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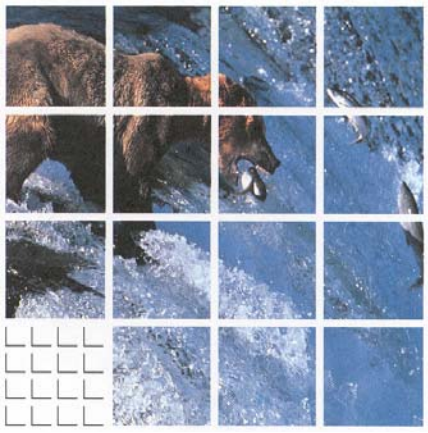
Draft Report

STANDARDIZATION AND REFINEMENT OF THE H295R CELL-BASED ASSAY TO IDENTIFY CHEMICAL MODULATORS OF STEROIDOGENESIS AND AROMATASE ACTIVITY

- Repeat of Inter-Laboratory Study -

Order No. 4W-2979-NBLX

Contract code: GS-10F-0041L



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March 2007

TABLE OF CONTENTS

1 ABBREVIATIONS

AOE	City University of Hong Kong
DIFVR	Danish Institute for Food and Veterinary Research
EC	Effective Concentration
GER	University of Heidelberg
IHCP	Institute for Health and Consumer Protection
LOEC	Lowest Observable Effect Concentration
MSU	Michigan State University
NOEC	No Observable Effect Concentration
OECD	Organisation for Economic Co-operation and Development
VMG NA	Validation Management Group for Non-animal Testing for Endocrine Disruptors
REACH	Registration, Evaluation and Authorisation of Chemicals
RTP	US Environmental Protection Agency
T	Testosterone
E2	17β-estradiol

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4 SUMMARY

This report summarizes the work completed to date on the repeat of the "Inter-Laboratory Comparison and Validation of the H295R Cell System to Test for Effects of Chemicals on Steroid Hormone Synthesis" that is part of the ongoing Project No. GS-10F-0041 "Development of an Assay Using the H295R Cell Line to Identify Chemical Modulators of Steroidogenesis and Aromatase Activity". All tasks that were originally outlined in the proposed work have been completed. Specifically, the methods and protocols that were used at each of the participating laboratories to measure the production of testosterone (T) and estradiol (E2) by H295R cells in the culture medium have been optimized and revised to meet performance criteria of the assay. The total number of participating laboratories has been increased to six while the number of test chemicals was reduced to three. Four of the six laboratories that participated in this study had already demonstrated the ability to perform within the quality criteria established in the original protocol of the first inter-laboratory study as reported in "Inter-Laboratory Comparison and Validation of the H295R Cell System to Test for Effects of Chemicals on Steroid Hormone Synthesis", submitted to EPA in February 2006.

The implementation of optimized protocols that included highly prescriptive methods to be used by the participating laboratories significantly improved the comparability of the data obtained with all three model chemicals (forskolin, fadrozole, prochloraz). Although some minor inter-laboratory differences were noted in both the basal hormone concentrations as well as in changes of hormone production measured in chemically exposed cells, these differences were negligible when compared to the differences observed during the first inter-laboratory comparison study. As noted in the previously submitted interim report "Influence of cell passage and freeze/thaw events on basal production of 17β-estradiol and testosterone by H295R cells", differences in absolute hormone production were mainly related to the use of cells from different cell passages in the experimental exposures. To correct for possible differences in absolute hormone concentrations between experiments and laboratories, the results were normalized to average solvent control hormone values and were given as a relative increase or decrease in hormone concentration. This normalization further improved the comparability of the data, and allowed for the correction of between-passage variability in most of the experiments. Another issue that was identified during these studies was the variability in relative hormone changes that was related to method detection limits that varied due to different sensitivities of hormone assays used at the various laboratories. Thus, the data was subjected to a second normalization procedure and expressed as percent-change relative to the maximum response observed within each experiment. This further eliminated much of the variation that was previously associated with the different method detection limits. However, it is important to note that data modified by the second normalization procedure does not allow one to evaluate the magnitude of hormone changes and it is recommended that one only use these data in combination with relative change data.

