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Evaluation of dietary dose administration as an alternative to oral gavage in the rodent uterotrophic and Hershberger assays

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Abbreviations: 4,4'-DDE, dichloro-2,2-bis(4-chlorophenyl) ethane; EE2,17α-ethinyl estradiol; LABP, levator ani-bulbocavernosus muscle TP, testosterone propionate.

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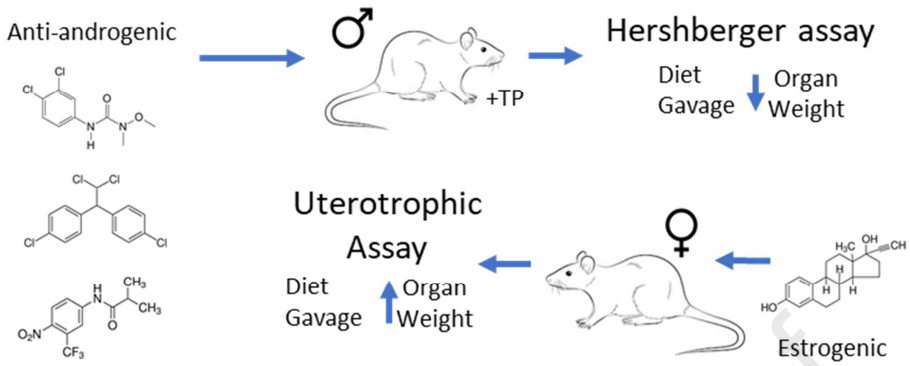
The rodent uterotrophic and Hershberger assays evaluate potential estrogenic and (anti)-androgenic effects, respectively. Both US EPA and OECD guidelines specify that test substance is administered daily either by subcutaneous injection or oral gavage. However, dietary administration is a relevant exposure route for agrochemical regulatory toxicology studies due to potential human intake via crop residues. In this study, equivalent doses of positive control chemicals administered via dietary and gavage routes of administration were compared in the uterotrophic (17α-ethinyl estradiol) and Hershberger (flutamide, linuron, dichloro-2,2-bis(4-chlorophenyl) ethane; 4,4'-DDE) assays in ovariectomized and castrated rats, respectively. For all positive control chemicals tested, statistically significant changes in organ weights and decreases in food consumption were observed by both routes of test substance administration. Decreased body weight gain observed for dietary linuron and 4,4'-DDE indicates that the maximum tolerated dose was exceeded. Hershberger dietary administration resulted in a similar blood exposure (AUC_{24}) for each positive control chemical when compared to gavage. Overall, the correlation in organ weight changes for both the uterotrophic and Hershberger assays suggest that dietary administration is an acceptable route of exposure with similar sensitivity to oral gavage dosing for evaluation of the endocrine potential of a test substance and represents a more appropriate route of test substance administration for most environmental exposure scenarios.

Keywords:

Androgenic; estrogenic; endocrine; Hershberger; toxicokinetics; uterotrophic

Graphical Abstract



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Text- 5127 words

1. INTRODUCTION

The rodent uterotrophic (OECD Test Guideline 440, USEPA OPPTS 890.1600) and Hershberger assays (OECD Test Guideline 441, USEPA OPPTS 890.1400) are two short-term *in vivo* studies that are widely accepted screening assays for identifying chemicals that have potential estrogenic and (anti)androgenic activity, respectively. The uterotrophic assay is designed to detect chemicals with potential estrogenic activity by measuring a chemical's ability to produce an increase in uterine weight after 3 days of dosing, whereas the Hershberger assay is designed to detect chemicals with the potential to act as androgen receptor agonists, antagonists, or 5α-reductase inhibitors by measuring the chemical's ability to alter male accessory sex organ weights after 10 days of dosing. For both the uterotrophic and Hershberger assays, test guidelines indicate that test substances should be administered daily by either subcutaneous injection or oral gavage. Animal welfare considerations as well as toxicological aspects such as the relevance to the route of human exposure, the physical/chemical properties, existing toxicological information, data on metabolism and kinetics (e.g., need to avoid first pass metabolism, better efficiency via a particular route), and especially the intended use of the assay (e.g., the US EPA Endocrine Disruptor Screening Tier 1 battery) should also be taken into consideration when choosing the route of administration. The test guidelines indicate that oral gavage is the surrogate model for ingestion while subcutaneous injection is the surrogate model for inhalation or dermal adsorption. However, the kinetics of systemic exposure between different routes such as oral gavage, dietary administration, and test substance administration via drinking water can be dramatic (Hannas *et al.*, 2016; Gayrard *et al.*, 2013; Sieli *et al.*, 2011; Atcha *et al.*, 2010). Based on the published literature, other routes of test substance administration such as dietary or

drinking water routes of administration are not routinely used. Instances of dietary test substance administration have been typically limited to evaluation of effects of diets containing phytoestrogens (Stroheker *et al.*, 2003; Minta *et al.*, 2013; Thigpen *et al.*, 2002), even though the dietary route of administration is a more appropriate route of test substance administration for most environmental exposure scenarios.

The primary benefit for administering a test substance by oral gavage or subcutaneous injection is the ability to deliver a precise dose at a specific time. However, both routes of test substance administration result in large variability in plasma/blood levels of the test substance. Test substance administration via a single bolus dose can lead to large spikes in blood levels (i.e., C_{max}), followed by long periods where there are relatively low blood concentrations (i.e., C_{min}) depending on the half-life of the test substance (Saghir et al., 2012). For example, Hannas *et al.* (2016) evaluated blood levels of two different test substances with short ($T_{1/2}$ = 1 hr) or long ($T_{1/2}$ = 13.5 hr) plasma half-lives after test substance administration by oral gavage or via diet in rabbits and found that plasma blood levels of the test substances had much more variability in rabbits dosed by oral gavage compared to those administered the test substance in diet. For the short half-life test substance ($T_{1/2}$ = 1 hr) the plasma levels varied by up to 6-fold when administered by diet compared to 368-fold when administered by oral gavage. Results obtained with the long half-life test substance ($T_{1/2}$ = 13.5 hr) showed less variability, where plasma levels were stable in rabbits exposed by the dietary route compared to a 3-fold variability in plasma levels for rabbits dosed via oral gavage. Yet, the concentration of test substance over time (AUC_{24}) is very similar between the dietary and gavage routes of administration, suggesting that these kinetic differences do not necessarily reduce the overall exposure to the test substance.

Clearly, test substance administration via the diet provides a more constant level of test substance exposure in comparison to a single bolus dose.

In recognition of the benefits of test substance administration by dietary exposure, the International Life Sciences Institute-Health and Environmental Sciences Institute Agricultural Chemical Safety Assessment (ILSI-HESI ACSA) technical committee recommended the use of dietary test substance administration over other routes of administration (Cooper *et al*., 2006; Conolly *et al.,* 1999). Others have also proposed changes to the route of administration for toxicology testing, particularly when evaluating potential adverse effects on endocrine signaling using studies such as the uterotrophic and Hershberger assays (Vandenberg *et al.,* 2014).

The objective of the current study was to compare differences in responses in the uterotrophic and Hershberger assays with selected positive control chemicals when administered by both the dietary and oral gavage routes of administration. The data demonstrate that the test substances were identified for their potential endocrine effects whether the test substance was administered by dietary exposure or oral gavage. Therefore, these results support the use of the dietary route of test substance administration for use in the uterotrophic and Hershberger assays.

2. MATERIAL AND METHODS

2.1. **Test substances.**

Flutamide (>99% purity) and dichloro-2,2-bis(4-chlorophenyl) ethane (4,4'-DDE; ≥ 97% purity) were purchased from Sigma-Aldrich (St. Louis, Missouri) and used as the test substance and analytical standards for these analyses. Linuron (99% purity) was purchased from Carbone Scientific (London, U.K.) and was used as the test substance and analytical standard. Testosterone propionate (TP; purity 96.5%) was purchased from Steraloids (Newport, Rhode

Island). The test substance 17α-ethinyl estradiol (EE2; 100% purity) was purchased from Selleck Chemicals (Houston, TX). The 17α-ethinyl estradiol (≥ 98% purity) used for analytical standards was purchased from Sigma-Aldrich (St. Louis, Missouri). Ethynyl Estradiol-2,4,16,16-d4 (Purity 97%) was obtained from Toronto Research Chemicals (TRC, Toronto, Canada).

2.2. **Test species & animal husbandry.**

Male and female Crl:CD®(SD) rats were acquired from Charles River Laboratories, Inc. (Raleigh, NC). Male rats were castrated at 45 days of age and at least 7 days prior to study start. Male rats were approximately 8 weeks of age at study start. Female rats were ovariectomized by the vendor at 56 days of age and 16 days prior to study start. Female rats were approximately 10 weeks of age at study start. The number of animals obtained for the Hershberger assay prevented running independent controls for both dietary and gavage administration. Therefore, only dietary controls were run.

