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ERRATUM

Erratum to: The OECD validation program of the H295R steroidogenesis assay: Phase 3. Final inter-laboratory validation study

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In the original article wrong unites were quoted in Table 3 (page 508) and Table 4 (page 510) as well as in the paragraph 3.2 Core chemical exposure experiments on page 509. Also in paragraph 2.3 Selection and testing of chemicals the link to the Supplemental Materials (ESM) was missing. The correct versions of the tables and the paragraph as well as the ESM link are provided below.

3.2 Core chemical exposure experiments

There were chemical-specific differences in the response of T production after exposure of H295R cells to the 12 core chemicals (Table 3). With a few exceptions, the observed chemical-specific responses of T production were comparable among laboratories and could be grouped into three different types of effects: inducers, inhibitors, and negative reference chemicals. Among the inducers, exposure to trilostane resulted in the greatest fold changes (>10-fold induction) in T concentra-

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tion when compared to SCs. The least fold changes were observed for the atrazine exposures where induction of T production was less than 1.5-fold with the exception of Lab 2, at which maximum induction was 2.4-fold. No effect on T production was observed after exposure to atrazine at Lab 6. Exposure to prochloraz resulted in a greater than 15-fold reduction of T production at the greatest concentration tested (100 μM) at all laboratories with the exception of Lab 4 where an up to 4.5-fold reduction was observed. The greater LOEC reported for Lab 2 is likely a function of the relatively great variation among replicate experiments at 0.01 μM (CV=35%). It is unclear why T production by cells was more sensitive to the exposure with prochloraz at Labs 1 and 3. However, a concentration-dependent response was observed starting at 0.01 μM, which is similar to the response patterns at the other labs. Therefore, it cannot be excluded that the significant reduction at 0.0001 and 0.001 μM represents an artifact. Exposure to the other inhibitors resulted in less than 4-fold changes in T production. When chemicals exhibited a less than 1.5-fold change in T production, they were categorized as negatives. This threshold was defined based on the average variation observed across all laboratories among replicate experiments. Some of these negative chemicals could have been categorized as inhibitors in individual cases (molinate: Lab 4; benomyl: Lab 1). However, even in situations where inhibition was observed at an individual laboratory, changes were always less than 2-fold and typically were not concentration-dependent. For instance, exposure to nonoxynol-9 resulted in a decrease in T concentrations at non-cytotoxic concentrations at two of five laboratories for which data was available. Relative to the SCs, inhibition of T production at Lab 1 was 29% (1 μM), while at Lab 2, it was 47% (10 μM). However, it should be noted that, at Lab 2, exposure to 10 μM nonoxynol-9 resulted in an average increase in cell viability (138% viable cells relative to the SCs), and thus the observed reduction in T production may be an artifact due to the correction for cell viability, especially as no such increase was observed by any of the other groups. The greatest letrozole concentration resulted in a significant decrease in T at all laboratories.

Table 3 Lowest observed effect concentrations (LOECs; measured by Dunnett's or Mann Whitney U test [mu]) and strength and direction of change (⇩= >0.5-fold; ⇩⇩ = 0.5- to >0.25-fold; ⇩⇩⇩ = 0.25- to >0.1-fold; ⇩⇩⇩⇩ = ≤0.1-fold; ⇧ = <2-fold; ⇧⇧ = 2- to <fold; ⇧⇧⇧ = 4- to <20-fold; ⇧⇧⇧⇧ = ≥20-fold) for testosterone (T) and estradiol (E2) after exposure to the twelve core chemicals. Ranges refer to maximum values measured in repeated experiments. nd – not detectable; — chemical not analyzed. Gray shaded cells – uncertainty due to interference of the antibody based hormone detection system with the test chemical

	Fold-Change (Testosterone)									
	Lab 1[a]		Lab 2		Lab 3		Lab 4		Lab 6	
	LOEC [μM]	*Max Change*	*LOEC [μM]*	*Max Change*	*LOEC [μM]*	*Max Change*	*LOEC [μM]*	*Max Change*	*LOEC [μM]*	*Max Change*
Prochloraz	0.0001	⇩⇩⇩⇩	0.1	⇩⇩⇩⇩	0.0001	⇩⇩⇩⇩	0.01	⇩⇩⇩⇩	0.01	⇩⇩⇩
Aminoglutethimide	100[d]	⇩⇩⇩	100[d]	⇩⇩	10	⇩⇩⇩	100[d]	⇩⇩	100[d]	⇩⇩⇩
Letrozole	100[d]	⇩⇩	100[d]	⇩⇩	100[a,d]	⇩⇩	100[d]	⇩⇩	100[d]	⇩⇩
Nonoxynol-9	10[c,d]	⇩	10[c,d]	⇩	nd[e]		10[c,d]	⇩	10[c,d]	⇩⇩
Molinate	nd		nd		100	⇩	nd		nd	
Benomyl	nd		nd		nd		nd [mu]		nd	
EDS	nd		nd		nd		nd		nd	
HCG	nd		nd		nd		nd		nd	
Paraben	10	⇧	nd		1	⇧	nd		nd	
Atrazine	100[d]	⇧	1	⇧	100[d]	⇧	nd		nd	
Forskolin	10	⇧⇧	1	⇧⇧	1	⇧	1	⇧⇧	1	⇧⇧
Trilostane	0.1 [mu]	⇧⇧⇧	0.01 [mu]	⇧⇧⇧	1 [mu]	⇧⇧⇧⇧	1 [mu]	⇧⇧⇧⇧	0.01 [mu]	⇧⇧⇧⇧
	Fold-Change (Estradiol)									
	Lab 1[a]		**Lab 2**		**Lab 3**		**Lab 4**		**Lab 6**	
	LOEC [μM]	*Max Change*	*LOEC [μM]*	*Max Change*	*LOEC [μM]*	*Max Change*	*LOEC [μM]*	*Max Change*	*LOEC [μM]*	*Max Change*
Letrozole	0.001	⇩⇩	0.001	⇩⇩	0.0001 [mu]	⇩⇩⇩	0.01	⇩⇩⇩	0.01	⇩⇩
Prochloraz	0.1	⇩⇩	1	⇩	0.1	⇩⇩	1	⇩⇩	0.1	⇩⇩
Aminoglutethimide	100[d]	⇩⇩	10 [mu]	⇩⇩	10	⇩⇩	100[b,d]	⇩⇩	100[d]	⇩⇩
Benomyl	nd		nd		nd[a]		nd[a]		nd	
EDS	nd		nd		nd		nd		nd	
Nonoxynol-9	nd		nd		nd		nd		nd	
HCG	nd		nd	⇧	nd[a]		nd[a]		nd	
Paraben	nd [mu]	⇧⇧⇧	10	⇧	10 [mu]	⇧⇧	nd	⇧⇧	nd	
Molinate	100[d]	⇧⇧ [mu]	100[d]	⇧	100[d] [mu]	⇧⇧	100[d] [mu]	⇧⇧	100[d]	⇧⇧
Atrazine	10	⇧⇧⇧	1 [mu]	⇧⇧⇧	1 [mu]	⇧⇧⇧⇧	10 [mu]	⇧⇧⇧	0.1	⇧
Forskolin	0.01 [mu]	⇧⇧⇧⇧	0.1 [mu]	⇧⇧⇧⇧	0.1 [mu]	⇧⇧⇧⇧	0.1 [mu]	⇧⇧⇧⇧	0.01 [mu]	⇧⇧
Trilostane	1 [mu]	⇧⇧⇧⇧	100	⇧	0.1 [mu]	⇧⇧⇧⇧	1 [mu]	⇧⇧⇧⇧	1 [mu]	⇧⇧⇧

[a] Only one experiment was conducted or considered for data evaluation

[b] Not statistically significant; $p = 0.051$

[c] Greatest concentration cytotoxic

[d] Effects occurred at greatest non-cytotoxic concentration; no dose-response

[e] Cytotoxicity observed at concentration at which effects occurred at other laboratories = 10

Table 4 Lowest observed effect concentrations (LOECs; measured by Dunnett's test) and strength and direction of change (⇓ = >0.5-fold; ⇓⇓ = 0.5- to >0.25-fold; ⇓⇓⇓ = 0.25- to >0.1-fold; ⇓⇓⇓⇓ = ≤0.1-fold; ⇑ = <2-fold; ⇑⇑ = 2- to <fold; ⇑⇑⇑ = 4- to <20-fold; ⇑⇑⇑⇑ = ≥20-fold) observed for the 16 test chemicals. nd – not detectable. Chemicals tested at the second laboratories (2nd Lab) were as follows; Lab2: Piperonyl butoxide, ketoconazole, prometon, DEHP, flutamide and danazol; Lab3: Bisphenol A, fenarimol, genistein, finasteride and dinitrophenol; Lab4: Spironolactone, mifepristone, tricrecyl phosphate, dimethoate and glyphosate

	Testosterone			
	LOEC [μM]		Max Change	
	1st Lab[b]	2nd Lab[c]	1st Lab	2nd Lab
Ketoconazole	1	1	⇓⇓⇓	⇓⇓⇓
Genistein	10	10	⇓⇓	⇓⇓⇓
Finasteride	10	100[d]	⇓⇓	⇓⇓
Bisphenol A	10	10	⇓⇓	⇓
Dinitrophenol	0.0001	100[d]	⇓	⇓⇓
Piperonyl butoxide	10	10	⇓	⇓
Spironolactone	1	1	⇓⇓⇓	⇓⇓
Fenarimol	nd	10	nd	⇓⇓
Danazol	nd	nd	nd	nd
DEHP	nd	nd	nd	nd
Dimethoate	nd	nd	nd	nd
Flutamide	nd	nd	nd	nd
Glyphosate	nd	nd	nd	nd
Prometon	nd	nd	nd	nd
Tricrecyl phosphate	10	nd	⇑	nd
Mifepristone	0.1	nd	⇑	nd

	Estradiol			
	LOEC [μM]		Max Change	
	1st Lab	2nd Lab	1st Lab	2nd Lab
Danazol	1	10	⇓⇓⇓	⇓⇓
Ketoconazole	10	10	⇓⇓	⇓⇓
Fenarimol	nd	1	nd	⇓⇓
Finasteride	nd	100[d]	nd	⇓
Glyphosate	nd	nd	nd	nd
Dinitrophenol	nd	nd	nd	nd
Spironolactone	nd	nd	nd	nd
Piperonyl butoxide	nd	nd	nd	nd
Dimethoate	10	nd	⇑⇑	nd
Flutamide	10	nd	⇑⇑	nd
Tricrecyl phosphate	10	nd	⇑⇑⇑	nd
Bisphenol A	10	1	⇑⇑	⇑⇑
DEHP	1[a]	1	⇑⇑	⇑⇑
Mifepristone	0.1	1	⇑⇑	⇑⇑
Prometon	100[d]	100[d]	⇑⇑⇑⇑	⇑⇑
Genistein	10	10	⇑⇑⇑⇑	⇑⇑⇑⇑

[a] considered because there was a clear concentration-response at all but the greatest concentration

[b] lead laboratory (Lab 1)

[c] participating laboratory (Labs 2,3 and 4)

[d] Effects occurred at greatest non-cytotoxic concentration; no dose-response

Hecker et al. (Environ Sci Pollut Res (2011) 18:503–515)

Supplemental Materials

TEST CHEMICALS

Core Chemicals

Out of the 28 chemicals, a core set of 12 compounds was established that were analyzed by all laboratories participating in the validation studies (Table S1). Selection of these core chemicals was made in accordance with accepted guidelines for the validation of screening type test systems (OECD 2005, Hartung 2004). All compounds included in this core set were previously reported to exhibit a specific type of direct or indirect interaction with the production of T and/or E2 as measured either by means of the H295R Steroidogenesis Assay or by other steroidogenic tests including tissue explant assays and/or *in vivo* studies. In addition, this core chemical test set included a number of negative chemicals that were not expected to elicit any effect on the endpoints measured here at non-cytotoxic concentrations. The inclusion of such negative chemicals is of importance because it allows evaluation of the specificity of a test system with regard to the endpoints of interest. The suite of positive chemicals utilized in this core chemical set was chosen to reflect different types and strengths of interactions with the production of the hormones analyzed here.