Overall, results obtained during these studies were highly comparable among laboratories and the variations observed were only minor and are deemed acceptable. Although there are still some unanswered questions regarding the complexity of the H295R cell

steroidogenic pathways, especially regarding the metabolic capacities of the cells, the results obtained during the second ring test indicate that the optimized H295R Steroidogenesis test system is highly reproducible, transferable, and provides a sensitive, reliable, economic and precise method to test for chemical effects on the production of T and E2. Currently, the most desirable improvement to the assay is to reduce the effort and time necessary to culture H295R cells to a stage at which they can be used in the assay. Studies are currently underway to improve this aspect, and are expected to be completed by March 2007. As soon as the protocol has been revised based on these improvements the H295R Steroidogenesis Assay will be ready for validation.

5 INTRODUCTION

Recently, studies have indicated potential links between the exposure to natural and human-made substances in the environment and adverse effects on the endocrine and reproductive systems of mammals, birds, reptiles, amphibians, and fish (EPA, 1997). In response to emerging concerns that these substances may alter the function of endocrine systems and result in adverse effects to human health, an OECD initiative to develop and validate *in vitro* and *in vivo* assays for the detection of chemicals that may interfere with the endocrine response was taken. Currently, studies are ongoing as part of the "Special Activity on the Testing and Assessment of Endocrine Disruptors" within the OECD Test Guidelines Program to review, develop, standardize, and validate a number of *in vitro* and *in vivo* toxicological assays for testing and assessment of chemicals concerning their potential to interact with the endocrine system of vertebrates.

To date, the focus of much of the effort to develop *in vitro* assays has focused on the development and validation of assays that assess the effects of substances mediated through binding to the estrogen receptor (ER), and androgen receptor (AR). In addition, *in vitro* assays have also focused on the development and characterization of transcriptional activation (TA) assays with the ER and AR in stably transfected cell lines. However, while most of the effort to date has been on developing assays that focus on steroid receptors and receptor-mediated pathways, there are a number of other non-receptor-mediated processes that may alter endocrine function. There are compounds that can modulate steroid hormone production or breakdown and cause endocrine disruption without acting as direct hormone mimics. These non-receptor-mediated effects are often exerted indirectly via effects on common signal transduction pathways. They can also act as direct or indirect stimulators or inhibitors of the enzymes involved in the production, transformation or elimination of steroid hormones. One cell line that has been shown to be useful as an *in vitro* model of steroidogenic pathways and processes is the human H295R adrenocarcinoma cell line.

The present project focused on the development and standardization of a cell-based pre-screening assay using this cell line to prioritize chemicals that act to alter steroidogenic process in humans and wildlife.

6 STUDY GOALS

The present project focused on the use of the H295R cell line as an *in vitro* assay to screen for the potential effects of chemicals on steroidogenesis. The H295R human adrenocortical carcinoma cell line is a subpopulation of H295 line that forms a monolayer in culture and has been shown to have the ability to express all the key enzymes necessary for steroidogenesis. These include CYP11A (cholesterol side-chain cleavage), CYP11B1 (steroid 11b-hydroxylase), CYP11B2 (aldosterone synthetase), CYP17 (steroid 17α-hydroxylase and/or 17,20 lyase), CYP19 (aromatase), CYP21B2 (steroid 21-hydroxylase), and 3β-hydroxysteroid dehydrogenase. The cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells, with the ability to produce the steroid hormones of each of the three phenotypically distinct zones found in the adult adrenal cortex. A major advantage of the H295R cell system is its multiplex potential, which allows multiple endpoints at multiple levels to be tested using the same assay. These endpoints include gene expression, hormone production and enzyme activities, all of which have been measured individually or in combination as a part of previous projects (Sanderson et al. 2000, Hilscherova et al. 2004, Zhang et al. 2005, Gracia et al. 2006, Hecker et al. 2006). Specifically, this project was intended to serve as a preliminary validation step in the development of a screening protocol based on the H295R cell line that measures the production of the steroid hormones testosterone (T), and 17β-estradiol (E2). The overall goal of this project was to evaluate the transferability, flexibility, and applicability of this assay across several laboratories and to characterize the ability of several model substances to alter steroid hormone production.