Upon arrival, rats were housed 2 per cage in solid-bottom caging with Shepherd's™ ALPHA-dri® bedding (Shephard Specialty Papers, Milford, NJ) (males), certified Sani Chips® hardwood bedding (P.J. Murphy, Montville, NJ) (females), and appropriate enrichment. Rats were fed Certified Rodent Diet #5002 (PMI Nutrition International, Inc., St. Louis, MO) (males) or Teklad 2016 Certified Meal (Envigo, Madison, WI) (females) with isoflavone content < 20 mg/kg, and provided with filtered water ad libitum. Animal rooms were maintained on a 12-hour light/dark cycle (fluorescent light), a temperature of 23 ± 3°C, and a relatively humidity of 50% ± 20%.

156 After a quarantine period of 3 days (males) or 1 week (females), rats that displayed
157 adequate weight gain and freedom from clinical signs were divided by computerized, stratified
158 randomization into groups so that there were no statistically significant differences among group
159 body weight means. Weight variation of selected animals did not exceed ± 20% of the mean
160 weight. During testing, all rats were weighed daily, and cage-side examinations were performed
161 to detect moribund or dead rats. At each weighing, rats were individually handled and examined
162 for abnormal behavior or appearance.

163 2.3. **Test sample preparation.**

164 For the uterotrophic assay, EE2 doses for gavage administration were selected based on
165 previously-conducted studies (Kanno, 2001). Equivalent dietary concentrations of EE2 were
166 selected based on daily food consumption data typically observed at the facility (Table 1) and
167 these concentrations correlated with dietary concentrations previously reported (Heneweer,
168 2007). For the Hershberger assay, doses for gavage administration of flutamide, linuron, and
169 4,4'-DDE were chosen based on previous studies (OECD, 2009; O'Connor et al., 1999, 2002;
170 Freyberger et al., 2007). Equivalent dietary doses were selected based on daily food
171 consumption data typically observed at the facility (Table 2).

172

Group	Test substance	Animals/Group	Route of Administration	Test substance concentration/dose
1	Control	6	Dietary	0 ppm
2	17α-ethinyl	6	Dietary	0.17 ppm
3	17α-ethinyl	6	Dietary	1.7 ppm
4	Control	6	Oral gavage	0 µg/kg/day [a]
5	17α-ethinyl	6	Oral gavage	10 µg/kg/day [a]
6	17α-ethinyl	6	Oral gavage	100 µg/kg/day [a]

173

174 **Table 1**: Uterotrophic Assay Study Design

175 [a] Test substance administered once daily by oral gavage on test days 1-5 in vehicle (corn
176 oil with 1% ethanol) at a dose volume of 2 mL/kg.

177

178

Group	Test substance	Animals/Group	Route of Administration	Test substance concentration/dose	Testosterone Propionate (mg/kg/day) [a]
1	Control	6	Dietary	0 ppm	0.4
2	Flutamide	6	Dietary	50 ppm	0.4
3	Linuron	6	Dietary	1500 ppm	0.4
4	4,4'-DDE	6	Dietary	2500 ppm	0.4
5	Flutamide	6	Oral	3 mg/kg/day [b]	0.4
6	Linuron	6	Oral	100 mg/kg/day	0.4
7	4,4'-DDE	6	Oral	160 mg/kg/day [b]	0.4

179

180 **Table 2**: Hershberger Assay Study Design

181 [a] Testosterone propionate administered once daily by subcutaneous injection on test days
182 1-10 in vehicle (corn oil with 1% ethanol) at a dose volume of 0.5 mL/kg.

183 [b] Test substance administered once daily by oral gavage on test days 1-10 in vehicle
184 (0.1% Tween in 0.5% methylcellulose prepared in deionized water) at a dose volume of 10
185 mL/kg.

186

187 For dietary test substance preparation, EE2, flutamide, 4,4'-DDE, and linuron were added
188 to diet and thoroughly mixed for at least 6 minutes to ensure homogeneous distribution in the

diet. EE2 and TP were dissolved in ethanol prior to dilution in corn oil. The final concentration of ethanol in the vehicle was 1%. For test substances administered by oral gavage, test substances were suspended or dissolved in vehicle and continuously mixed during the dosing period to ensure homogeneity of the dosing solutions.

Hershberger assay test substance preparations were adjusted for purity and homogeneity, and stability of the test substance in the diet or dose vehicle were verified. Test substance preparation stability and homogeneity was not confirmed in the uterotrophic assay. Where daily doses were administered, individual animal dose volumes were based on the daily body weight and administered at approximately the same time each day except for the last day of dosing (test day 10) for male rats, which was stratified across groups and spread across several dosing periods.

2.4. Statistical Analyses**.**

Preliminary tests were conducted for homogeneity of variance (Levene, 1960) and normality (Shapiro and Wilk, 1965). A one-way analysis of variance (Snedecor and Cochran, 1967) followed by Dunnett's test (Dunnett, 1964) were conducted if data were normally distributed and had homogeneity of variance. For data that did not show homogeneity of variances, a robust version of Dunnett's test (Dunnett, 1980) was used. For all statistical analyses, significance was judged at $p < 0.05$.

2.5. **Uterotrophic Assay**

Study design. The uterotrophic study was conducted at BASi (Gaithersburg, MD). This study was approved by the Institutional Animal Care and Use Committee established in the test facility and was performed in accordance with the animal welfare by-laws of the facility, which is

accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. This study was conducted in accordance with relevant test guidelines (OECD Test Guideline 440; USEPA OPPTS 890.1600). Although not conducted in full compliance of Good Laboratory Practices (GLPs), the study was conducted in a GLP-compliant facility in the spirit of GLP compliance.

The study consisted of 6 groups of 6 ovariectomized female rats (Table 1). Evidence of estrus was evaluated on each animal daily, beginning ten days after ovariectomy surgery and for at least five consecutive days prior to randomization, in order to verify complete ovariectomy. Estrus was not detected in any animals that were used for the study, indicating complete ovariectomy. Dosing by oral gavage or by incorporation into diet was performed for 5 consecutive days (test days 1-5) following release from quarantine. While the test guideline only requires dosing for 3 days, this increased test duration is permissible according to the test guidelines. Parameters evaluated daily during the study included mortality, physical examinations, body weights, food consumption, and vaginal cytology. On the morning of test day 6, approximately 24 hours after the last administered oral gavage dose or from the time diet was presented to the rats, all rats were euthanized by carbon dioxide inhalation followed by exsanguination. At necropsy, all animals were evaluated for gross observations of toxicity and the uterus was weighed (wet and blotted weights).

Toxicokinetic analyses. On test day 5, blood was collected for toxicokinetic analyses from all rats. Rats were not fasted prior to blood collection. At each timepoint (06:00 hr and after 4, 8, and 12 hours for groups 1-3; 1, 4, 8, and 12 hr post-dose for groups 4-6), 50 µl of blood was collected from the lateral tail vein and placed into potassium EDTA tubes. All samples were

capped, vortexed, placed on dry ice, and stored frozen at approximately -80°C until evaluated. However, the method for detection (Figure S1) was not sensitive enough for EE2 quantification in this study.

Figure S1

Hershberger assay diet and blood sample analyses. An aliquot of 4 gram of each diet sample was extracted with 40 mL acetonitrile by grinding at 1400 stokes/min for 5 minutes, then followed by centrifugation at 4250 rpm for 20 minutes. The supernatant was analyzed for diet concentration determination by UHPLC coupled with tandem mass spectrometry (LC/MS/MS) by multiple reaction monitoring (MRM). Blood samples were evaluated for concentrations of flutamide, hydroxyflutamide, and linuron using LC/MS/MS. Briefly, 30 µL of blood sample was mixed with 90 µL of 0.1 M $ZnSO_4$ and extracted with 360 µL of acetonitrile containing 0.1% formic acid by protein precipitation. The extracts were injected into the LC/MS/MS for concentration determination. The UHPLC system was an Agilent 1290 infinity directly connected to electrospray ionization mass spectrometer, AB Sciex Qtrap 5500. The column used was Phenomenex Kinetex ®XB-C18, 100Å, 2.1×30 mm, 2.6 µm and kept at 30°C with a gradient separation. The gradient started from 5% mobile phase B (acetonitrile containing 0.1% formic acid) and 95% mobile phase A (water containing 0.1% formic acid), linearly raised to 95% mobile phase B at 3 minutes and stepped back down to 5% mobile phase B for equilibrium. The mass spectrometer was operated at negative mode monitoring MRM transitions for Flutamide (275→205) and Hydroxyflutamide (291→205), and at positive mode for monitoring Linuron (249→133). Analyst® software was used for calibration curve construction and sample concentration determination. 4,4-DDE was evaluated with gas chromatography coupled with MS detection (GC/MS, Agilent 7890A gas chromatograph with 5975C inert XL EC/CI MSD) by selected ion monitoring (SIM). The GC column used was Agilent J&W DB-1701, 30 m × 250 µm × 0.25 µm. The oven temperature was hold at 100°C for 0.5 minutes, then raised at 25°C/minute to 280°C and hold for 2 min with a total run time of 9.7 min. SIM was set to monitoring ion of 318. John- Check if you think this whole section needs to be included.