***Table S1**: Core chemicals and their hypothesized mode of action selected for the H295R Steroidogenesis Assay validation studies. Due to the nature of the validation studies, conduct of experiments using coded chemicals, here we do not distinguish between core and supplementary chemicals. Chemicals are sorted in alphabetical order.*

Name	CAS #	Mode of action	Product class	Effect type
Aminoglutethimide	125-84-8	Inhibits CYP19 aromatase and other cytochrome P450 enzymes	Pharmaceutical (phased out)	Medium to weak inhibitor of T and E2 production.
Atrazine	1912-24-9	Aromatase inducer in vitro	Herbicide	Weak inducer of E2 production.
Benomyl	17804-35-2	Aromatase inducer in vitro	Fungicide	Weak inhibitor of T production; Weak inducer or negative for E2 production. Has been shown to induce aromatase activity in human ovarian tumor cells (KGN).
Ethane dimethane sulfonate (EDS)	4672-49-5	Cytotoxic		No effect expected at non-cytotoxic concentrations.
Forskolin	66575-29-9	Cyclic-AMP second messenger system	Pharmaceutical	Strong inducer of T and E2 production.
Human chorionic gonadotropin (hcG)	9002-61-3	Binds to GtH receptor	Peptide hormone	No effect on T and E2 production in H295R cells.
Letrozole	112809-51-5	Specifically inhibits catalytic aromatase activity.	Pharmacytical	Strong inhibitor of E2 production. Weak inhibitor of T production.

Molinate	2212-67-1	Anti-cholinesterase/ neurotoxicant. **Note:** In vitro, molinate is a poor inhibitor of esterase activity, whereas molinate sulfoxide, a major metabolite of molinate in rats, and molinate sulfone were shown to be potent inhibitors of esterase activity, suggesting that metabolic activation of molinate is required in vivo.	Pesticide	Weak inducer of E2 and negative/weak inhibitor of T production.
Nonoxynol-9	26027-38-3	Unknown	Excipients, Pharmaceutical aid [surfactant], Pharmaceutical aid [wetting and or solubilizing agent], Spermaticide	Unknown.
Paraben (Butyl paraben)	94-26-8	ER binder	Preservative in food, cosmetics, toiletries, pharmaceutical.	Weak inducer of E2, and weak inhibitor of T production.
Prochloraz	67747-09-5	General inhibitor of microsomal cytochrome P450 mixed function oxidases.	Fungicide	Strong inhibitor of T and E2 production.
Trilostane	13647-35-3	3B-HSD competitive inhibitor	Pharmaceutical, used in treatment of Cushings disease	Strong inducer of T and E2 production.

Supplementary chemicals

In addition to the 12 core chemicals described in the previous section, 16 compounds were selected for additional testing (Table S2). To reduce the burden on each laboratory, the 16 chemicals were divided into 3-subsets of 4-6 chemicals each and each subset was be tested by two laboratories.

Selection of these additional 16 chemicals was made based on the range of putative effects, as well as general toxic properties and technical feasibility (e.g. availability of the compound, ownership rights, etc.). The types of effects were categorized as strong, medium, and weak inducers and inhibitors of production of testosterone, estradiol, or both hormones as well as negative compounds. All decisions were discussed and made in agreement with the US-EPA and the OECD advisory group.

Table S2: *Supplemental chemicals and their hypothesized mode of action selected for the H295R Steroidogenesis Assay validation studies. Due to the nature of the validation studies, conduct of experiments using coded chemicals, here we do not distinguish between core and supplementary chemicals. Chemicals are sorted in alphabetical order.*

Name	CAS #	Mode of action	Product class	Effect type
2,4-Dinotrophenol	51-28-5	Cell toxicant: phosphorylation uncoupler	Industrial chemical	No known endocrine function other than cell toxicity and altered bioenergetics.
Bisphenol A	80-05-7	Cyclic-AMP second messenger system; proported ER binder	Monomer in polycarbonate plastics	Unknown. Some evidence that alters Progesterone in vitro, but mechanism may or may not be c-AMP second messenger system. For all steroidogenesis assay, will need to be specific for endpoint of assay. Tested positive for ER binding in vitro and in uterrotrophic assay.
Danazol	17230-88-5	3HSD; P450c17 (17 hydrolase/C17-20 lyase); 17KSR	Agricultural Chemical, Antineoplastic agents, Contraceptives, postcoital, synthetic, Drug / Therapeutic Agent	Unknown
Di (2-ethylhexyl) phthalate (DEHP)	117-81-7	Inhibits FSH-stimulated c*AMP* accumulation. Effects have been demonstrated at the level of P450scc and aromatase. **Note:** Compound that has been hypothesized to be active is the metabolite MEHP, not DEHP.	Polyvinyl additive	Metabolite monoethylhexyl phthalate (MEHP) has been shown to suppress aromatase and estradiol production in female rat primary granulosa cells. Parent compound is not considered active.
Fenarimol	60168-88-9	Aromatase inhibition	Fungicide	Shown to inhibit aromatase (CYP19) in vitro, evidence from in vivo studies not as unequivocal
Finasteride	98319-26-7	5-a reductase inhibitor	Pharmaceutical, therapeutic agent for prostrate cancer, hirsutism, and alopecia	Unknown
Flutamide	13311-84-7	P450c17 (17 hydrolase/C17-20lyase)	Pharmaceutical	Unknown
Genistein	446-72-0	Anti-oxidant, topoisomerase inhibitor/ tyrosine kinase inhibitor	Pharmaceutical	Weak inducer of E2 and weak inhibitor of T production. Weak estrogen receptor agonist
Glyphosate (Roundup)	1071-83-6		Herbicide	Unknown. Has not shown to conclusively affect reproduction in laboratory in vivo studies.

Ketoconazole	65277-42-1	Inhibiting the microsomal cytochrome P450 mixed function oxidases. This drug inhibits 17 alpha-hydroxylase, C17-20 lyase, and the cholesterol-side-chain cleavage enzyme	Fungicide	Strong inhibitor of T production; Medium inhibitor of E2 production; Induces progesterone production.
Piperonyl butoxide	51-03-6	Cytochrome P450 inhibitor	Pesticide synergist	Unknown. This compound is used to inhibit several P450s involved in metabolism but not necessarily steroidogenesis.
Prometon	1610-18-0	Photosynthetic inhibitor	Wide-spectrum herbicide	Weak inducer of E2 production; Negative for T.
RU-486/mifepristone	84371-65-3	Negative for ER very weakly positive for AR at high conc., blocking the progesterone receptor, incr. levels of EST.	Pharmaceutical	Unknown
Spironolactone	52-01-7	Antiandrogen action through inhibition of 17α hydroxlase;Glucocorticoid & PXR-ligand	Pharmaceutical	Unknown
Dimethoate	60-51-5	StAR Protein	Organophosphate pesticide	Unknown
Tricrecyl phosphate	1330-78-5	Cholesterol esterase inhibitor	Flame retardant, nonflammable fluid, solvent, plasticizer	Unknown

TESTING OF CHEMICALS

Four of the five laboratories (1, 2, 3 and 4) had participated in the pre-validation studies, and therefore, were considered experienced with regard to the assay. The last laboratory (Lab 6), however, never conducted the H295R Steroidogenesis Assay prior to this validation study, and thus, was considered to provide information regarding the true transferability of the assay protocol. With very few exceptions (see subsequent sections) the performance of this laboratory was comparable to that of the other groups. Therefore, the data set presented here from the four laboratories can be assumed to be representative of the performance of the H295R Steroidogenesis Assay.

Cell Viability

Out of the 12 chemicals tested only four compounds were found to be cytotoxic (Figures S1 & S2). Benomyl, paraben, and prochloraz were cytotoxic at only the greatest dose tested (100 μM) with the exception of benomyl when measured at Labs 1 and 6 where cytotoxicity occurred either at the two greatest concentrations or where no cytotoxicity was observed, respectively. For nonoxyenol-9, effects on cell viability were observed at 10 μM for 2 out 4 laboratories that evaluated this compound. As a result, cytotoxic concentrations for these chemicals were excluded from further data analysis. An increase in cell viability greater than

that observed in the solvent controls was observed at 4 out of 5 laboratories in the forskolin experiments (Fig. S1). Maximum inductions in cell viability relative to the controls observed for this chemical were 126 and 136% at the greatest dose at Labs 1 and 2, respectively, and 137% at Lab 6 at 10μM. In addition to the forskolin exposures, a greater than 20% increase in cell viability was observed for Lab 2 experiments with nonoxynol-9 (10 μM), trilostane (100 μM) and prochloraz (10 and 100 μM). This trend was opposite to that reported by the other groups. It is assumed that there might have been a technical problem with the cell viability assay because the hormone concentration data obtained for these doses behaved in a manner that was similar to those reported by the other groups. To verify this hypothesis, the cell viability experiments for prochloraz, benomyl, and trilostane were re-run by this laboratory. The data obtained during this second set of experiments was in accordance with those obtained by the other groups (Figure S2). Finally, Lab 6 observed an increase in cell viability with molinate for doses greater or equal to 1μM, a trend that was not observed by the other groups. It is unclear what the reason for this increase in cell viability was.