7 STUDY OBJECTIVES

This inter-laboratory comparison and validation study was conducted to evaluate the performance of the optimized H295R cell culture and exposure protocols (APPENDICES I & II) in different laboratory settings. The study protocol was revised based on a series of optimization efforts conducted in response to issues and problems encountered during an earlier study to pre-validate the H295R Steroidogenesis Assay.

The specific objectives of this study were as follows:

1. Test the optimized H295R Steroidogenesis protocol with six independent laboratories;
2. Compare the specific changes in the production of T and E2 in response to three model compounds with different mode of interactions with steroidogenic pathways (two inhibitors, one inducer);
3. Assess the transferability, reproducibility, sensitivity and applicability of the assay;
4. Identify and optimize appropriate data evaluation procedures;
5. Identify remaining issues and propose further procedures/studies to optimize the assay;
6. Optimize the H295R Steroidogenesis Assay protocol based on the above aspects.

8 STUDY DESIGN

A detailed description of the study design and expected outcomes is outlined in the study protocols (APPENDICES I & II). Briefly, the participating laboratories were to test the effects of three model chemicals having different mode of interactions with steroidogenic pathways (**Table 8.1**). Cells for the experiments were to be cultured for five passages using new a NCI-H295R batch from ATCC (Cat# CRL-2128) prior to initiation of the exposure studies (see optimized H295R cell culture and exposure protocols for a detailed description and discussion; APPENDICES I & II). A total number of three experiments were conducted by each laboratory using subsequent passages (5-8). Each experiment consisted of four separate 24-well plates: one quality control (QC) and three model chemical exposure plates. The QC plate served as an internal control for each experiment. Chemicals were tested at six different doses in triplicate per plate. A summary of the steps that were conducted by each laboratory is provided (**Table 8.2**). The layout of both QC and exposure plates is illustrated (**Figures 8.1** and **8.2**).

Table 8.1: *Model test chemicals and their mode of action.*

Chemical	**Reported Mode of Action**
Prochloraz (PRO)	Imidiazol fungicide: Potent inhibitor of aromatase; Also capable of affecting other P450-dependent enzymes
Forskolin (FOR)	General inducer: Stimulating adenylcyclase and increasing cAMP levels in adrenal cells
Fadrozole (FAD)	Specific inhibitor of aromatase catalytic activity

Table 8.2: *Step-by-step description of procedures in the H295R inter-laboratory exposure experiments. For a detailed description of the cell passage labeling see APPENDIX I.*

Step	**Description**	**Time (~)**
1	Order new NCI-H295R batch from ATCC (Cat# CRL-2128).	1 week
2	Culture cells for five passages following the procedures listed in the culture protocol. **Note:** Split cells into only two plates rather than three plates at each passage change to reduce time for each passage to reach confluency.	4 weeks
3	Freeze down cell passages A/04/04/E – A/04/04/P.	
4	Grow cell passages A/04/04/A – A/04/04/D for one more generation.	5 days
5	Combine cells from passages A/05/05/A – A/05/05/D into one vial and seed at a density of 300,000 cells per mL (1 mL per well) into four different 24-well plates (see exposure protocol), and incubate at 37°C and 5% CO_2 for 24 hours.	24 h

