979).

In a ***developmental toxicity*** study conducted with linuron in Sprague-Dawley rats, dietary doses of 5.0, 12.1, or 49.8 mg/kg/day were administered on days 6-15 of gestation. The NOELs for maternal systemic toxicity and developmental toxicity were 12.1mg/kg/day. The LOEL of 49.8 mg/kg/day for maternal systemic toxic effects was based upon decreased body weight and food consumption values. The developmental toxicity LOEL of 49.8 mg/kg/day was based on increases in post-implantation loss and increases in the litter and fetal incidences of resorptions. (E.I. du Pont de Nemours and Co., Inc., 1978).

More recently, in a ***reproductive toxicity study***, pregnant female Sprague-Dawley rats were fed linuron at 12.5, 25, 50, 75 mg/kg/day on gestational days 13-18 to determine the impact of *in utero* linuron on fetal testis gene expression and testosterone production. Researchers reported no overt fetal or maternal

toxicity; no significant differences in maternal weight, litter size, or litter loss were observed. Although a slight decrease in maternal weight gain was observed at 25mg/kg/day, it was not significant (Wilson 2009).

In a ***developmental toxicity study***, maternal NOEL for rabbits was reported at 5 mg/kg/day. Developmental toxicity NOEL was 25mg/kg. At 100 mg/kg/day, an increase in abortion rate and depression in liver and liver body weight ratio was observed. Developmental LEL was reported at 5 mg/kg/day and manifested as a decrease in fetal body weights and litter size and a statistically significant trend in elevation of total skull alterations (E.I. du Pont de Nemours and Co., Inc., 1986).

Reproductive and developmental data clearly show that linuron exposure can cause maternal and fetal toxicity at levels much lower than levels causing acute toxicity.

Estrogenic activity

To determine *in vitro* estrogenic activity, ***MCF7 human breast cancer cells*** were exposed to linuron at concentrations 0.001, 0.1, 1, and 10μM. After 6 days of exposure, no differences in cell proliferation responses (fold increases) were seen in any treatment, indicating no estrogenic activity (Vinggaard 1999).

A recombinant yeast screen was used to evaluate estrogenic activity of linuron *in vitro*. Linuron at 0.01, 0.1, 1, 10, 100, 1000μM had no estrogenic activity (Orton 2009).

Efficacy studies to evaluate the LUMI-CELL© ER assay showed that linuron did not affect estrogens in vitro (Gordon et al 2003, Gordon et al 2004).

Using several cell types and using several different methods, linuron showed ***no estrogenic activity*** *in vitro*.

Androgenic Activity:

In validation studies for ***male pubertal assay***, linuron was used at 50 and 100mg/kg/day (EPA 2007). As expected, linuron delayed puberty at both 50 and the 100 mg/kg/day dose levels, as evidenced by delayed acquisition of preputial separation. All of the androgen dependent tissue weights showed statistically significant decreases at both doses with the exception of the testes, which was statistically significant only at the higher dose. T4 decreased at the high dose only, and TSH decreased at the low dose only. Pituitary weight decreased significantly at both doses, and liver and kidney decreased significantly at the high dose only.

In validation studies (Charles River Laboratories) intact male rats were dosed at 50, 100, and 150 mg/kg/day for 15 days. Significant reductions in absolute weight of accessory sex glands were seen at all dose levels and absolute epididymal weight was reduced significantly. Thyroid weight was increased significantly at 50mg/kg/day and weight increase was inversely related to dose. Testosterone, T4, and T3 blood concentrations were significantly less than the control group in all three treated groups (Lech 2006).

In ***a multi-generation*** study conducted by du Pont (1984), linuron was fed to Charles River Crl:CD rats at 0, 25, 125, or 625 ppm. There was an increase in testicular interstitial-cell adenomas and hyperplasia in treated males of both generations compared with controls. The effect was most apparent at 125ppm.