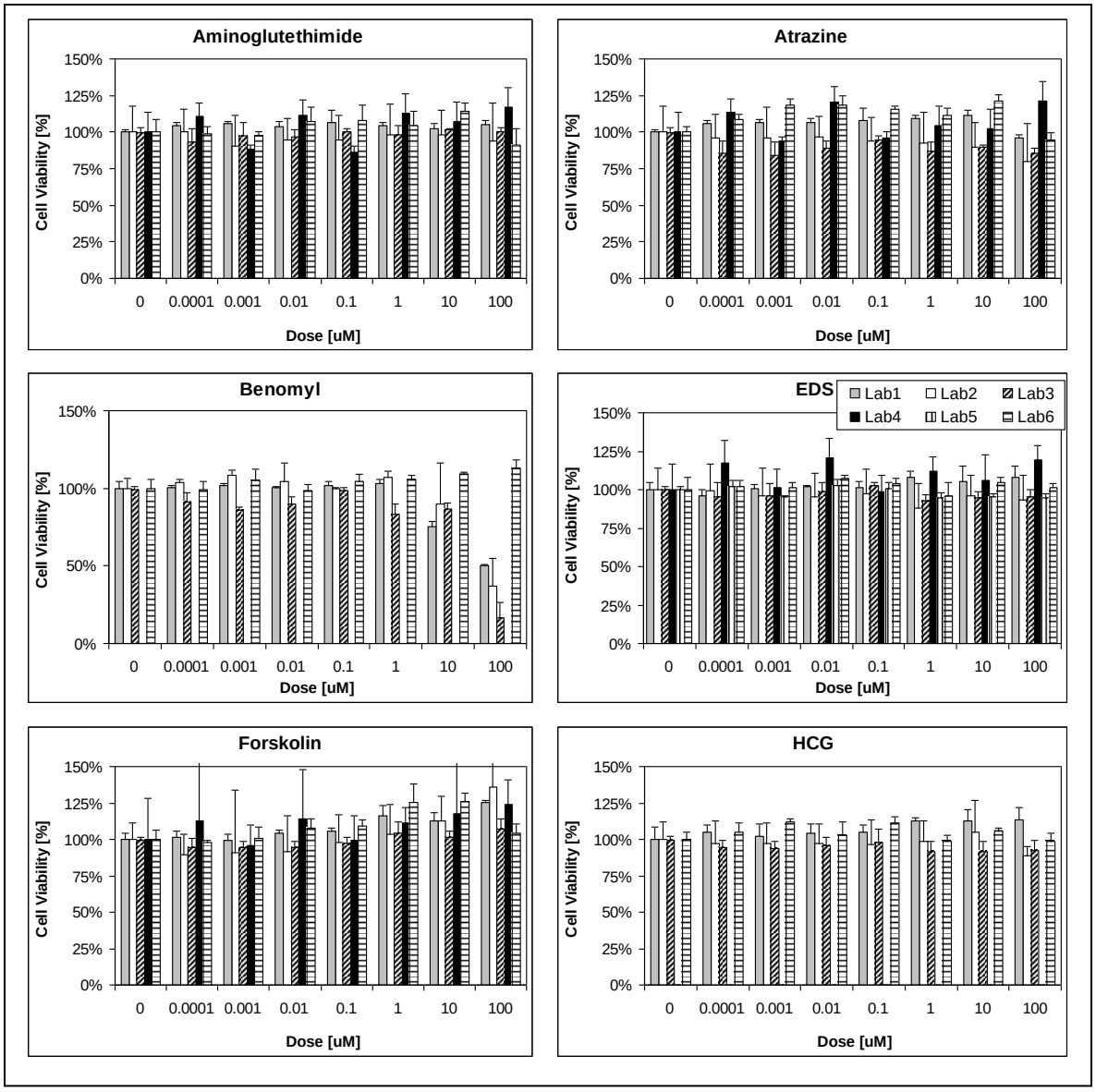


Figure S1: *Comparison of cell viability among laboratories (Lab) after exposure to Aminoglutethimide, Atrazine, Benomyl, EDS, Forskolin and HCG. Cell viability is expressed relative to the solvent controls (SC = 100%) in each plate. Error bars = 1 x SD. Lab4: No data available for HCG and benomyl. Lab6: No data available for aminoglythetimide, atrazine, forskolin and HCG.*

Given the variations (slight inductions or reductions in the number of viable cells), it was decided to normalize all data from wells with cell viabilities greater 80% for cell viability by dividing the hormone response by the relative viability (SC=1) in each well. All data from wells with cell viability of equal to or less than 80% was not considered for further evaluation due to potential interference through cytotoxicity.

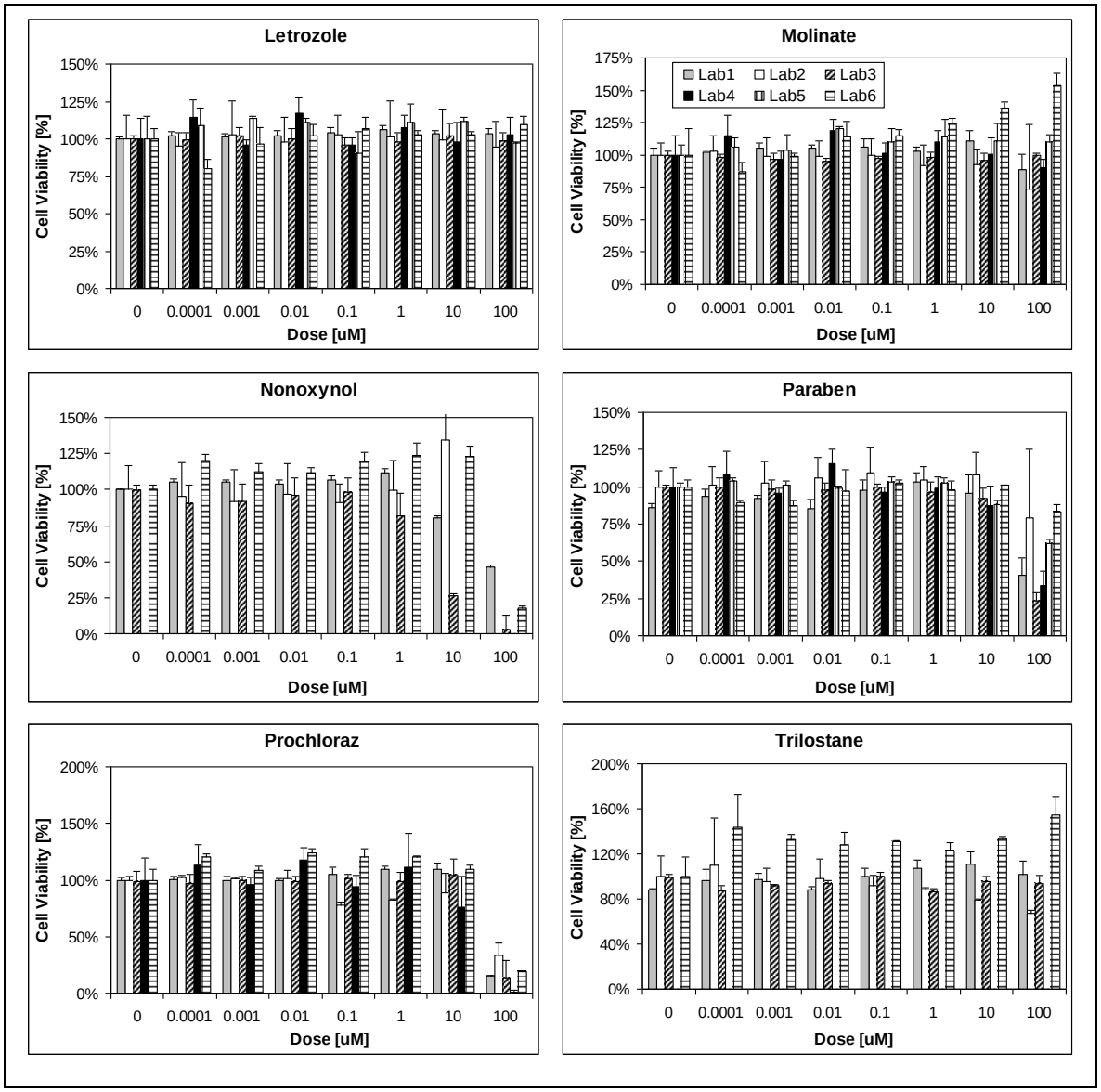


Figure S2: *Comparison of cell viability among laboratories (Lab) after exposure to Letrozole, Molinate, Nonoxynol-9, Paraben, Prochloraz and Trilostane. Cell viability is expressed relative to the solvent controls (SC = 100%) in each plate. Error bars = 1 x SD. Lab4: No data available for nonoxynol-9 and trilostane. Lab6: No data available for nonoxynol-9, prochloraz and trilostane.*

Relative changes after Exposure to Core Chemicals

Testosterone

There were marked differences in the response of T production after exposure of H295R cells to the 12 core chemicals (Figures S3 – S5). With few exceptions, the effects observed were comparable among laboratories and could be grouped in three different types of effects: inducers (Figure S3), inhibitors (Figure S4) and negative reference chemicals (Figure S5). Among the inducers, exposure to trilostane resulted in greatest fold changes (>10-fold induction) in T concentration when compared to solvent controls. The least fold-changes were observed for the atrazine exposures where induction of T production all were less than 1.5-fold with the exception of Labs 2 and 6, at which maximum inductions were 2.4- and 1.5-fold, respectively. Exposure to prochloraz resulted in greater15-fold reductions of T production at the greatest dose tested (100 μM) at all laboratories with the exception of Lab 4 where up to 4.5-fold reductions were observed. Exposure to the other inhibitors resulted in less than 4-fold changes in T production.

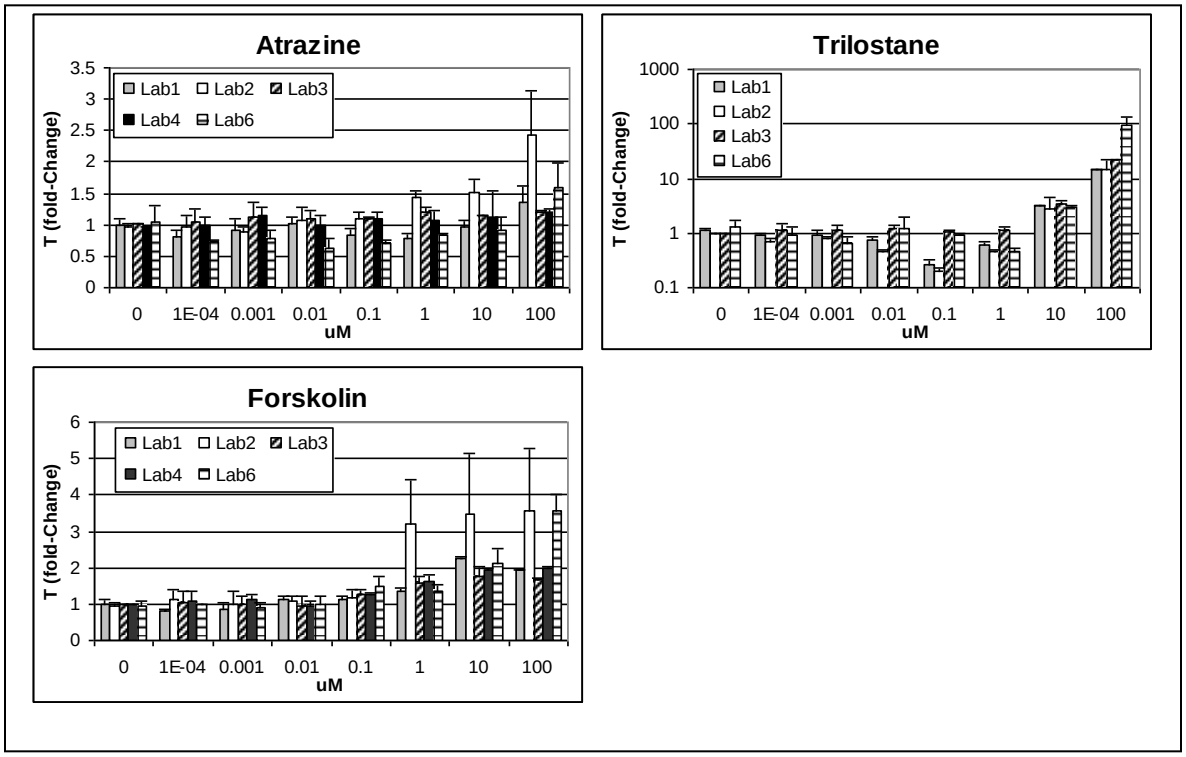


Figure S3: *Comparison of changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to forskolin, trilostane, atrazine and paraben. Error bars = 1x standard deviation. Bars represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Lab3 – only two replicate experiments were conducted). Lab4: No data available for trilostane. Lab6: No data available for atrazine, forskolin and trilostane.*

When chemicals exhibited a less than 1.5-fold change in T production they were categorized as negatives (Fig. S5). Some of these negative chemicals could have been categorized as inhibitors in individual cases (Molinate: Lab 4; Benomyl: Lab1). However, even in situations where an inhibition was observed at an individual laboratory, this change was always less than 2-fold, and typically did not follow a dose-dependent trend. In the case of nonoxynol-9, a decrease in T concentrations at non-cytotoxic concentrations at two of four laboratories for which data was available was observed. Inhibitions were 29 and 47% relative to the SCs for Labs 1 (1 μM) and 2 (10 μM), respectively. However, it should be noted that at Lab 2 10μM nonoxynol-9 cause an average increase in cell viability of 38%, and thus, the observed reduction in T production may be an artifact due to the correction for cell viability, especially as no such increase was observed at any of the other groups.