	Continue growing other cell passages (A/05/05/E – A/05/05/H).	
6	After 24 h replace medium with fresh medium and dose cells at concentrations of chemicals listed in Table 2.	
7	Incubate dosed cells for 48 h at 37°C and 5% CO_2.	48 h
8	After 48 h of incubation time remove medium from cells and place in appropriately labeled Eppendorf vials (two vials per sample, each of which receives 480 µL of sample), and place samples on ice. Total number of samples equals 2 x 96.	
9	Conduct cell viability assay immediately after removal of medium from wells (see exposure protocol).	
10	Freeze one sub-set of 96 samples at -80°C. **Note:** The purpose of this is to preserve a sub-set of each sample as a backup. The other sub-sample will be processed immediately after termination of the experiment without freezing it down.	
11	Extract second sub-set of samples immediately after termination of experiment following the procedures listed in the exposure protocol. **Note:** In the case that the laboratory cannot run the tritiated standard for recovery with each sample, a recovery of 100% will be assumed for all further calculations. However, this will likely result in a slight overestimation of the absolute hormone amount reported.	1 day
12	Store dried samples for a maximum of three days at 4°C before analysis!	1-3 days
13	Measure hormone concentrations in medium extract following the instructions provided by manufacturer of the specific test kits used.	1 day
14	Enter hormone raw data into an Excel spreadsheet and calculate pg hormone per mL medium by correcting for dilution. **Note:** Please provide a description of all dilution factors, standard curves and all individual data points (measurements).	1 day
15	Combine cells from next cell passage (A/06/06/A – A/06/06/D) into one vial and seed at a density of 300,000 cells per mL (1 mL per well) into four different 24-well plates (see exposure protocol), and incubate at 37°C and 5% CO^2 for 24 hours. Continue growing other cell passages (A/07/07/E – A/07/07/H).	5 days
16	Repeat steps 6-14.	4-6 days
17	Combine cells from next cell passage (A/07/07/A – A/07/07/D) into one vial and seed at a density of 300,000 cells per mL (1 mL per well) into four different 24-well plates (see exposure protocol), and incubate at 37°C and 5% CO_2 for 24 hours.	5 days
18	Repeat steps 6-14.	4-6 days

	1	2	3	4	5	6
A	Blank [a]	Blank [a]	Blank [a]	Blank [a] + MeOH [b]	Blank [a] + MeOH [b]	Blank [a] + MeOH [b]
B	DMSO 1µL	DMSO 1µL	DMSO 1µL	DMSO 1µL + MeOH [b]	DMSO 1µL + MeOH [b]	DMSO 1µL + MeOH [b]
C	FOR 1µM (Stock 4)	FOR 0.1µM (Stock 6)	FOR 10µM (Stock 2)	PRO 0.3µM (Stock 3)	PRO 0.03µM (Stock 5)	PRO 3µM (Stock 1)
D	FOR 1µM (Stock 4)	FOR 0.1µM (Stock 6)	FOR 10µM (Stock 2)	PRO 0.3µM (Stock 3)	PRO 0.03µM (Stock 5)	PRO 3µM (Stock 1)

[a] Blank wells receive medium only.
[b] Methanol (MeOH) will be added **after** the exposure is terminated and the medium is removed from these wells

Figure 8.1: *Quality control plate layout for testing performance of H295R cells exposed to known inhibitors (Pro = prochloraz) and stimulators (For = forskolin) of E2 and T production. A 70% methanol solution will be added to all "MeOH" wells after termination of the exposure experiment and removal of medium (see Live/Dead® Product Insert). Letters indicate rows and numbers indicate columns in a 24-well cell culture plate.*

	1	2	3	4	5	6
A	DMSO 1µL	DMSO 1µL	FOR 1µM (Stock 4)	Stock 3 1µL	Stock 3 1µL	Stock 3 1µL
B	Blank	Blank	PRO 0.3µM (Stock 3)	Stock 4 1µL	Stock 4 1µL	Stock 4 1µL
C	Stock 1 1µL	Stock1 1µL	Stock 1 1µL	Stock 5 1µL	Stock 5 1µL	Stock 5 1µL
D	Stock 2 1µL	Stock 2 1µL	Stock 2 1µL	Stock 6 1µL	Stock 6 1µL	Stock 6 1µL

Blanks: Nothing should be added to these wells.
Stock 1-6: 1 µL of appropriate stock solution needs to be added to each well.

Figure 8.2: *"Test plate" dosing schematic for the exposure of H295R to test chemicals in a 24-well plate. Dosing is calculated based on a total volume of 1 mL per well. Letters indicate rows and numbers indicate columns in a 24-well cell culture plate.*

8.1 Methods

8.1.1 Cell Culture & Exposure

All procedures pertaining to the care and culture of H295R cells in preparation for their use in the H295R Steroidogenesis assay are described in detail in the H295R culture protocol (APPENDIX I). Methods required for conducting the exposure experiments are listed and discussed in the H295R exposure protocol (APPENDIX II). Hormone detection systems utilized by the participating laboratories were mass spectrometry (LC/MS) and antibody-based assays (radioimmunoassays [RIA] and enzyme-linked immunoassays [ELISA]).