Uterotrophic assay blood sample analysis. Prior to analysis, each blood sample was thawed and diluted 1:1 with HPLC grade water. 20 µL of each sample was partitioned against 200 µL of HPLC grade ethyl acetate and the organic layer transferred to a 1.5 mL Eppendorf tube. Each tube was then taken to dryness under a low flow of nitrogen gas and the dried residue reconstituted with 20 µL of a solution composed of 60% methanol, 40% water and 1 ng/mL of 2,4,16,16-d4 EE2 utilized as an internal standard . After vortex mixing for 30s to ensure homogeneity, the samples were analyzed for EE2 via LC/MS/MS (Agilent 1290 Infinity II system running 3mM ammonium fluoride as solvent A and methanol as solvent B; Restek Raptor Biphenyl 3.0 mm x 10 cm, 2.7 µm). The gradient program started at 60% methanol and progressed to 99% over 4 minutes. Following a 2.5 minute isocratic hold at 99% methanol, the gradient returned its starting condition to re-equilibrate the column. This system was coupled to an ABSciex QTRAP 6500+ configured with an ESI source operated in negative ion mode and utilized multiple reaction monitoring (MRM) to quantify the residue of EE2. The method performance was validated at 40 pg EE2/mL of whole blood (LLOQ), with a mean recovery of 80 ± 11% and individual recoveries falling between 73 and 93% (N=5). The method LOD was estimated to be 5 - 10 pg EE2/mL of whole blood.

2.6. **Hershberger Assay**

Study design. The Hershberger study was conducted at DuPont Haskell Laboratory for Health & Environmental Science (Newark, DE). This study was approved by the Institutional Animal Care and Use Committee established in the test facility and was performed in accordance with the animal welfare bylaws of the facility, which is accredited by AAALAC International. This study was conducted in accordance with relevant test guidelines (OECD Test Guideline 441; USEPA OPPTS 890.1400). Although not conducted in full compliance of Good Laboratory Practices (GLPs), the study was conducted in a GLP-compliant facility in the spirit of GLP compliance.

The study consisted of 7 groups of 6 castrated male rats (Table 2). Dosing by subcutaneous injection (TP), oral gavage, and/or by incorporation into diet was performed for 10 consecutive days (test days 1-10) following release from quarantine. Parameters evaluated daily during the study included mortality, physical examinations, body weights, and food consumption. On the morning of test day 11, approximately 24 hours after the last administered oral gavage dose or from the time diet was presented to the rats, all rats were euthanized by exsanguination under isoflurane anesthesia. At necropsy, all animals were evaluated for gross observations of toxicity and the following tissues were weighed: liver, ventral prostate, seminal vesicle (plus fluids and coagulating glands), levator ani-bulbocavernosus muscle, paired Cowper's glands and the glans penis.

Toxicokinetic analyses. On test day 9, blood was collected for toxicokinetic analyses from all rats. Rats were not fasted prior to blood collection. At each timepoint (06:00 am, 10:00 am, 14:00 pm, and 18:00 pm for dietary groups, [similar to Saghir, 2006]; 0.25, 0.5, 1, 2, 4, 8,

12, and 24 hr post-dose for gavage groups) 50 µl of blood was collected from the lateral tail vein and placed into tubes containing potassium ethylenediaminetetraacetic acid (EDTA). The sample was immediately transferred to 1 mL tubes containing 50 µL of HPLC-grade water, and all samples were capped, vortexed, placed on wet ice, and stored frozen at approximately -70°C until evaluated. Diet and blood analysis was conducted according to Figure S1. The concentration time course data for blood from each animal was analyzed using a commercially available software program (Phoenix® WinNonlin®, Pharsight – A Certara™ Company, St. Louis, MO, U.S.A.) to determine area-under-the-curve over 24 hours (AUC_{24}, h x ng/mL), peak concentration (C_{max}, ng/mL), and dose normalized values for AUC_{24} and C_{max}.

3. RESULTS

3.1. Estrogenic effects of positive control chemical by gavage administration in the uterotrophic assay

EE2 was used as the positive control chemical for evaluation of responses in the uterotrophic assay when the test substance was administered by oral gavage or via the diet. By gavage administration, EE2 was administered at 10 (low dose) and 100 (high dose) µg/kg/day. Mean terminal body weights were statistically significantly reduced at 100 µg/kg/day EE2 when compared to the control group (-11%), while the 5% reduction in body weight observed at the 10 µg/kg/day EE2 dose did not reach statistical significance (Table 3A). Body weight loss (group absolute body weight gain compared to the group body weight at the study initiation) of 1.7% was observed at 100 µg/kg/day EE2. However, body weight gain at 10 µg/kg/day was not significantly different from control. There was also a statistically significant, dose-dependent

282 decrease in food consumption at both 10 and 100 µg/kg/day EE2 (-24 and -35%, respectively)
283 when compared to the control group.

A

Uterotrophic Assay		Mean Daily Intake (µg/kg/day)	Body Weight (g)	Body Weight versus control (%)	Body Weight Gain (g)	Body Weight Gain (%)	Food Consumption (g/day)	Food Consumption versus control (%)
Control	Gavage	-	311.7 ± 20.4	-	25.3 ± 6.5	8.9	19.2 ± 1.6	-
EE2 (low)		10	294.8 ± 21.7	-5.4	9.2 ± 2.1 *	3.2	14.6 ± 1.6 *	-23.6
EE2 (high)		100	278.4 ± 16.1 *	-10.7	-4.9 ± 5.9 *	-1.7	12.5 ± 0.6 *	-34.9
control	Diet	-	310.2 ± 18.8	-	24.2 ± 6.4	8.4	19.1 ± 1.0	-
EE2 (low)		9.3	282.5 ± 12.3 *	-8.9	-2.9 ± 7.2	-1.0	12.9 ± 0.5 *	-32.5
EE2 (high)		77.6	267.0 ± 13.1 *	-13.9	-14.9 ± 6.9	-5.24	10.5 ± 0.5 *	-45.0

B

Hershberger Assay	Route	Mean Daily Intake (mg/kg)	Body Weight (g)	Body Weight versus control (%)	Body Weight Gain (g)	Body Weight Gain (%)	Food Consumption (g/day)	Food Consumption versus control (%)
Control	Diet	-	351.2 ± 19.0	-	95.2 ±10.7	37.2	26.3 ± 1.1	-
Flutamide	Gavage	3	325.3 ± 25.5	-7.4	73.5 ± 10.2	29.2	23.2 ± 1.2 *	-11.7
Linuron		100	302.3 ± 28.4 *	-13.9	48.6 ± 16.2 *	19.2	18.1 ± 2.7 *	-31.0
4,4'-DDE		160	309.3 ± 29.4	-11.9	56.2 ± 14.6 *	22.2	20.4 ± 0.7 *	-22.2
Control	Diet	-	351.2 ± 19.0	-	95.2 ±10.7	37.2	26.3 ± 1.1	-
Flutamide		4.1	335.5 ± 27.0	-4.5	80.8 ± 11.6	31.7	24.2 ± 1.8	-7.8
Linuron		71.3	252.5 ± 15.6 *	-28.1	-1.6 ± 14.2 *	-0.6	11.4 ± 1.3 *	-56.7
4,4'-DDE		142.6	271.0 ± 26.5 *	-22.8	17.5 ± 17.3 *	6.9	14.5 ± 1.5 *	-44.8

Table 3
Summary of in-life parameters as mean ± standard deviation for the (A) Uterotrophic and (B) Hershberger assays. Six ovariectomized female rats in the uterotrophic assay and 6 castrated rats in the Hershberger assay were used per treatment group. A single group of 4 castrated rats administered in the diet were used for the Hershberger controls. * Statistically significant ($p < 0.05$) using Dunnett's test.

284

285 Following gavage administration of EE2, the absolute and relative wet/blotted uterine weights
286 were statistically significantly increased at 100 µg/kg/day (Fig. 1A/B, Table S1A) when
287 compared to the control group. Mean absolute (wet and blotted) uterine weights were increased
288 by 223% and 225% compared to the control group. Similarly, mean relative (wet and blotted)
289 uterine weights were increased by 248% and 249% compared to the control group. The uterine
290 weights (wet and blotted) at 10 µg/kg/day EE2 were similar to control values.