Variation between laboratories did not exceed 2-fold for a given dose with the exception of trilostane. Among group CVs for inducers were always less than 1.5-fold (no trilostane).

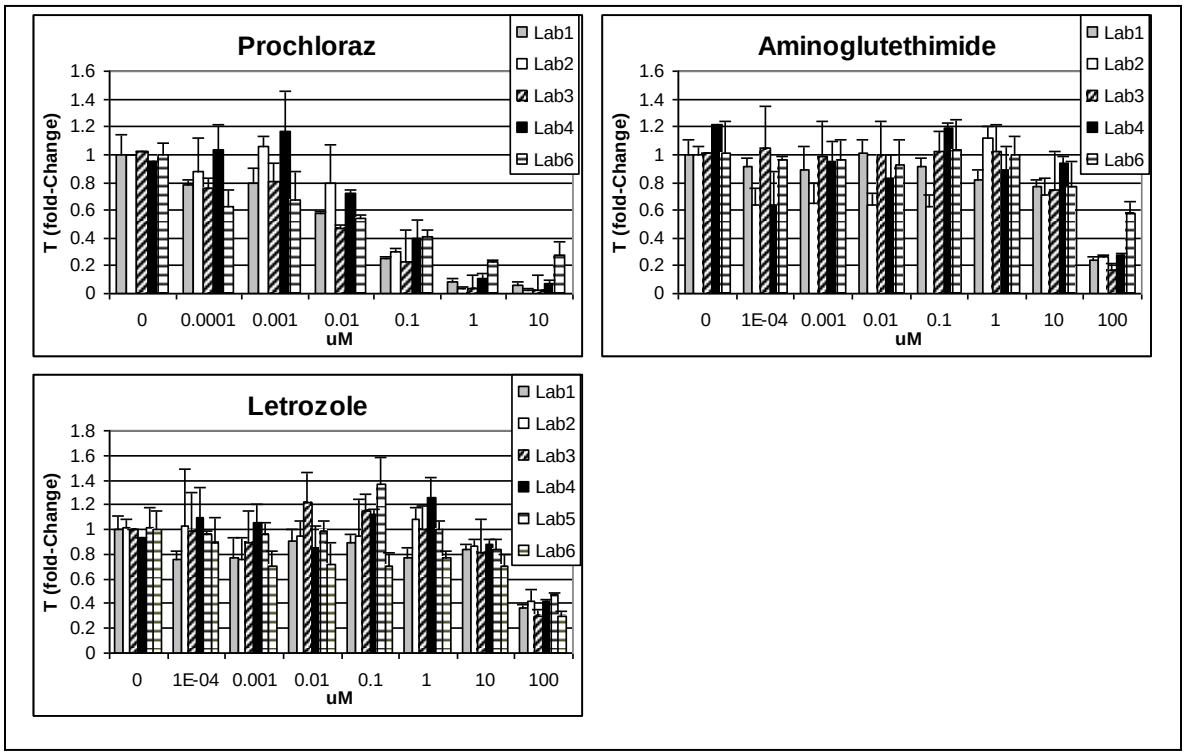


Figure S4: *Comparison of changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to prochloraz, aminoglutethimide, and letrozole. Error bars = 1x standard deviation. Bars represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Lab3 – only two replicate experiments were conducted). Lab6: No data available for prochloraz and aminogluthetimide.*

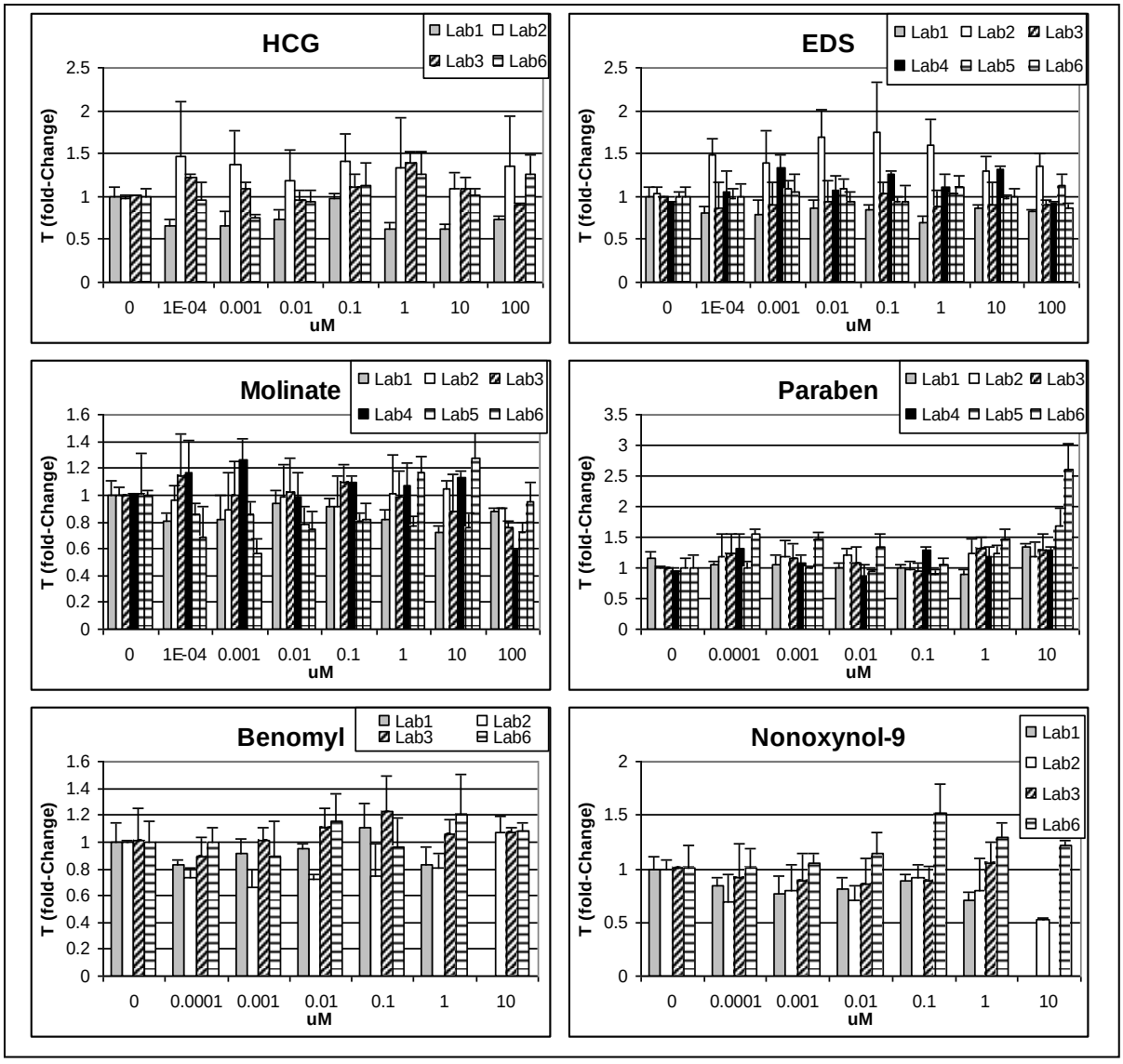
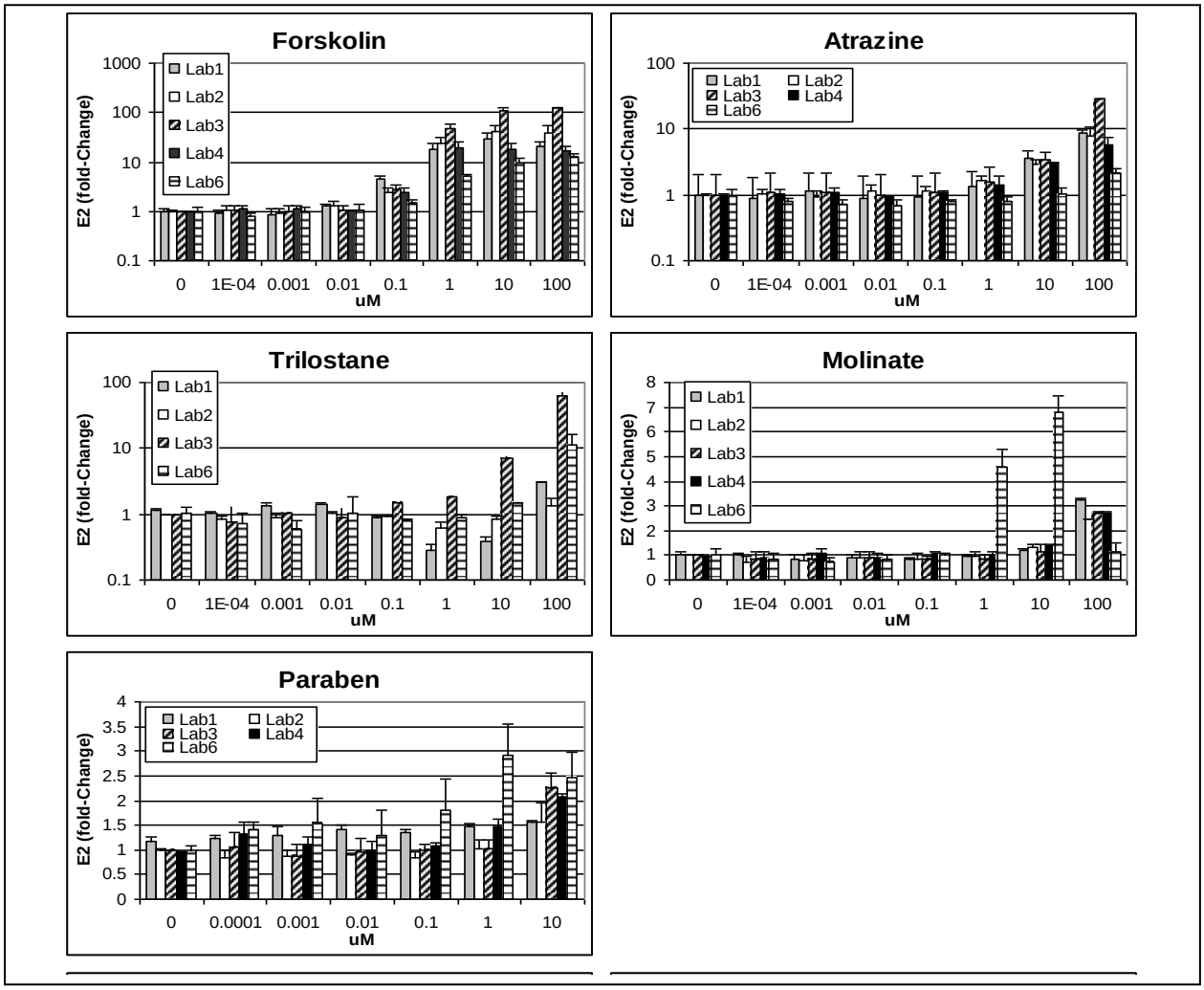


Figure S5: *Comparison of changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to HCG, EDS, molinat, benomyl and nonoxynol-9. Error bars = 1x standard deviation. Bars represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Lab3 – only two replicate experiments were conducted). Lab4: No data available for HCG, benomyl and nonoxynol-9. Lab6: No data available for HCG, benomyl and nonoxynol-9.*

Estradiol

Significant differences in the response of E2 production was observed for H295R cells exposed to 12 core chemicals (Figures S6 – S8). The direction of effects for each chemical was comparable among laboratories with the exception of the Lab2 trilostane data. Overall, the types of effect were slightly different than those observed for T with the majority of the chemicals acting as inducers of E2 production (Figure S6). Three chemicals inhibited E2 concentrations (letrozole, prochloraz and aminoglutethimide; Figure S7) while HCG, EDS, benomyl and nonoxynol-9 (Figure S8) did not elicit any clear (> 1.5-fold) effects at non-cytotoxic



doses.