Cell viability/cytotoxicity was assessed using either the *LIVE/DEAD®* Viability/Cytotoxicity Kit (Invitrogen, L-3224; MSU, RTP, DIFVR, AOE) or the MTT assay (GER). The cytotoxicity testing was conducted in the same plate in which the chemical exposure took place, and were conducted immediately after termination of the exposure experiments (see H295R exposure protocol, APPENDIX II).

While the methods for the culture and maintenance of the H295R cells and the conduct of the exposure experiments were highly prescriptive, there were still some minor differences between the procedures used by the different participating laboratories. These differences were as follows:

1. The German laboratory (GER) did not have the required permits to conduct work with radioactive isotopes such as tritium. Therefore, extraction of medium was conducted without the addition of a ^{3}H-labeled hormone as an internal spike to correct for recovery. For all of the extracts of the German group a recovery rate of 100% was assumed. Hormone extraction efficiency is typically not less than 80% of the nominal concentration as determined in over 100 extraction experiments at the MSU laboratory. Thus, the hormone concentrations determined by the GER laboratory may represent a slight overestimation of the "true" values but this was not considered of relevance to the overall outcome of the studies.
2. The US-EPA endocrinology laboratory (RTP) did not extract medium samples prior to use in the radioimmunoassay used by this group. It was stated that the methods used would not require an extraction step due to the very low cross-reactivities of the antibodies with common hormone metabolites and conjugates.

It should also be noted here that the Hong Kong (AOE) laboratory had no previous experience in conducting the assay.

8.1.2 Data Evaluation & Statistical Approach

All data were expressed as mean +/- standard deviation (SD) or when appropriate, as standard error of the mean (SEM). For relative increase/decrease evaluations, results were normalized to the mean solvent control (SC) value for each assay (i.e., each 24-well plate of cells used to test a given chemical), and results were expressed as % change relative to the SC.

Changes relative to the maximum response for each chemical were calculated in a three-step process. First, the mean of the solvent controls was subtracted from each well on the plate. Second, the solvent control-adjusted replicate well value was divided by the average maximum value for a chemical that had been adjusted by the SC. Finally, the derived value was multiplied by -100 for chemical-related decreases in hormone concentrations or by +100 for chemical-related increases in hormone concentrations.

To evaluate the statistical significance of chemical treatments on hormone production, a parametric Dunnett's test was used to compare treatment-related differences to those observed in the solvent controls (SCs). Effective concentrations (EC_{50}s and EC_{20}s) were calculated using a probit model. All statistical analyses were conducted using SAS 9.0 (SAS Institute, Cary, NC, USA), SYSTAT 11 (SYSTAT Software Inc., Point Richmond, CA) or Microsoft Excel. Differences were considered significant at $p < 0.05$.

9 RESULTS

Detailed descriptions of results are given in APPENDICES III-VI. Here we summarize and evaluate the results obtained during the studies at the participating laboratories.

In general, each laboratory performed within the given quality criteria framework for cell culture, hormone detection systems, and exposure conditions as described in the optimized study protocols (APPENDICES I & II). However, since no data were available from the CERI laboratory at the time this report was written, the CERI data will be amended to this report when available. In the following sections a summary of each specific criterion will be given for each aspect of the H295R test system that was characterized.

9.1 Cell culture

All participating laboratories demonstrated that cell growth characteristics such as doubling times, cell viability and time to achieve confluence were within the given range of 3-5 days for doubling time and 5-7 days for time to reach confluency as described in the protocols (APPENDICES I & II).