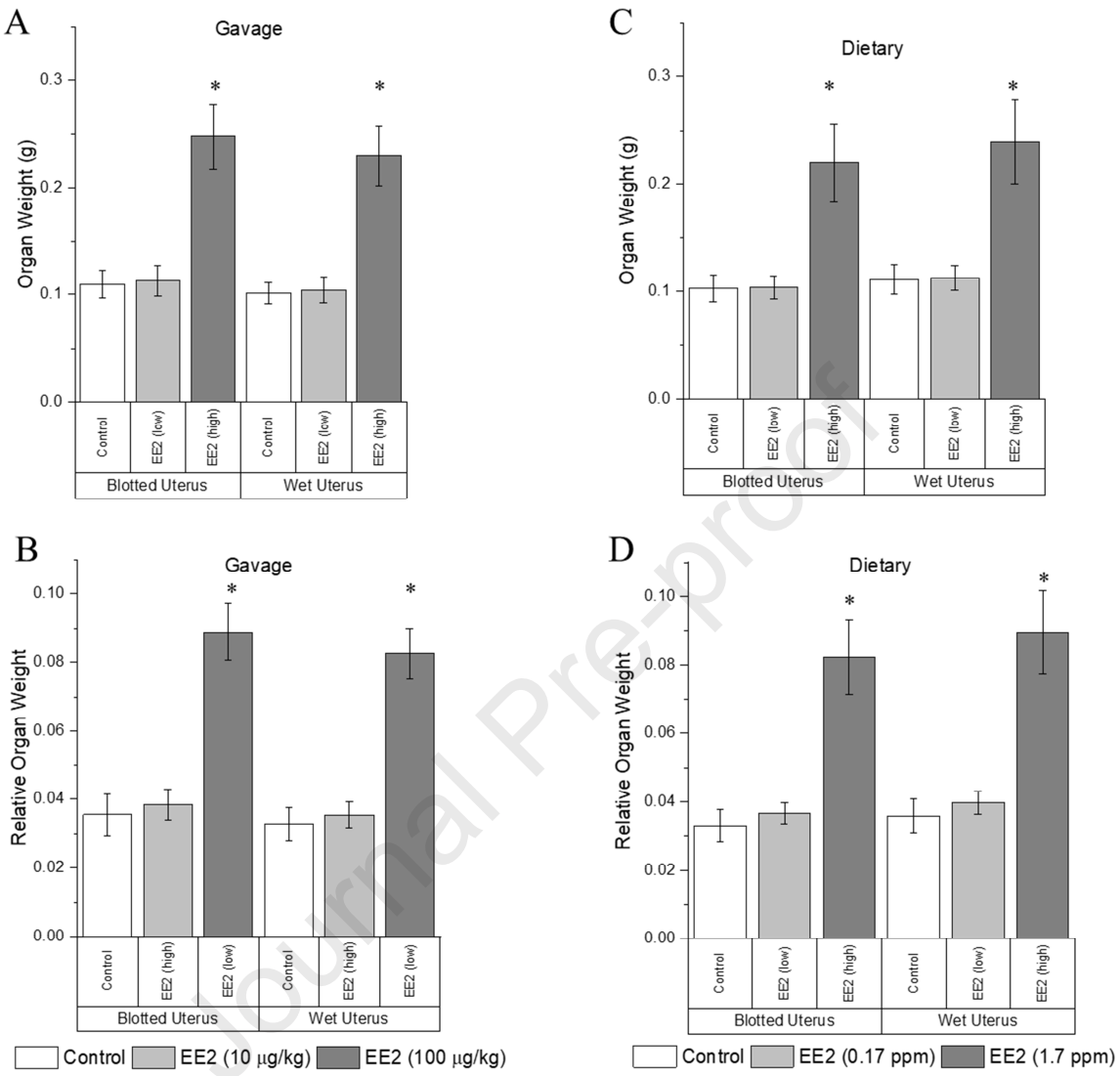

Fig. 1 Absolute (A,C) and relative (B,D) uterus weights by gavage and dietary administration of EE2 over 5 days in ovariectamized rats. (n= 6 rats/group) * Statistically significant ($p < 0.05$) using Dunnett's test.

291

Uterotrophic Assay	Route	Wet Uterus (g)		Wet Uterus/BW		Blotted Uterus (g)		Blotted Uterus/BW		n =
Control (0 ug/kg)	Gavage	0.1097	± 0.0131	0.0355	± 0.0061	0.1012	± 0.0098	0.0327	± 0.0048	6
17α-ethynyl estradiol (10 ug/kg)		0.1132	± 0.0143	0.0384	± 0.0046	0.1041	± 0.0120	0.0354	± 0.0038	6
17α-ethynyl estradiol (100 ug/kg)		0.2478	± 0.0302 *	0.0890	± 0.0084 *	0.2301	± 0.0277 *	0.0826	± 0.0074 *	6
Control (0 ppm)	Diet	0.1108	± 0.0138	0.0358	± 0.0050	0.1022	± 0.0125	0.0331	± 0.0045	6
17α-ethynyl estradiol (0.17 ppm)		0.1123	± 0.0117	0.0397	± 0.0034	0.1033	± 0.0107	0.0365	± 0.0031	6
17α-ethynyl estradiol (1.7 ppm)		0.2395	± 0.0390 *	0.0895	± 0.0122 *	0.2201	± 0.0361 *	0.0822	± 0.0109 *	6

Table S1
Summary of absolute and relative mean uterus weights ± standard deviation for the uterotrophic assay.
* Statistically significant ($p < 0.05$) using Dunnett's test.

3.2. **Estrogenic effects of positive control chemical by dietary administration in the uterotrophic assay**

Mean daily intake for EE2 was 9.3 and 77.6 µg/kg/day in the low (0.17 ppm) and high (1.7 ppm) groups, respectively (Table 3A). By dietary administration, mean terminal body weights were statistically significantly reduced at 0.17 and 1.7 ppm EE2 compared to the vehicle control group (-9 and -14%, respectively). In addition, animals lost weight in the high dose group (-5%). Food consumption was also statistically significantly reduced at 0.17 and 1.7 ppm (-33 and -45%, respectively) (Table 3A).

Following dietary administration of EE2, the absolute and relative wet/blotted uterine weights were statistically significantly increased at 1.7 ppm EE2 (Fig. 1C/D, Table S1) compared to the control group. Mean absolute (wet and blotted) uterine weights were increased by 216% and 215% compared to the control group. Similarly, mean relative (wet and blotted) uterine weights were increased by 250% and 249% compared to the control group. The uterine weights (wet and blotted) at 0.17 ppm EE2 were similar to control values.

	Gavage				Diet			
	Control (0 mg/kg)	Flutamide (3 mg/kg)	Linuron (100 mg/kg)	4,4'-DDE (160 mg/kg)	Control (0 ppm)	Flutamide (50 ppm)	Linuron (1500 ppm)	4,4'-DDE (2500 ppm)
Cowper Gland (g)	0.0380 ± 0.0049	0.0184 ± 0.0029 *	0.0267 ± 0.0063 *	0.0216 ± 0.0024 *	0.0380 ± 0.0049	0.0147 ± 0.0036 *	0.0240 ± 0.0045 *	0.0183 ± 0.0051 *
Cowper Gland /Term BW (%)	0.0108 ± 0.0013	0.0057 ± 0.001	0.0088 ± 0.0018	0.0070 ± 0.0006 *	0.0108 ± 0.0013	0.0044 ± 0.0009 *	0.0095 ± 0.0018	0.0067 ± 0.0015 *
Glans Penis (g)	0.0938 ± 0.0028	0.0724 ± 0.0093	0.0793 ± 0.0100	0.0788 ± 0.0102	0.0938 ± 0.0028	0.0642 ± 0.0085 *	0.0769 ± 0.0070 *	0.0727 ± 0.0073 *
Glans Penis/ Term BW (%)	0.0268 ± 0.0020	0.0224 ± 0.0038	0.0264 ± 0.0042	0.0256 ± 0.0030 *	0.0268 ± 0.0020	0.0192 ± 0.0029 *	0.0305 ± 0.0022	0.0270 ± 0.0033
LABC (g)	0.6064 ± 0.1085	0.3482 ± 0.0684 *	0.3805 ± 0.0738 *	0.3114 ± 0.0572 *	0.6064 ± 0.1085	0.3101 ± 0.0520 *	0.3797 ± 0.0603 *	0.2885 ± 0.0602 *
LABC/Term BW (%)	0.1732 ± 0.0339	0.1072 ± 0.0212 *	0.1252 ± 0.0174 *	0.1006 ± 0.0152 *	0.1732 ± 0.0339	0.0924 ± 0.0136 *	0.1507 ± 0.0234	0.1072 ± 0.0238 *
Liver Wt (g)	14.40 ± 1.420	13.46 ± 1.36	12.61 ± 2.16	22.70 ± 2.70 *	14.40 ± 1.42	13.91 ± 1.07	10.03 ± 1.17 *	19.71 ± 2.40 *
Liver /Term BW (%)	4.093 ± 0.226	4.137 ± 0.285	4.149 ± 0.426	7.327 ± 0.225 *	4.093 ± 0.226	4.148 ± 0.119	3.966 ± 0.329	7.270 ± 0.426 *
Sem Ves (g)	0.7065 ± 0.1416	0.2613 ± 0.0230 *	0.4271 ± 0.0667 *	0.3255 ± 0.0826 *	0.7065 ± 0.1416	0.1618 ± 0.0385 *	0.4903 ± 0.1016 *	0.3065 ± 0.0814 *
Sem Ves/ Term BW (%)	0.2006 ± 0.0337	0.0807 ± 0.0092 *	0.1411 ± 0.0156 *	0.1043 ± 0.0186 *	0.2006 ± 0.0337	0.0489 ± 0.0141 *	0.1943 ± 0.0401	0.1145 ± 0.0334 *
Ventral Prostate (g)	0.1930 ± 0.0311	0.0692 ± 0.0074 *	0.1053 ± 0.0317 *	0.0749 ± 0.0207 *	0.1930 ± 0.0311	0.0330 ± 0.0096 *	0.1248 ± 0.0097 *	0.0886 ± 0.0225 *
Vent Pros	0.0547 ± 0.0058	0.0214 ± 0.0026 *	0.0349 ± 0.0100 *	0.0241 ± 0.0062 *	0.0547 ± 0.0058	0.0100 ± 0.0033 *	0.0495 ± 0.0044	0.0328 ± 0.0081 *
n=	4	6	6	6	4	6	6	6

Table S2

Summary of absolute and relative mean organ weights ± standard deviation for the Hershberger assay.