Figure S6: *Comparison of changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to forskolin, atrazine, trilostane,molinate,and paraben. Error bars = 1x standard deviation. Bars represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Lab3 – only two replicate experiments were conducted). Lab4: No E2 data available for trilostane. Lab6: No data available.*

The strength of the response to the exposure with chemicals that increased production of E2 ranged between 20-fold or greater (Forskolin) to <3-fold (Paraben). The dose at which effects occurred was not related to the magnitude of the response. While forskolin resulted in increases in E2 production at doses greater or equal to 0.1 μM exposure to other inducers typically did not reveal effects at doses less than 1 μM. Exposure to letrozole and prochloraz resulted in marked reductions of E2 at doses greater 0.001 and 0.1 μM, respectively. Exposure to aminoglutethimide, in contrast, only caused a clear reduction in E2 concentrations at the greatest dose tested.

Variation between laboratories did not exceed 2-fold for a given dose with the exception of trilostane.

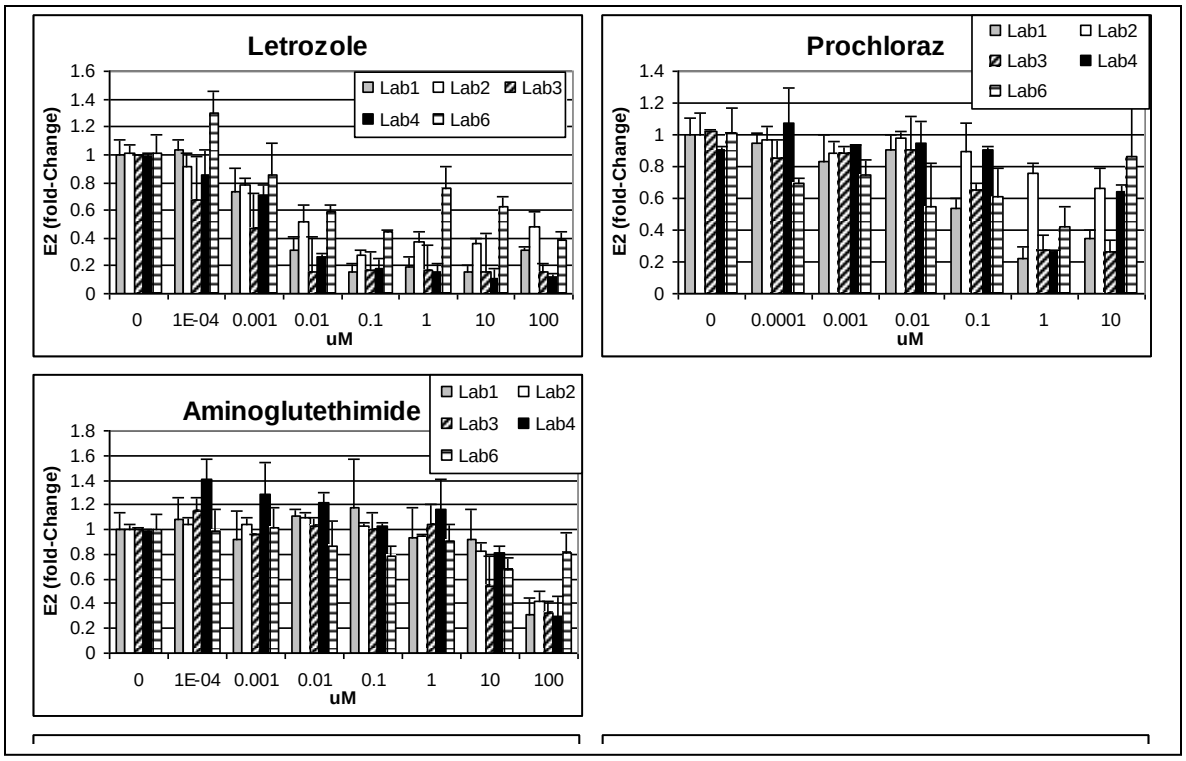


Figure S7: *Comparison of changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to letrozole, prochloraz and aminoglutethimide, letrozole. Error bars = 1x standard deviation. Bars represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Lab3 – only two replicate experiments were conducted). Lab6: No E2 data available.*

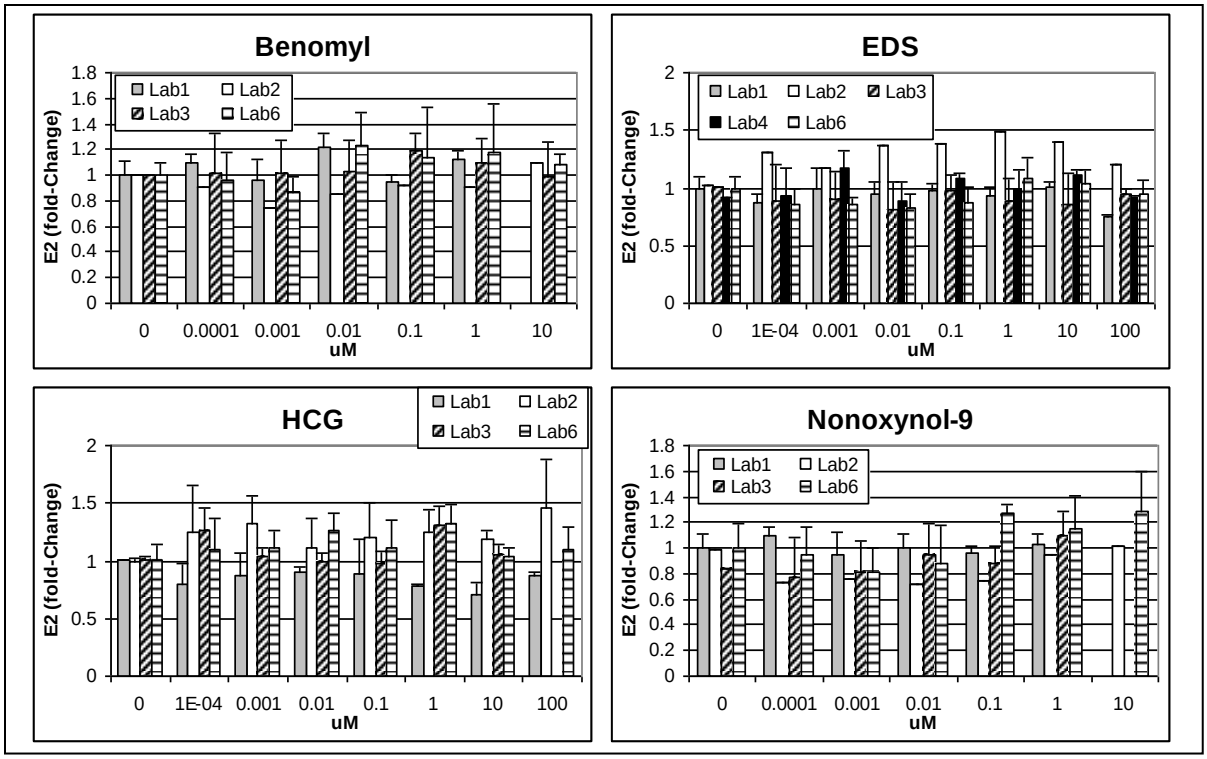


Figure S8: *Comparison of changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to HCG, EDS, and benomyl. Error bars = 1x standard deviation. Bars represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Lab3 – only two replicate experiments were conducted). Lab4: No data available for benomyl, , HCG and nonoxynol-9. Lab6: No E2 data available.*

APPLICATION OF THE H295R STEROIDOGENESIS ASSAY TO A SELECTION OF SUPLEMENTARY CHEMICALS

Of the 16 chemicals all were analyzed by the lead laboratory, while three additional laboratories (Lab2, 3 and 4) each analyzed 5 to 6 chemicals so that data sets are available from two different laboratories for each of the test compounds. As for the experiments with the 12 core chemicals, data obtained from wells with less or equal to 80% cell viability was excluded from further analysis. While over 47% of all chemicals showed reductions in cell viability of greater or equal to 20%, no single compound revealed cytotoxicity at doses less than 100μM (Figures S9 – S11). Also, in most cases, effects (decreases) in cell viability were comparable among laboratories with the exception of tricrescyl phosphate and spironolactone when measured at Lab 4 and fenarimol at Lab3. The trend in cell viability change at Labs 1 and 3 for fenarimol was the same (decrease) but while at Lab 1 a greater than 90% decrease was observed, cytotoxicity at Lab3 only reached 23% at the greatest dose. The differences in cell viability after exposure to tricrescyl phosphate between Labs1 and 4 was likely due to an issue with the SCs at Lab 4, which were approximately 50% less than those observed for the least doses of this chemical. When excluding the SCs of this group, the trend in cytotoxicity with increasing doses was comparable among these groups. It is unclear why there was no cytotoxicity observed for spironolactone at the greatest dose at Lab 4.

As for the above described 12 core chemicals, the H295R Steroidogenesis Assay allowed distinguishing between inducers and inhibitors of different strength/potency for both T and E2 (Figures S12 - S17). Five and four of the 17 compounds (24 and 29%, respectively) tested negative in the assay for T and E2, respectively. DEHP, dimethoate, flutamide, glyphosate and prometon did not elicit significant dose dependent responses for T, and glyphosate, dinitrophenol, piperonyl butoxide and spironolactone did not reveal any dose-dependent effects on E2 production. One exception was dinitrophenol, which was identified as a significant inhibitor of T at all doses tested. However, no dose-response trend was recognizable and the magnitude of the effect was weak. Therefore, it is possible that this response represents an artifact. Compared to the 12 core chemicals, there was greater variation among the responses observed at different laboratories. Approximately 24 and 35% of the chemicals showed a significant response for T and E2, respectively, at only on of the two laboratories at which they were tested. It is unclear what the reasons for these differences are but it should be noted that in 7 out of the 10 cases where such incongruencies were observed these were associated with one group (Lab 4). Six of these 7 compounds were identified as inducers by the 1st lab. Also, at the same laboratory some of the cell viability data revealed no effects where significant decreases were observed at the other group (tricrescyl phosphate and spironolactone) indicating that there may have been some issues related to dosing. When excluding this group there were only a and 2 chemicals for T and E2, respectively, where the data obtained at different laboratories did not match.

Regardless of these remaining uncertainties, it could be demonstrated that the H295R Steroidogenesis Assay protocol successfully identified chemicals with unknown modes of interaction with sex steroid synthesis as inducers and inhibitors of T and E2 production. Some

of the chemicals identified as inhibitors of T showed a biphasic response with typically slight increases in hormone production up to concentrations of 0.1 to 1 μM. However, with the exception of genistein exposure experiments none of these changes exceeded 1.5-fold. This phenomenon did not affect the final categorization of a chemical. It is hypothesized that these minor changes are likely to be a compensatory mechanism, reflecting the integrative nature of the H295R Steroidogenesis Assay rendering a more realistic assay with regard to the identification of potential in vivo inducers/inhibitors of T and E2 production.

The specificity of the assay could be demonstrated by the relatively great number of chemicals that tested negative for the interference with the production of either T or E2 or both hormones.