Linuron was included in validation studies for the weanling and castrated versions of the ***Hershberger assay***, (Freyberger 2007, Owens 2007, Moon 2009a, Moon 2009b). From Phase 1 studies, the OECD concluded that the Hershberger protocol was not affected by differences in the rat strain, diet, caging, and routine laboratory procedures, but modest differences were seen depending on the age of the animals that had been castrated (OECD 2002a, Owens et al 2006). Phase 2 (41 studies) assessed the reliability and relevance of the assay and the alternative OECD Hershberger assay was regarded as a reliable method for detecting androgenic and anti-androgenic compounds (Shin 2007). Sixteen laboratories from 7 nations participated in Phase 2. During phase 2, ***anti-androgen activity*** of linuron was observed at doses of 3, 10, 30, and 100 mg/kg/day (Owens 2007). In phase 3, linuron reduced male sex organ weights at doses of 10 and 100 mg/kg/day (Moon 2008).

In an *in vitro* study using PALM cell line, there was a modest indication of ***androgen antagonism*** although the concentration relationship was unclear. Concentrations ranged from 1e -09 to 1e -05 (Freyberger 2009).

In a ***reproductive*** study pregnant female Sprague Dawley rats were dosed at 12.5, 25, 50, 75 mg/kg to determine the impact of *in utero* linuron on fetal ***testis gene expression*** and ***testosterone production***. *Ex vivo* testosterone production was significantly reduced at all levels tested when analyzed on an individual fetus basis. When analyzed on a per-litter basis, only 50 and 75 mg doses showed significant testosterone reduction. Linuron treatment did not affect ins13, cyp17a, cyp11a, or stAR mRNA expression. Testes were incubated and exposed to additional linuron to evaluate testosterone production effects *in vitro*. ***Testosterone production*** was greatly ***reduced*** at 30mg and above. Progesterone production was not affected (Wilson 2009).

Four separate *in vivo* studies were conducted to determine effects of linuron on androgen-dependent organs: Hershberger, transgenerational (testis and epididymis), adult castrated testosterone implanted, and adult castrated testosterone implanted for gene transcription *in situ* (Lambright 2000). Linuron significantly reduced androgen-dependent seminal vesicle and ventral prostate gland weights, while significantly increasing adrenal weight. Animals exposed to linuron *in utero* had significant epididymal and testicular malformations; more than 50% of test animals exhibited agenesis or atrophy of one or both.

The anti-androgenic activity of Linuron has been thoroughly documented using the exact Tier 1 protocols for the Hershberger and male pubertal assays as well as in other *in vivo* and *in vitro* studies. No further testing in mammalian Tier 1 assays is warranted.

Thyroid Activity:

In a TR-mediated transcriptional activation study with human hepatocarcinoma cells (HepG2), linuron behaved as a TR angonist, stimulating the thyroid hormone receptors (Schmutzler 2007).

Fish and Birds: In a combination *in vitro* and *in vivo* study, stickleback fish kidney cells and whole fish, which produce a protein (spiggin) in response to androgenic stimulation, were exposed to linuron and protein production amounts were documented. *In vitro*, linuron induced a significant *reduction* of spiggin

production indicating ***anti-androgenic activity*** at a concentration of 25ng/L. *In vivo*, linuron significantly *reduced* spiggin production (again indicating ***anti-androgenic activity***) at doses 100 and 250µg/L (Jolly 2009).

In another study, reproductive output from fathead minnow pair mating assay was used to determine ***reproductive effects*** in fish. A significant reduction in eggs spawned was seen at linuron an exposure concentration of 1000µg/L (Thorpe et al 2006).

Male fathead minnows were exposed to linuron to determine effects on secondary sex characteristics, specifically the fat pad weight on male minnows. After 21 days of exposure, linuron levels of 220µg/L significantly reduced the fat pad weight indicating an ***anti-androgenic effect*** (Thorpe et al 2006).

In avian ***reproductive toxicity*** studies with both bobwhite quail and mallard ducks, LOEL was 300ppm and NOEL was 100ppm for reproductive endpoints (RED 1995).

Conclusion:

Linuron has an enormous library of toxicity data that clearly indicate ***anti-androgenic activity*** both *in vitro* and *in vivo*. It affects male reproductive organs and sexual development in mammals and fish. LOELs and NOELs for EDSP endpoints have been well documented and there is no sound scientific reason to conduct more tests to evaluate the endpoints slated for EDSP. New data would be redundant and unnecessary.

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