* Statistically significant ($p < 0.05$) using Dunnett's test.

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3.3. **Anti-androgenic effects of positive control chemicals by gavage administration in the Hershberger assay**

For the Hershberger assay, flutamide, linuron, and 4,4'-DDE were used as the positive control chemicals for evaluation of the difference in responses when the test substances were administered by oral gavage or via the diet. By gavage administration, no effects on terminal body weights were observed for flutamide while there was a 14% (statistically significant) and 12% (not statistically significant) reduction in mean terminal body weight for animals dosed by gavage with linuron and 4,4'-DDE (Table 3B) when compared to the control group. However, body weight gain compared to body weight at study initiation (29, 19 and 22%) and food consumption (-12, -31 and -22%) were reduced compared to controls for flutamide, linuron, and 4,4'-DDE, respectively, with all of these changes except body weight gain for animal receiving flutamide achieving statistical significance. Absolute (58%) and relative (79%) liver weights were statistically significantly increased when compared to the control group following 4,4'-DDE administration, but were not affected by linuron or flutamide treatment (Table S2).

Gavage administration of all three positive control chemicals reduced TP-induced secondary sex organ weight increases. When compared to organ weights from the control group, flutamide caused a statistically significant decrease in absolute (-52, -43, -63, -64%, respectively) and relative (-48, -38, -60 and -61%, respectively) Cowper's gland, LABC, seminal vesicle, and ventral prostate gland weights after gavage administration (Fig. 2A/B). A similar profile was observed with linuron, where statistically significant decreases in absolute Cowper's gland, LABC, seminal vesicle, and ventral prostate gland weights (30, 37, 40 and 46%, respectively) were observed after gavage administration. For linuron, statistically significant decreases in relative organ weights were also observed for the LABC, seminal vesicle, and ventral prostate

gland (-28, -30 and -36%) when compared to the control group. For 4,4'-DDE, statistically significant decreases in absolute (-43, -49, -54 and -61%, respectively) and relative (-35, -42, -48 and -56%, respectively) Cowper's gland, LABC, seminal vesicle and ventral prostate gland weights were observed after gavage administration (Fig. 2A/B). In addition, a statistically significant decrease in the relative glans penis weight was observed with treatment of 4,4'-DDE.

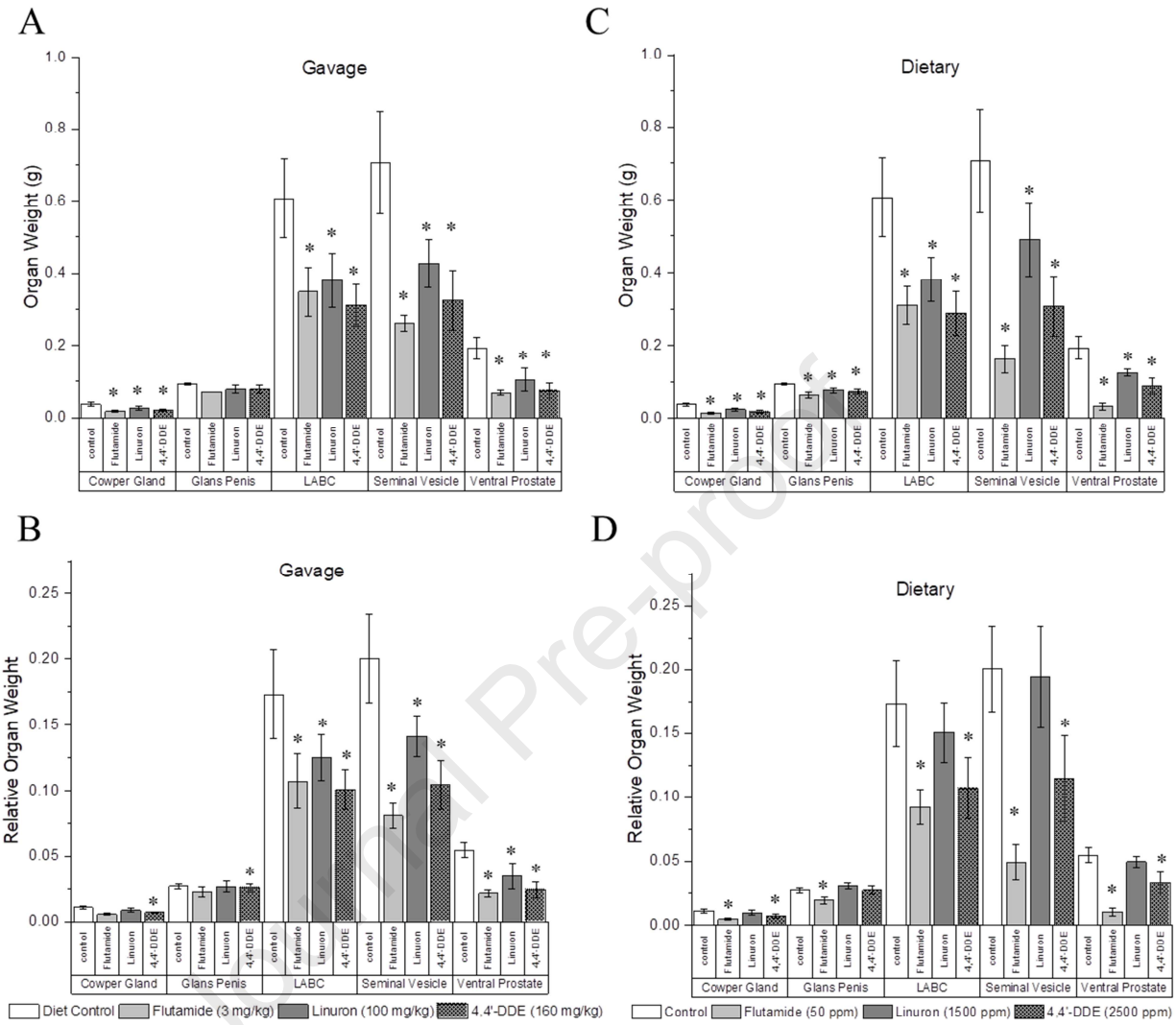

Fig. 2 Absolute (A,C) and relative (B,D) organ weights by gavage and dietary administration of flutamide, linuron and 4,4'-DDE over 10 days in castrated rats. Control rats for both gavage and dietary administration were dosed by dietary administration, n=4 rats/group. For all positive control chemicals, n= 6 rats/group. LABC = levator ani plus bulbocarvernosus muscle. * Statistically significant ($p < 0.05$) using Dunnett's test.

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3.4. **Anti-androgenic effects of positive control chemicals by dietary administration in the Hershberger assay**

The mean daily intake for flutamide, linuron, and 4,4-DDE was 4.0, 71.3, and 142.5 mg/kg/day, respectively (Table 3) when each were administered at dietary concentrations of 150 ppm, 1500 ppm, and 2500 ppm, respectively. All anti-androgen positive control chemicals reduced all of the TP-induced secondary sex organ weight increases by dietary administration (Fig. 2C/D). No statistically significant effects on terminal body weight were observed for flutamide, while there was a 28% and 23% reduction in mean terminal body weights for linuron and 4,4'-DDE, respectively. Overall body weight gains as compared to body weight at study initiation were also reduced by dietary administration of linuron and 4,4'-DDE (-0.6 and 6.9%, respectively). Food consumption was not significantly impacted with dietary administration of flutamide but was significantly reduced with linuron and 4,4'-DDE (-57 and -45%, respectively). Absolute (37%) and relative (78%) liver weights were increased significantly following 4,4'-DDE treatment compared to control. The absolute liver weight was decreased by 30% after linuron treatment, although no statistically significant change in relative liver weight was observed. Dietary administration with flutamide resulted in no statistically significant changes in either absolute or relative liver organ weights.

3.5. **Toxicokinetic evaluation in the uterotrophic and Hershberger Assays**

In the uterotrophic assay, blood from all rats including control group was collected on test day 5. Due to the low dosing concentration of EE2 required to see a positive response in this assay system (10 and 100 µg/kg/day), blood concentrations were below the limit of detection (5-10 pg EE2/mL of whole blood) and could not be evaluated for toxicokinetic parameters.