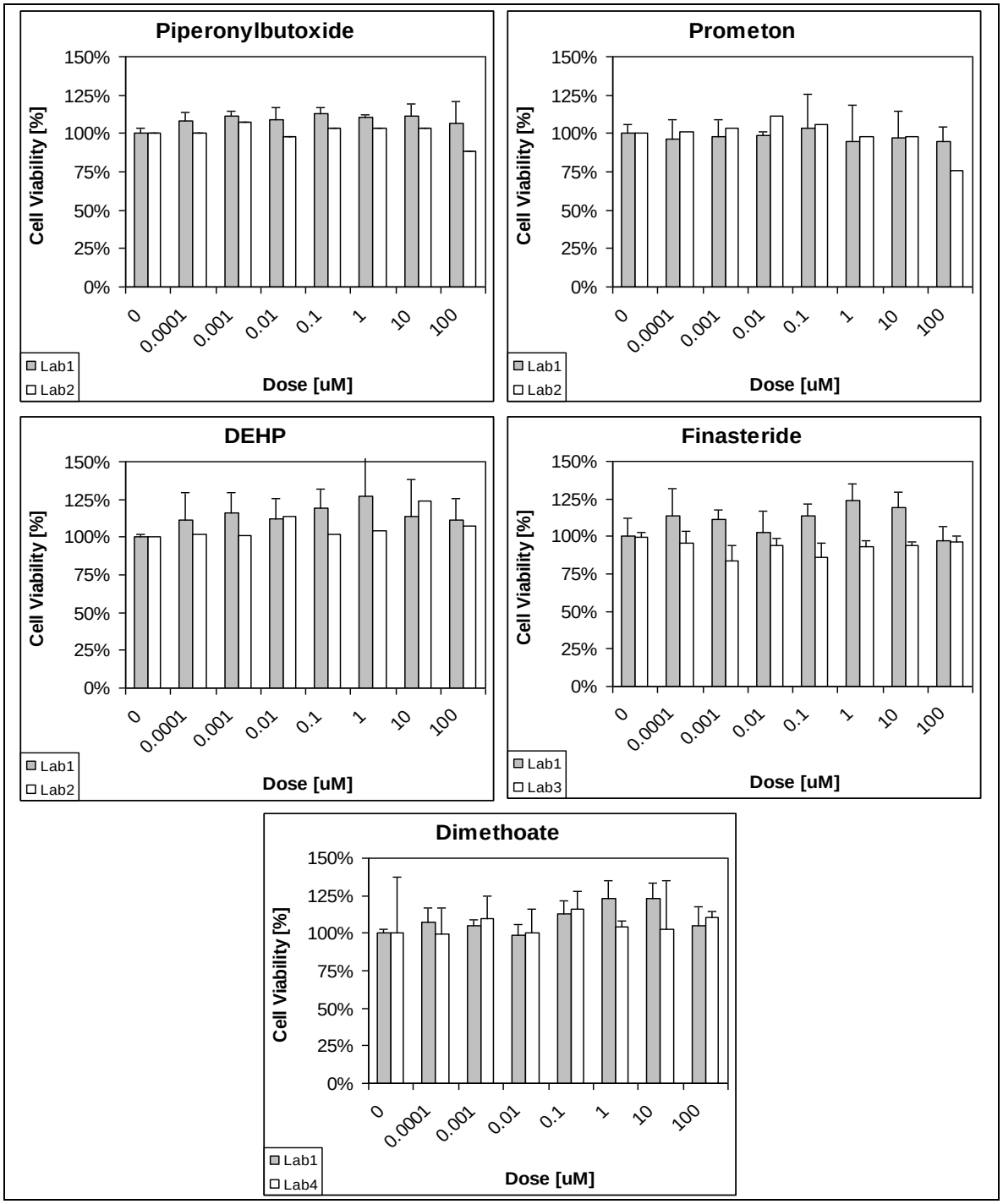


Figure S9: *Cell viability after exposure to piperonly butoxide, prometon, DEHP, finasteride, and dimethoate. Cell viability is expressed relative to the solvent controls (SC = 100%) in each plate. Error bars = 1 x SD. Note: Lab2 did re-measure cell viability due to technical issues that were encountered during the initial experiments. Therefore, only one experimental data set is available from this lab, resulting in the lack of error bars for the cell viability results of this group.*

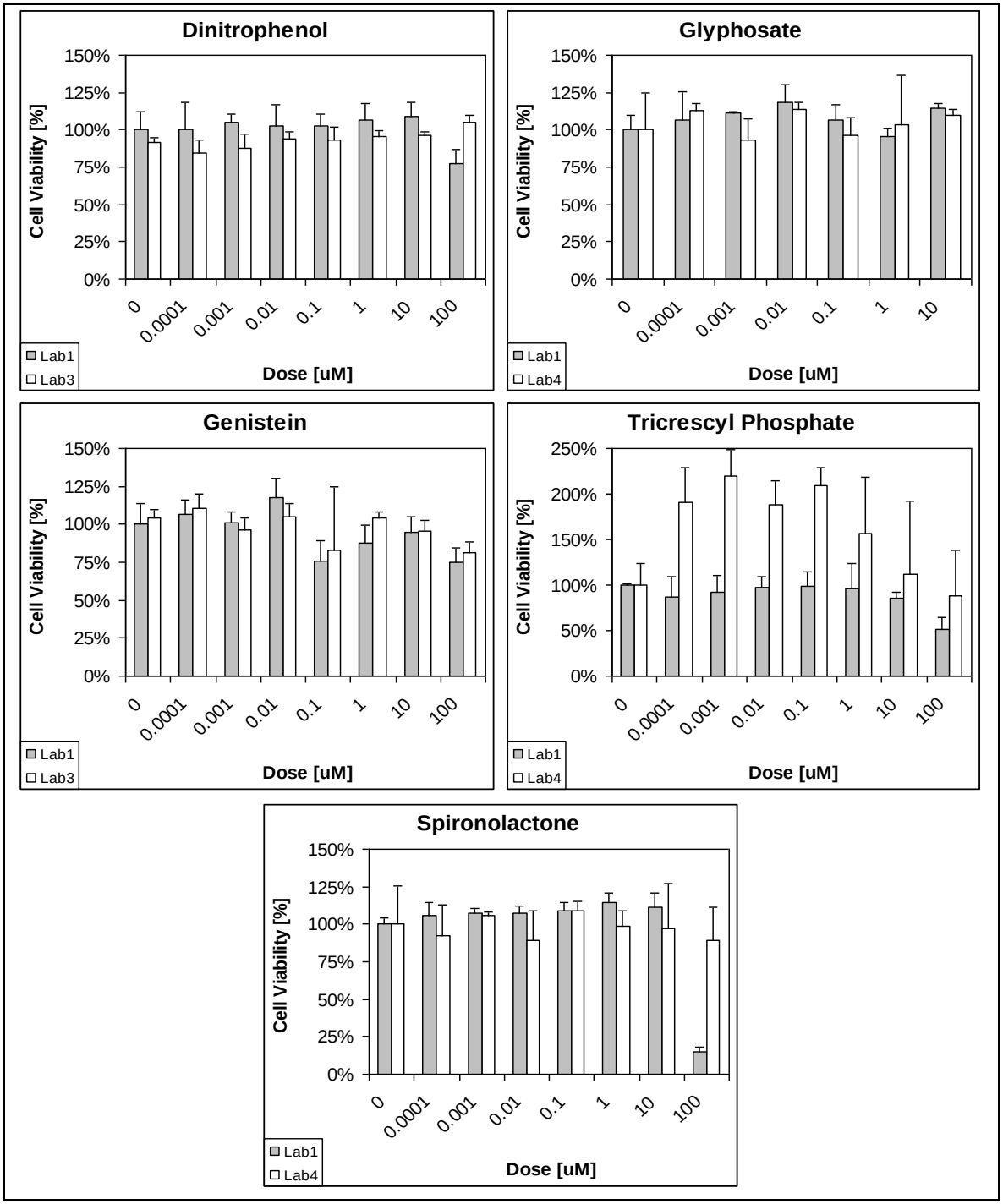


Figure S10: *Cell viability after exposure to dinitrophenol, glyphosate, genistein ,tricrescyl phosphate, and spironolactone. Cell viability is expressed relative to the solvent controls (SC = 100%) in each plate. Error bars = 1 x SD. Note: Lab2 did re-measure cell viability due to technical issues that were encountered during the initial experiments. Therefore, only one experimental data set is available from this lab, resulting in the lack of error bars for the cell viability results of this group.*

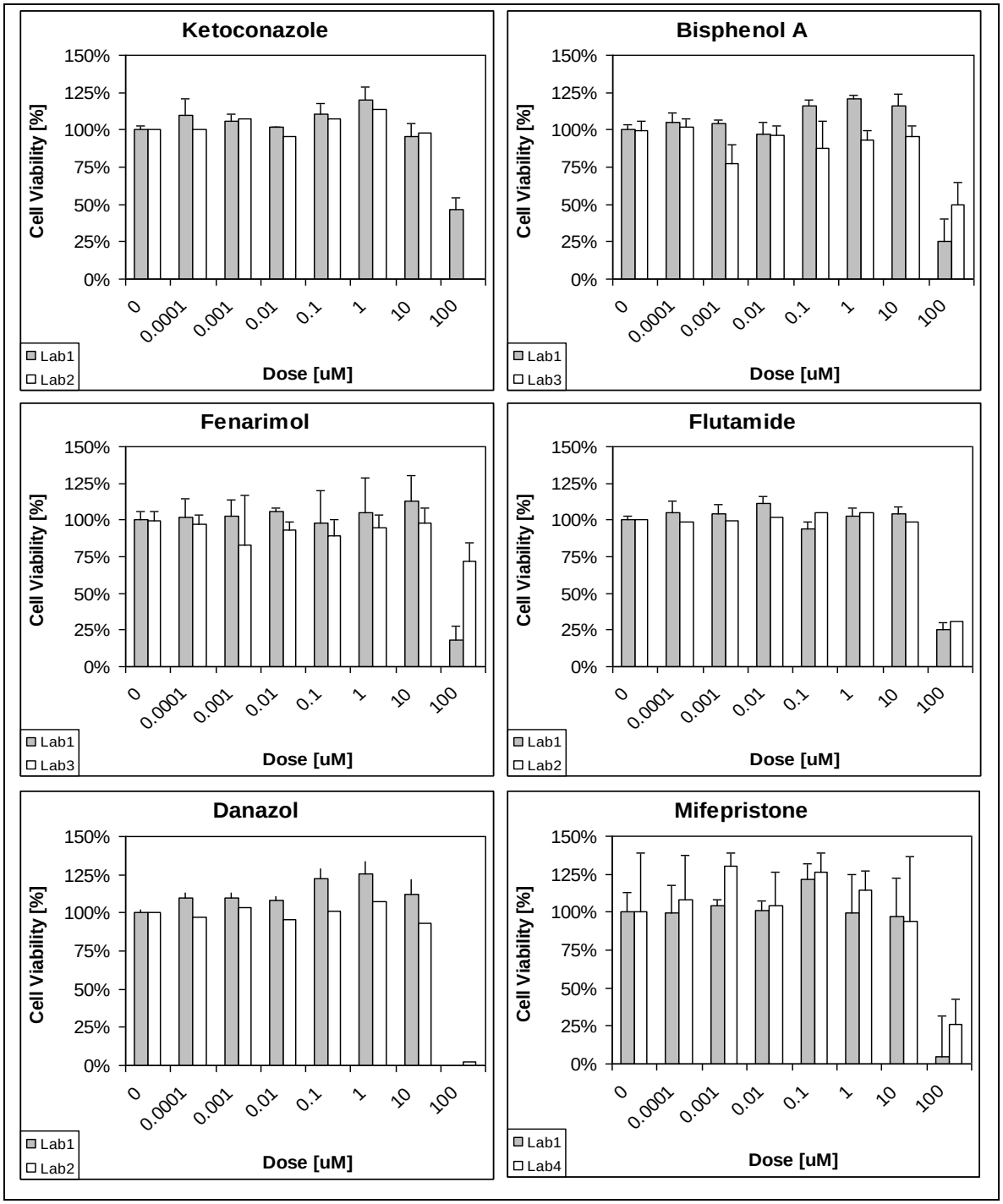


Figure S11: *Cell viability after exposure to ketoconazole, bisphenol A, fenarimol, flutamide, danazol and mifepristone. Cell viability is expressed relative to the solvent controls (SC = 100%) in each plate. Error bars = 1 x SD. Note: Lab2 did re-measure cell viability due to technical issues that were encountered during the initial experiments. Therefore, only one experimental data set is available from this lab, resulting in the lack of error bars for the cell viability results of this group.*