9.2 Basal Hormone Production

The comparison of basal hormone production by H295R cells revealed some variability between laboratories (**Figure 9.1**). Maximum (5942 +/- 786 pg/ml; mean +/- SD) and minimum (373 +/- 92 pg/ml) T values were observed for passages 7 at the GER and AOE laboratories, respectively. However, with the exception of the AOE group the overall variation of T among laboratories and between different cell passages was relatively low (mean +/- SD = 3711 +/- 910 pg/ml; CV = 25%). As was observed for T concentrations, there were marked differences in basal E2 concentrations among the participating laboratories. Minimum (30 +/- 6 pg/ml; Passage 5) and maximum (138 +/- 38 pg/ml; Passage 5) E2 concentrations were measured at the GER and AOE laboratories, respectively. In general, concentrations of E2 did not vary excessively (mean +/- SD = 79 +/- 27; CV = 34%) among laboratories or cell passages. At each laboratory the within-experiment variability was low for both T and E2 production (**Table 9.1**). There was an increase in E2 concentrations in medium as a function of increasing cell passage at all but the AOE laboratories. In contrast, with the exception of the GER laboratory no such trends were observed for T concentrations with increasing cell passage.

Given the differences observed for some of the hormones at or between some of the laboratories, it was decided that the data in the subsequent document would be presented as both absolute values and relative changes.

Table 9.1: *Within- and across-passage coefficients of variation (CV) of T and E2. Within-passage CV: calculated based on variation between replicates of each experiment; Across-passage CV: calculated based on variation between average responses of all experiments.*

	MSU		GER		RTP		DIFVR		AOE	
Passage	T	E2	T	E2	T	E2	T	E2	T	E2
5	9%	7%	13%	11%	-	-	27%	12%	10%	28%
6	26%	22%	7%	7%	23%	58%	28%	24%	22%	6%
7	12%	9%	13%	30%	2%	13%	-	-	42%	16%
All	31%	44%	30%	48%	15%	12%	26%	27%	60%	38%