In the Hershberger assay, blood from all rats was collected on test day 9 and concentrations of linuron, 4,4'-DDE, or the flutamide metabolite hydroxyflutamide were measured, including the control group. All three positive control chemicals were not detected in the blood samples collected from control group. Figure 3 shows the toxicokinetic profiles of each positive control chemical over a 24-hour time period following dietary and oral gavage administration. Oral gavage resulted in a typical absorption profile with blood concentrations climbing to a maximum (C_{max}) and then declining to a C_{min} for each positive control chemical. In contrast, dietary intake resulted in steadier blood concentrations over the 24-hour time course. Hydroxyflutamide mean blood concentrations from dietary administration range from 270 to 534 ng/mL (2-fold change) vs 34.3 to 572 ng/mL (17-fold change) by gavage. Linuron mean blood concentrations from dietary administration ranged from 269 to 537 ng/mL (2-fold change) vs 46.3 to 2350 ng/mL (51-fold change) by gavage. 4,4'-DDE mean blood concentrations from dietary administration ranged from 23700 to 24800 ng/mL (~1-fold change) vs 144200 to 32900 ng/mL (2.3-fold change) by gavage. The mean daily dietary intakes were 1.35-, 0.713-, and 0.894-fold of the gavage dose for the three corresponding positive control substances, respectively (Table S3). The dietary dose-normalized C_{max} values (Figure 4A) were 62.7%, 49.2%, and 86.0% of the corresponding dose-normalized C_{max} values from oral gavage administration for hydroxyflutamide, linuron, and 4,4-DDE, respectively.

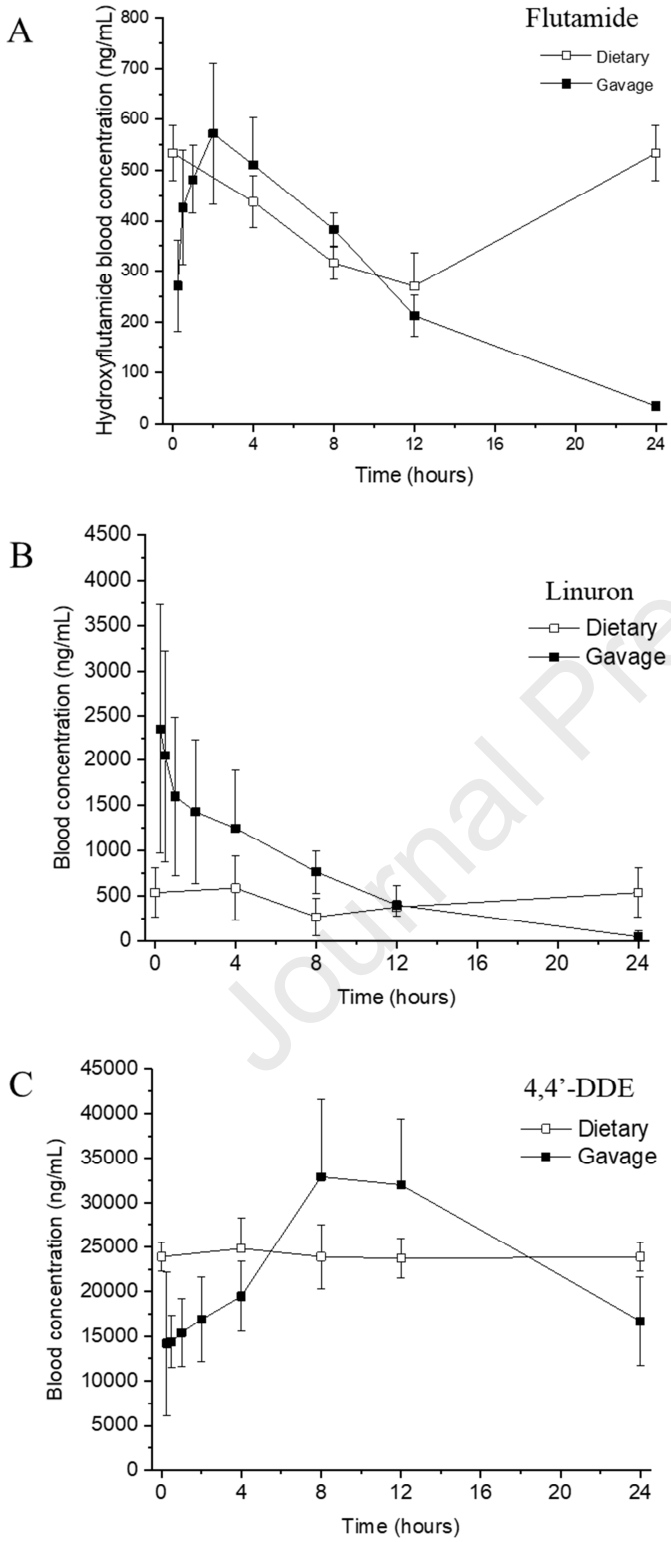

Fig. 3 Hershberger assay blood concentrations in non-fasted rats over 24 hours on day 9 of dosing via dietary and gavage administration for: (A) flutamide (hydroxyflutamide metabolite) at 4.1 (diet) and 3 (gavage) mg/kg/day , (B) linuron at 71.3 (diet) and 100 (gavage) mg/kg/day and (C) 4,4'-DDE at 143 (gavage) and 160 (diet) mg/kg/day.

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	Blood concentration (ng/mL)						Blood concentration (ng/mL)					
	Flutamide		Linuron		4,4'-DDE		Flutamide		Linuron		4,4'-DDE	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	Toxicokinetic parameters						Toxicokinetic parameters					
Dietary concentration (ppm)	50		1500		2500							
Gavage dose (mg/kg/day)							3		100		160	
Mean daily intake day 1 to 11 (mg/kg/day)	4.06	0.11	71.3	10.5	143	9						
Day 9, AUC_{24} (h*ng/mL)	9440	1140	10800	1520	575000	48400	6410	409	15000	4890	593000	114000
Day 9, AUC_{24}/dose (h*kg*ng/mL/mg)	2320	241	155	42	4040	315	2140	136	150	49	3710	713
Day 9, C_{max} (ng/mL)	534	54	844	193	26100	3010	628	59	2500	1230	34300	9030
Day 9, C_{max}/dose (kg*ng/mL/mg)	131	11	12.3	4.2	184	22	209	20	25	12.3	214	57
Day 9, AUC_{12} (h*ng/mL)	4620	467	5260	1840	290000	30400						

Table S3
Summary of Hershberger assay toxicokinetic data as represented in figure 3 for flutamide (hydroxyflutamide metabolite), linuron and 4,4'-DDE.

The mean dose-normalized area under the curve for the 24-hour timeframe (AUC_{24}) from dietary intake was 108%, 103%, and 109% of the corresponding mean dose-normalized AUC_{24} from oral gavage for hydroxyflutamide (2320 ± 241 vs 2140 ± 136 ng.hr.kg/mL/mg), linuron (155 ± 42 vs 150 ± 49 ng.hr.kg/mL/mg), or 4,4'-DDE (4040 ± 315 vs 3710 ± 713 ng.hr.kg/mL/mg), respectively (Figure 4B and Table S3). The dose normalized AUC_{24} from dietary intake was not significantly different from the oral gavage for all three testing compounds, illustrating that both dietary and oral gavage administration resulted in essentially equivalent systemic exposure. These results suggest that the extent of absorption for the three positive control substances is independent of the route of dose administration.