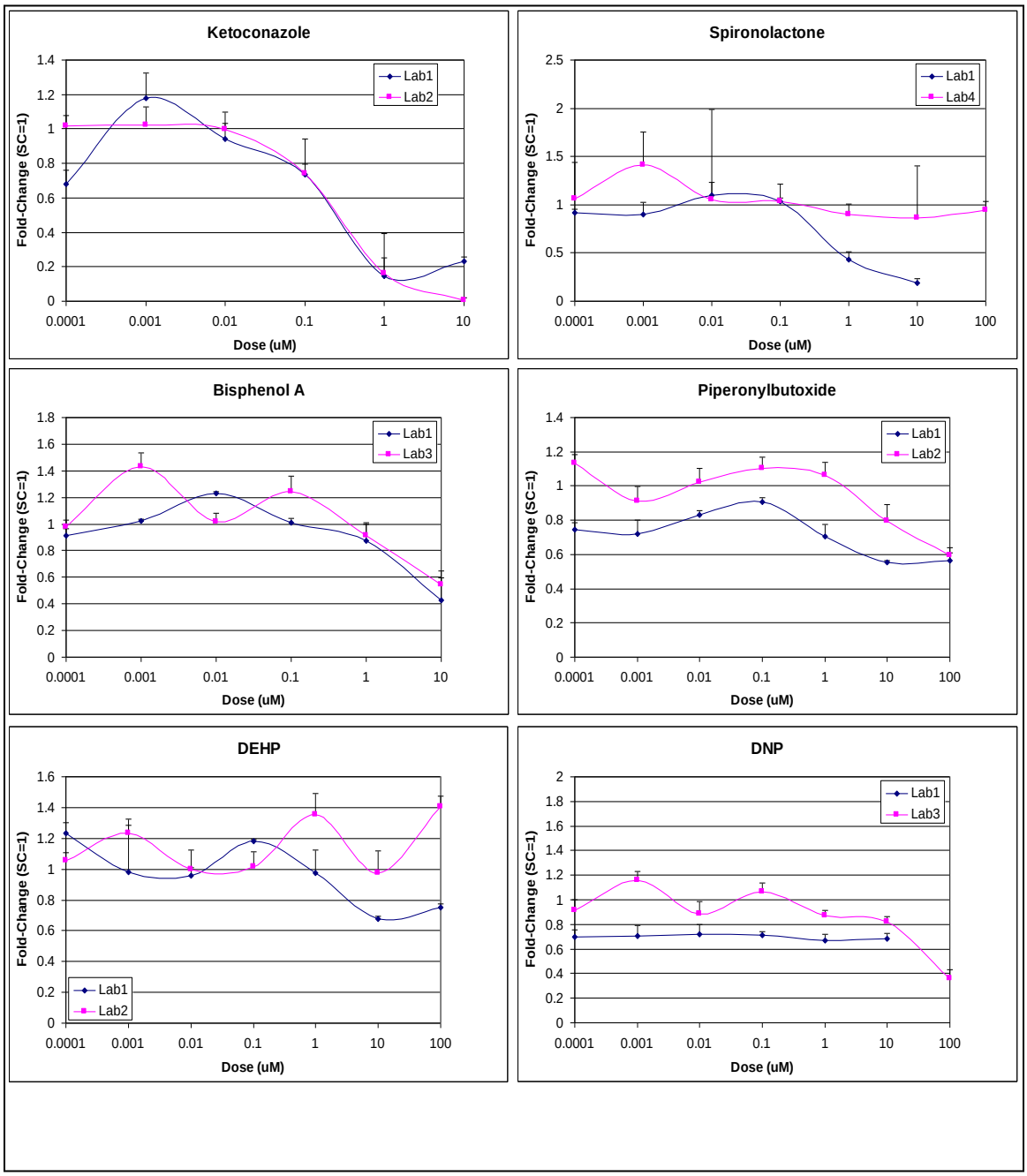


Figure S12: *Changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to ketoconazole, spironolactone, bisphenol A, piperonyl butoxide, DEHP, and dinitrophenol (DNP). Error bars = 1x standard deviation.*

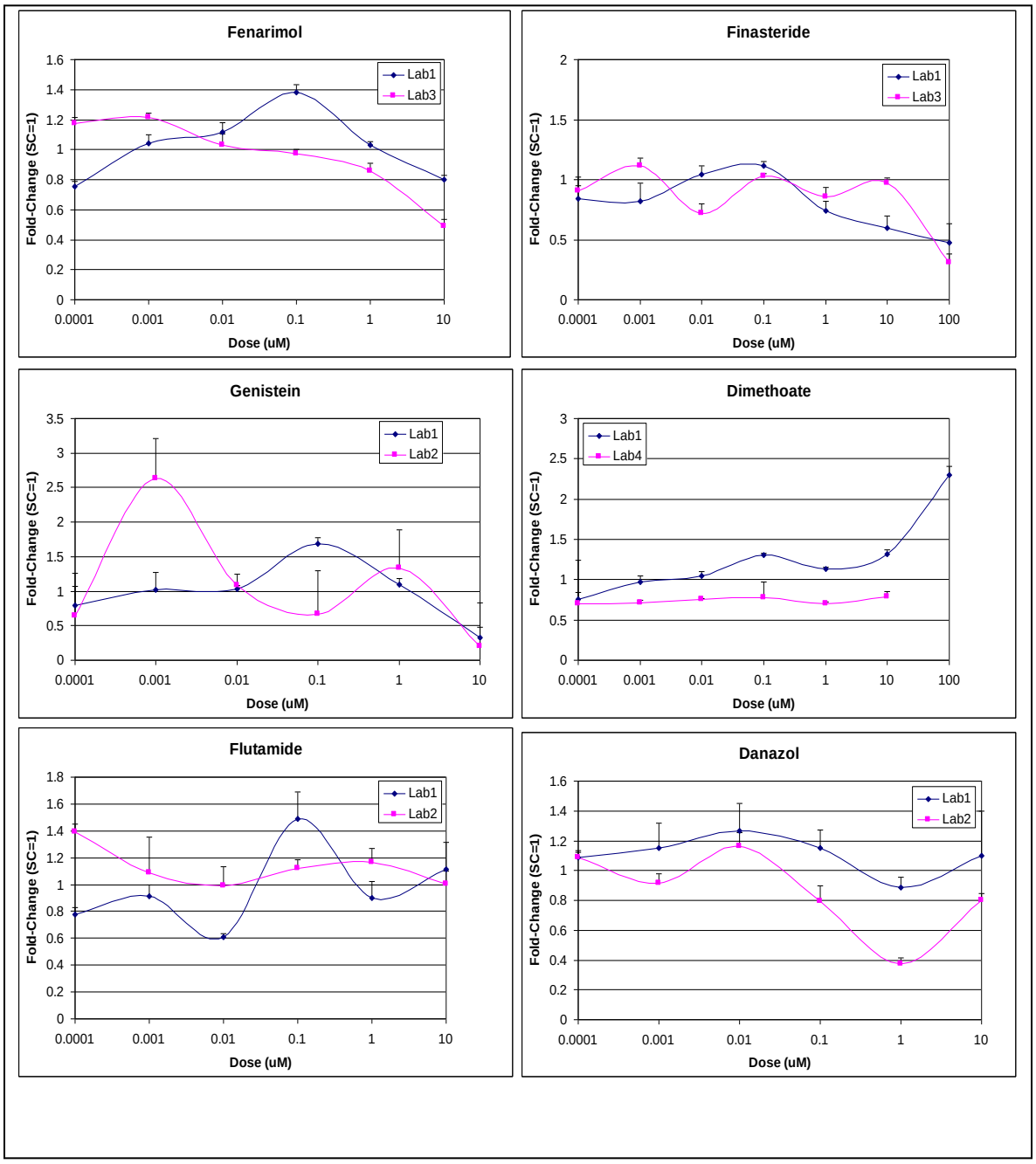


Figure S13: *Changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to fenarimole, finasteride, genistein, dimethoate, flutamide, and danazol. Error bars = 1x standard deviation.*

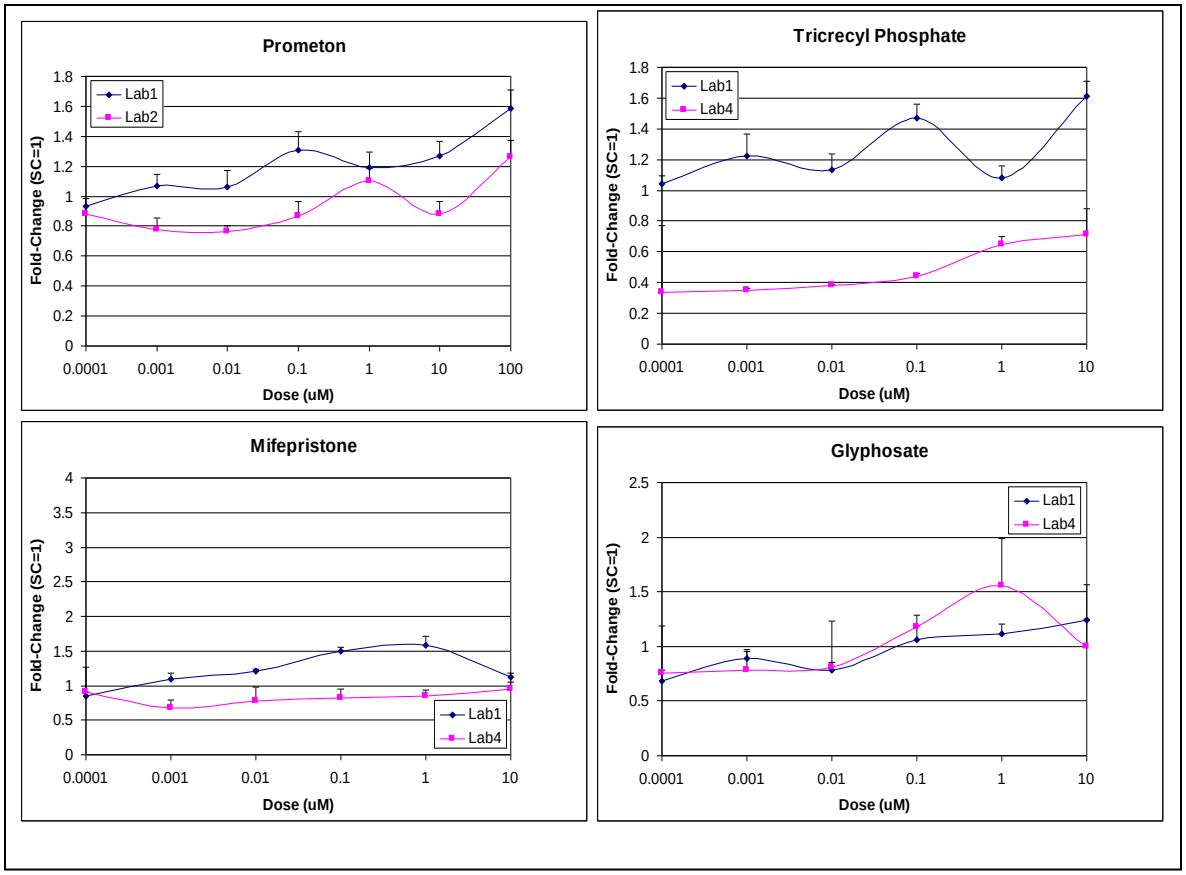


Figure S14: *Changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to prometon, tricrescyl phosphate, mifepristone, and glyphosate. Error bars = 1x standard deviation.*