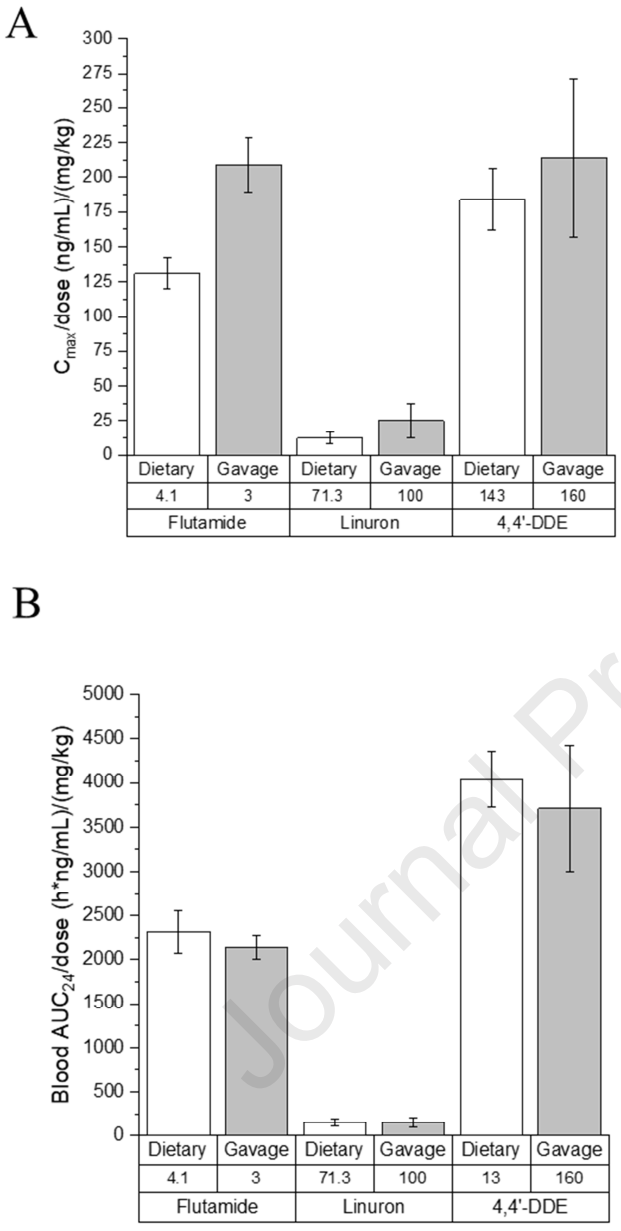

Fig. 4 Hershberger assay (A) dose normalized maximum blood concentrations (Cmax) and (B) dose normalized area-under-the-curve values (AUC_{24h}) in non-fasted rats on day 9 of dosing via dietary and gavage administration for flutamide (hydroxyflutamide metabolite), linuron, and 4,4'-DDE. Dosing concentrations are mg/kg/day.

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4. DISCUSSION

For both the uterotrophic and Hershberger assays, the test guidelines specify that test substances are to be administered daily by either subcutaneous injection or oral gavage. However, the guidelines also state that the relevance to humans should be considered when determining the route of administration. In the present study, positive control chemicals were administered by either diet administration or oral gavage in order to assess the sensitivity of each administration method for identifying endocrine active test substances in both the uterotrophic and Hershberger assays.

In the Hershberger study, significant body weight decreases in excess of a typical maximum tolerated dose (10% difference in terminal body weight compared to the control group) were observed for linuron and 4,4'-DDE after dietary administration. Body weight decreases were also observed by gavage administration, and were also in excess of the MTD, but to a lesser extent. Consistent with the body weight decrements observed, food consumption was reduced two-fold when compared to gavage administration, suggesting that there may be an effect on palatability that may be contributing to the more severe body weight effects observed after dietary administration. For the Hershberger assay, the test guidelines specify that the highest dose should not cause a reduction in the terminal body weight greater than 10% of control weight (OECD, 2009; EPA, 2009a). Previous studies in male Wistar rats have shown that the weights of secondary sex organs such as the epididymis, ventral prostate, and seminal vesicles in males can be impacted by body weight loss exceeding 10% of control (Laws, 2007; O'Connor, 2000). Therefore, the potential for reduced food consumption and body weight losses must be considered when using the dietary route of exposure for the Hershberger assay, as potential

differences in food consumption and/or body weight decrements can be significant between the different routes of exposure. Despite the body weight losses observed after dietary administration of linuron and 4,4'-DDE, the results from the Hershberger assay demonstrate that positive controls chemicals can be correctly identified when the test substances is administered by either gavage or via the diet, and the sensitivity for both routes of exposure were similar.

In the uterotrophic assay, body weight decreases were also observed at the highest dose of EE2, although it was comparable for both the gavage and dietary routes of administration, and food consumption was also comparable for both routes of test substance administration. In contrast to the Hershberger assay, the uterotrophic assay test guidelines indicates that the body and uterine weights are not correlated (OECD, 2007; EPA, 2009b). When body weight loss was evaluated in female Wistar rats, statistically significant decreases in the wet and blotted uterine weight were not observed, although a trend was observed when the body weight loss was equal to 40% (Laws *et al*. al., 2007). While there is still potential for confound results in the uterotrophic assay under conditions of severe body weight loss, the short duration of the test helps to reduce the likelihood of exceeding a typical maximum-tolerated dose. While body weight effects were not a factor in the current study, the results from the uterotrophic assay were similar to those from the Hershberger assay and demonstrate that positive control chemicals can be correctly identified when administered by either gavage or diet. Therefore, the sensitivity for both routes of exposure are essentially equivalent.

While the test guidelines indicate that oral gavage is the surrogate model for ingestion, kinetics between different routes of oral exposure such as gavage and dietary exposure can be dramatic (Hannas *et al.*, 2016; Gayrard *et al.*, 2013; Sieli *et al.*, 2011; Atcha *et al.*, 2010). By measuring

436 the concentration of the test substance in the blood, toxicokinetic analysis can generate both
437 AUC_{24} and C_{max} values, and differences in these measurements provide insight into the
438 endocrine findings. In this study toxicokinetic data was generated by collecting blood and
439 evaluating positive control chemical concentrations at specific timepoints. In the uterotrophic
440 assay, the dose required to elicit an estrogenic effect were in the µg/kg/day range. Although the
441 EE2 limit of detection for the developed analytical method was 5-10 pg/mL, at the timepoints
442 evaluated with the volume of blood collected, EE2 was not quantifiable. Therefore, we were
443 unable to correlate administered dose via either dosing regimen with toxicokinetic parameters of
444 exposure.

445 In contrast, due to the higher dose levels administered in the Hershberger assay, toxicokinetic
446 parameters could be evaluated. The daily exposure by dietary and gavage administration was
447 similar for flutamide, linuron, and 4,4'-DDE. Overall, a higher C_{max} was observed when each of
448 the positive control chemicals was administered by gavage. The C_{max} normalized by dose for
449 flutamide and 4,4'-DDE for gavage administration was within 1.6-fold of the dose-normalized
450 C_{max} for dietary administration, while the dose-normalized C_{max} of linuron was 2-fold greater by
451 gavage relative to the corresponding dose-normalized C_{max} by diet. When comparing blood
452 concentrations across all 3 positive control chemicals, blood concentrations of the test substances
453 after dietary administration remained relatively stable over the 24-hour measurement period.

454 *In vitro* assays such as the estrogen and androgen receptor binding assays and
455 corresponding transcriptional activation assays can be helpful to determine the likelihood of
456 identifying a response in the uterotrophic and Hershberger assays. Blood concentration data from
457 the toxicokinetic analysis can support these predictions. In a very simple *in vitro* to *in vivo*

458 extrapolation, the EC_{50} value in an *in vitro* binding experiment can be compared to the blood
459 concentrations achieved through dosing *in vivo*. If the test substance (ligand) reaches a
460 concentration *in vivo* at which test substance is available to bind to the receptor, a response may
461 be elicited, assuming that the cascade of events in the endocrine pathway occur. The degree of
462 effect will depend on the potency of the test substance and the duration that the test substance
463 concentration is maintained (Salahudeen and Nishtala, 2017). Therefore, the choice for route of
464 administration in these endocrine assays is important, as test substance toxicokinetics may lead
465 to varying organ weight changes based on the blood concentration of the test substance that is
466 achieved *in vivo*.

467 When conducting repeat dose studies such as the uterotrophic and Hershberger assays, it
468 is important to reduce the potential for effects that could confound the ability to interpret apical
469 outcomes. For example, initial exposures by gavage (i.e., high C_{max}) may induce acute toxicity
470 leading to adverse effects in test animals that would not be observed by diet administration
471 where variations between C_{max} and C_{min} are more limited. Dosing via oral gavage may also
472 result in generalized stress and increased risk of injury to the animal as a result of perforation of
473 the esophagus (Reviewed in Vandenberg *et al*., 2014; Balcombe *et al*., 2004). Further, dosing by
474 oral gavage results in bypass of initial digestion and absorption that occurs via mucosal surfaces
475 of the oral cavity that may impact the overall systemic exposure (Madhav *et al.*, 2012). At the
476 same time, the palatability of test diets can result in significant decreases in food consumption
477 and subsequent body weight reductions. Multiple factors therefore must be considered when
478 selecting the most appropriate route of administration.

479 The objective of the current study was to compare the differences in responses observed
480 in the uterotrophic and Hershberger assays with several positive control chemicals by both the

dietary and oral gavage routes of administration. For both the uterotrophic assay and the Hershberger assay, the data demonstrate that the test substances were identified for their potential endocrine effects whether the test substances were administered by diet or oral gavage. In addition, the effects that were observed after both administration methods showed similar magnitudes of change and an overall similarity in the sensitivity for detecting endocrine effects. The pharmacokinetic data from the Hershberger assay show that the daily systemic exposures by dietary and gavage administration were nearly equivalent for all three test substances, which is consistent with the similar degree in organ weight responses observed by both routes. Taken together, the results of this study illustrate that the dietary route of exposure is a valid alternative dosing method for both the uterotrophic and Hershberger assays and should be considered for test substances where the dietary route is more relevant for assessing the potential for human exposure.

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598 Figure Legends:

599 Fig. 1 Absolute (A,C) and relative (B,D) uterus weights by gavage and dietary administration of
600 EE2 over 5 days in ovariectomized rats. (n= 6 rats/group) * Statistically significant ($p < 0.05$)
601 using Dunnett's test.

602 Fig. 2 Absolute (A,C) and relative (B,D) organ weights by gavage and dietary administration of
603 flutamide, linuron and 4,4'-DDE over 10 days in castrated rats. Control rats for both gavage and
604 dietary administration were dosed by dietary administration, n=4 rats/group. For all positive
605 control chemicals, n= 6 rats/group. LABC = levator ani plus bulbocarvernosus muscle. *
606 Statistically significant ($p < 0.05$) using Dunnett's test.

607 Fig. 3 Hershberger assay blood concentrations in non-fasted rats over 24 hours on day 9 of
608 dosing via dietary and gavage administration for: (A) flutamide (hydroxyflutamide metabolite) at
609 4.1 (diet) and 3 (gavage) mg/kg/day , (B) linuron at 71.3 (diet) and 100 (gavage) mg/kg/day and
610 (C) 4,4'-DDE at 143 (gavage) and 160 (diet) mg/kg/day.

611 Fig. 4 Hershberger assay (A) dose normalized maximum blood concentrations (Cmax) and (B)
612 dose normalized area-under-the-curve values (AUC_{24h}) in non-fasted rats on day 9 of dosing via
613 dietary and gavage administration for flutamide (hydroxyflutamide metabolite), linuron, and
614 4,4'-DDE. Dosing concentrations are mg/kg/day.

615 Fig. S1: Hershberger assay diet and blood sample analyses.

616 An aliquot of 4 gram of each diet sample was extracted with 40 mL acetonitrile by grinding at
617 1400 stokes/min for 5 minutes, then followed by centrifugation at 4250 rpm for 20 minutes. The

618 supernatant was analyzed for diet concentration determination by UHPLC coupled with tandem
619 mass spectrometry (LC/MS/MS) by multiple reaction monitoring (MRM). Blood samples were
620 evaluated for concentrations of flutamide, hydroxyflutamide, and linuron using LC/MS/MS.
621 Briefly, 30 µL of blood sample was mixed with 90 µL of 0.1 M $ZnSO_4$ and extracted with 360
622 µL of acetonitrile containing 0.1% formic acid by protein precipitation. The extracts were
623 injected into the LC/MS/MS for concentration determination. The UHPLC system was an
624 Agilent 1290 infinity directly connected to electrospray ionization mass spectrometer, AB Sciex
625 Qtrap 5500. The column used was Phenomenex Kinetex ®XB-C18, 100Å, 2.1×30 mm, 2.6 µm
626 and kept at 30°C with a gradient separation. The gradient started from 5% mobile phase B
627 (acetonitrile containing 0.1% formic acid) and 95% mobile phase A (water containing 0.1%
628 formic acid), linearly raised to 95% mobile phase B at 3 minutes and stepped back down to 5%
629 mobile phase B for equilibrium. The mass spectrometer was operated at negative mode
630 monitoring MRM transitions for Flutamide (275→205) and Hydroxyflutamide (291→205), and
631 at positive mode for monitoring Linuron (249→133). Analyst® software was used for
632 calibration curve construction and sample concentration determination. 4,4-DDE was evaluated
633 with gas chromatography coupled with MS detection (GC/MS, Agilent 7890A gas
634 chromatograph with 5975C inert XL EC/CI MSD) by selected ion monitoring (SIM). The GC
635 column used was Agilent J&W DB-1701, 30 m × 250 µm × 0.25 µm. The oven temperature was
636 hold at 100°C for 0.5 minutes, then raised at 25°C/minute to 280°C and hold for 2 min with a
637 total run time of 9.7 min. SIM was set to monitoring ion of 318.

638 Uterotrophic assay blood sample analysis.

Prior to analysis, each blood sample was thawed and diluted 1:1 with HPLC grade water. 20 µL of each sample was partitioned against 200 µL of HPLC grade ethyl acetate and the organic layer transferred to a 1.5 mL Eppendorf tube. Each tube was then taken to dryness under a low flow of nitrogen gas and the dried residue reconstituted with 20 µL of a solution composed of 60% methanol, 40% water and 1 ng/mL of 2,4,16,16-d4 EE2 utilized as an internal standard. After vortex mixing for 30s to ensure homogeneity, the samples were analyzed for EE2 via LC/MS/MS (Agilent 1290 Infinity II system running 3mM ammonium fluoride as solvent A and methanol as solvent B; Restek Raptor Biphenyl 3.0 mm x 10 cm, 2.7 µm). The gradient program started at 60% methanol and progressed to 99% over 4 minutes. Following a 2.5 minute isocratic hold at 99% methanol, the gradient returned its starting condition to re-equilibrate the column. This system was coupled to an ABSciex QTRAP 6500+ configured with an ESI source operated in negative ion mode and utilized multiple reaction monitoring (MRM) to quantify the residue of EE2. The method performance was validated at 40 pg EE2/mL of whole blood (LLOQ), with a mean recovery of 80 ± 11% and individual recoveries falling between 73 and 93% (N=5). The method LOD was estimated to be 5 - 10 pg EE2/mL of whole blood.

A

Uterotrophic Assay		Dose (µg/kg bw/day)	Terminal Body Weight (g)	Body Weight versus control (%)	Body Weight Gain (g)	Body Weight Gain (%)	Food Consumption (g/day)	Food Consumption versus control (%)
Control	Gavage	-	311.7 ± 20.4	-	25.3 ± 6.5	8.9	19.2 ± 1.6	-
EE2 (low)		10	294.8 ± 21.7	-5.4	9.2 ± 2.1 *	3.2	14.6 ± 1.6 *	-23.6
EE2 (high)		100	278.4 ± 16.1 *	-10.7	-4.9 ± 5.9 *	-1.7	12.5 ± 0.6 *	-34.9
control	Diet	-	310.2 ± 18.8	-	24.2 ± 6.4	8.4	19.1 ± 1.0	-
EE2 (low)		9.3	282.5 ± 12.3 *	-8.9	-2.9 ± 7.2	-1.0	12.9 ± 0.5 *	-32.5
EE2 (high)		77.6	267.0 ± 13.1 *	-13.9	-14.9 ± 6.9	-5.24	10.5 ± 0.5 *	-45.0

B

Hershberger Assay	Route	Dose (mg/kg bw/day)	Terminal Body Weight (g)	Body Weight versus control (%)	Body Weight Gain (g)	Body Weight Gain (%)	Food Consumption (g/day)	Food Consumption versus control (%)
Control	Diet	-	351.2 ± 19.0	-	95.2 ±10.7	37.2	26.3 ± 1.1	-
Flutamide	Gavage	3	325.3 ± 25.5	-7.4	73.5 ± 10.2	29.2	23.2 ± 1.2 *	-11.7
Linuron		100	302.3 ± 28.4 *	-13.9	48.6 ± 16.2 *	19.2	18.1 ± 2.7 *	-31.0
4,4'-DDE		160	309.3 ± 29.4	-11.9	56.2 ± 14.6 *	22.2	20.4 ± 0.7 *	-22.2
Control	Diet	-	351.2 ± 19.0	-	95.2 ±10.7	37.2	26.3 ± 1.1	-
Flutamide		4.1	335.5 ± 27.0	-4.5	80.8 ± 11.6	31.7	24.2 ± 1.8	-7.8
Linuron		71.3	252.5 ± 15.6 *	-28.1	-1.6 ± 14.2 *	-0.6	11.4 ± 1.3 *	-56.7
4,4'-DDE		142.6	271.0 ± 26.5 *	-22.8	17.5 ± 17.3 *	6.9	14.5 ± 1.5 *	-44.8

Table 3

Summary of in-life parameters as mean ± standard deviation for the (A) Uterotrophic and (B) Hershberger assays. Six ovariectomized female rats in the uterotrophic assay and 6 castrated rats in the Hershberger assay were used per treatment group. A single group of 4 castrated rats administered in the diet were used for the Hershberger controls. * Statistically significant ($p < 0.05$) using Dunnett's test.

Evaluation of dietary dose administration as an alternative to oral gavage in the rodent uterotrophic and Hershberger assays

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Highlights:

- Hershberger and uterotrophic assay guidelines specify test substance is administered by subcutaneous injection or gavage
- Positive control chemical evaluation suggests dietary administration is also an acceptable route of exposure
- Dietary administration is a more appropriate environmental exposure scenarios with potential human intake via crop residues

Evaluation of dietary dose administration as an alternative to oral gavage in the rodent uterotrophic and Hershberger assays

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Lauren Markell, John C. O'Connor, Brian Sayers, Robert Mingoia were responsible for the study design, data collection, analysis and interpretation of the data. Ruijuan Luo and Joseph Klems provided additional analysis once the *in vivo* experiments had been conducted. FMC Corporation provided internal clearance to submit this manuscript to Regulatory Toxicology and Pharmacology.

Declaration of interests:

Evaluation of dietary dose administration as an alternative to oral gavage in the rodent uterotrophic and Hershberger assays

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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