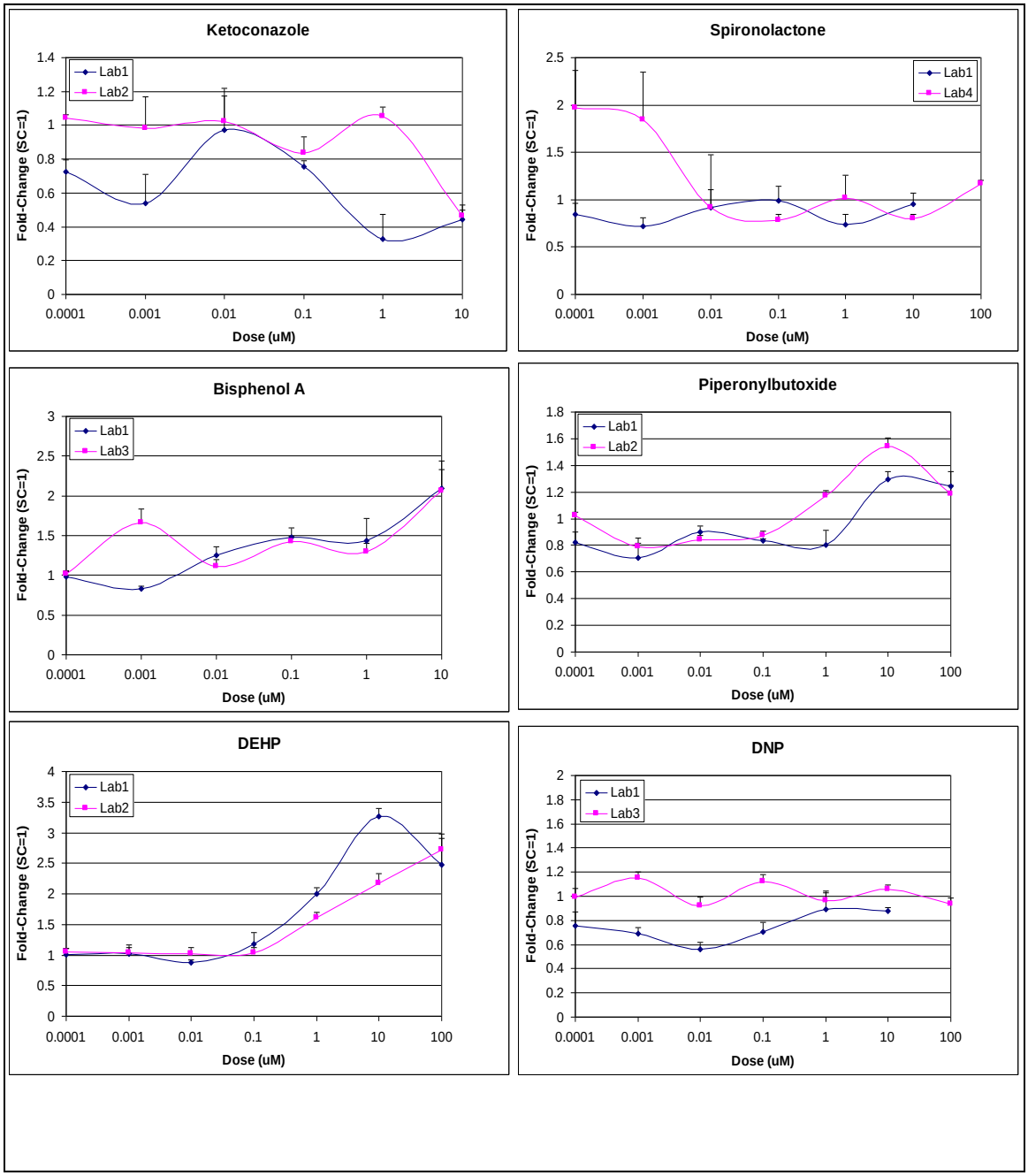


Figure S15: *Changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to ketoconazole, spironolactone, bisphenol A, piperonyl butoxide, DEHP, and dinitrophenol (DNP). Error bars = 1x standard deviation.*

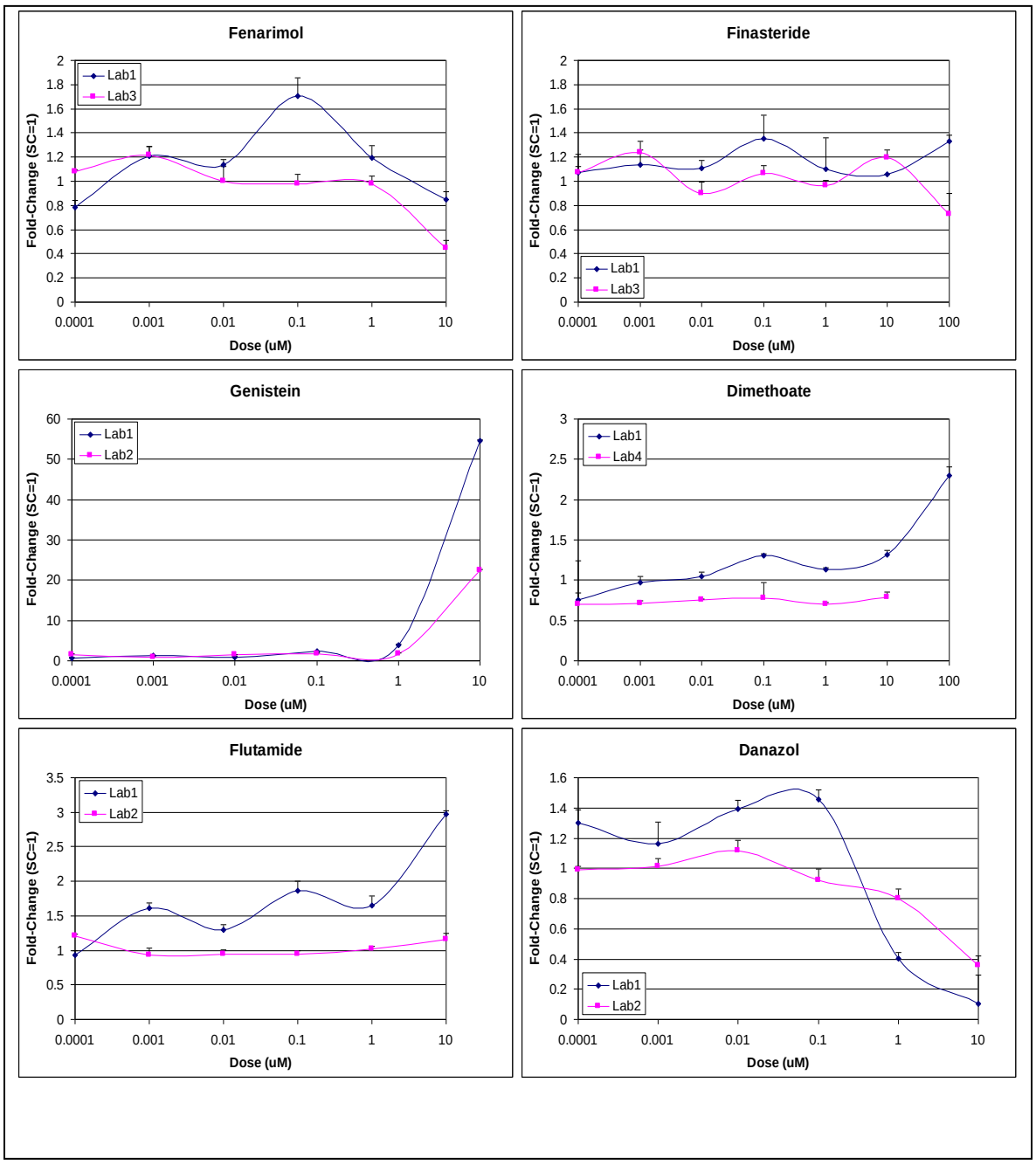


Figure S16: *Changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to fenarimole, finasteride, genistein, dimethoate, flutamide, and danazol. Error bars = 1x standard deviation.*

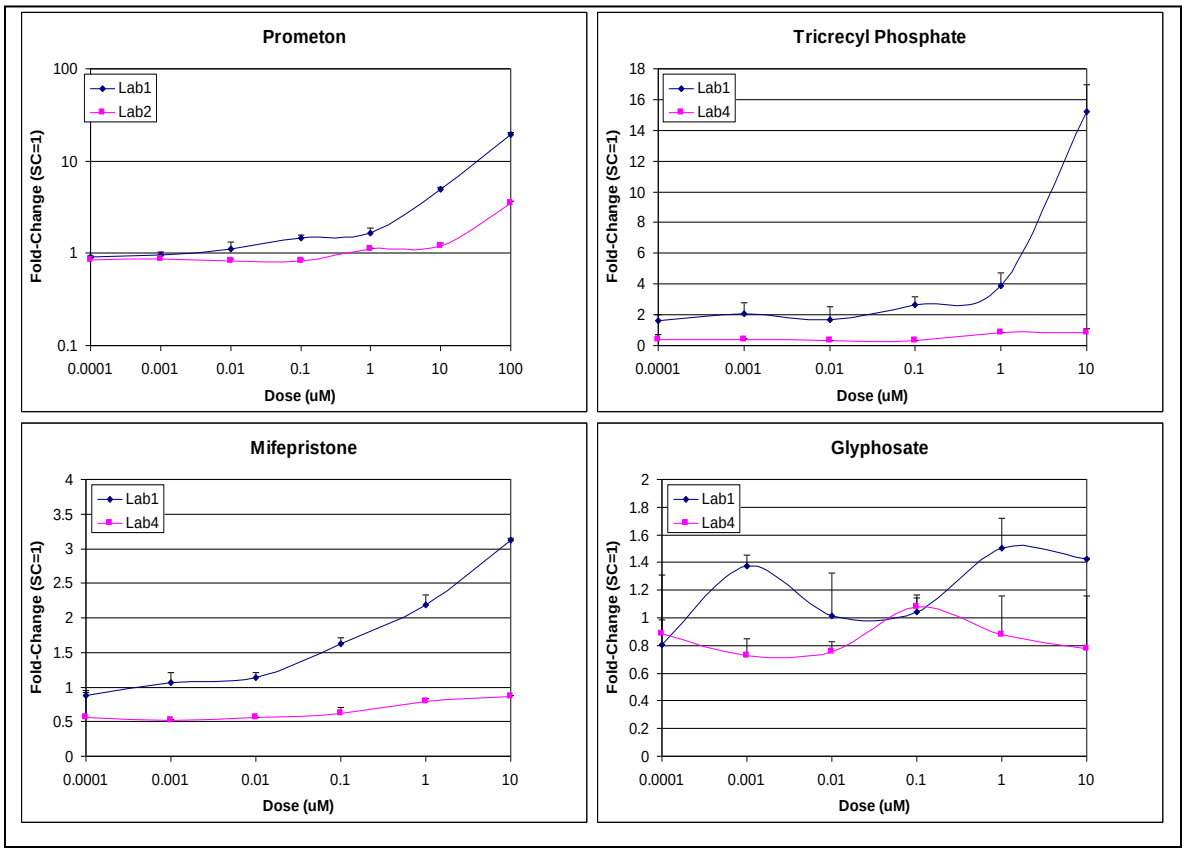


Figure S17: *Changes in the concentrations ofestradiol (E2) relative to the solvent controls (SC=1) after exposure to prometon, tricrescyl phosphate, mifepristone, and glyphosate. Error bars = 1x standard deviation*.

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