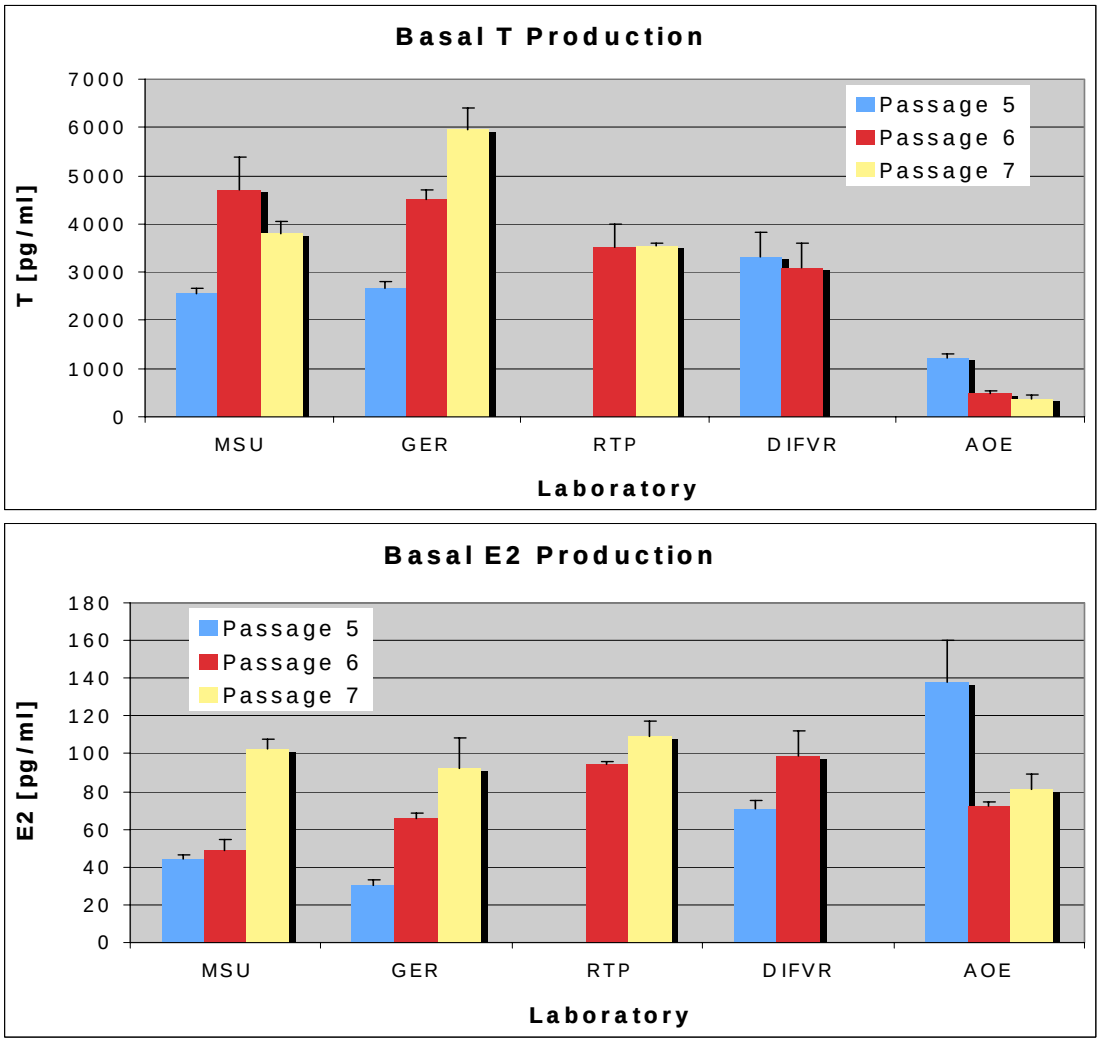

Figure 9.1: Basal production (pg/ml) of testosterone (T) and 17β-estradiol (E2) by different passages in cells cultured at five different laboratories. Error bar = Standard Error (SEM).

Exposure Experiments

9.2.1 Cytotoxicity/Cell Viability

Cell viability/cytotoxicity was determined to identify potential effects of chemical doses on cell health that could impact hormone production and give a false positive response. To control for potential inter-experiment variations, cell viability/cytotoxicity measures were conducted in all exposure plates and in each well after the medium had been removed at the end of an experiment. This method required the complete removal of medium from all wells prior to the addition of the cell viability test buffer to each well. However, in some of the initial experiments conducted at several of the laboratories the removal of culture medium resulted in the drying out of some of the wells. As a result cell viability/cytotoxicity data are not available for all experiments and laboratories. However, at least two full cell viability/cytotoxicity data sets are available from each laboratory with the exception of the AOE laboratory. These data will be amended to this report when available. For this report it was assumed that the available data is representative for all experiments.

No significant decreases in cell viability were observed for cells exposed to any of the model chemicals and/or treatments (**Figures 9.2 – 9.5**). Interestingly, the least percentage of viable cells was observed in the blanks from the forskolin and fadrozole experiments. This result was due to some very low (~ 30%) values associated with the second series of experiments and was probably the result of some drying out of several blank wells that resulted from the early removal of medium from wells at the termination of an experiment. The least number of viable cells associated with a chemical treatment was 77% that was observed in cells treated with the greatest fadrozole concentration in studies conducted at MSU. With the exception of 0.03 and 3 μM prochloraz treatments from the GER laboratory, there were no other significant negative effects of chemical treatment on cell viability. Cell viability in all other chemical treatments and/or laboratories was greater than 80%.

Notably, some of the chemical treatments resulted in a slight increase in cell viability at several of the laboratories. With the exception of the DIFVR and MSU groups, there was an increase in cell viability that occurred in the cells exposed to forskolin. The greatest increase (> 30%) was observed at a forskolin concentration of 0.3 μM at the GER laboratory. For the other laboratories, cell viability in cells exposed to forskolin did not exceed 120%. In addition, in studies conducted by the DIFVR group there was a significant increase ($p < 0.05$) in cell viability at the three greatest fadrozole concentrations (**Figure 9.4**). With the exception of the GER laboratory where the least fadrozole concentration resulted in increased cell viability, no other increases in cell viability were observed in the studies conducted at any of the other laboratories.

Although some effects on cell viability occurred in the studies conducted by the participating laboratories, these were minor in nature and were not considered relevant in the evaluation of the experimental hormone data.

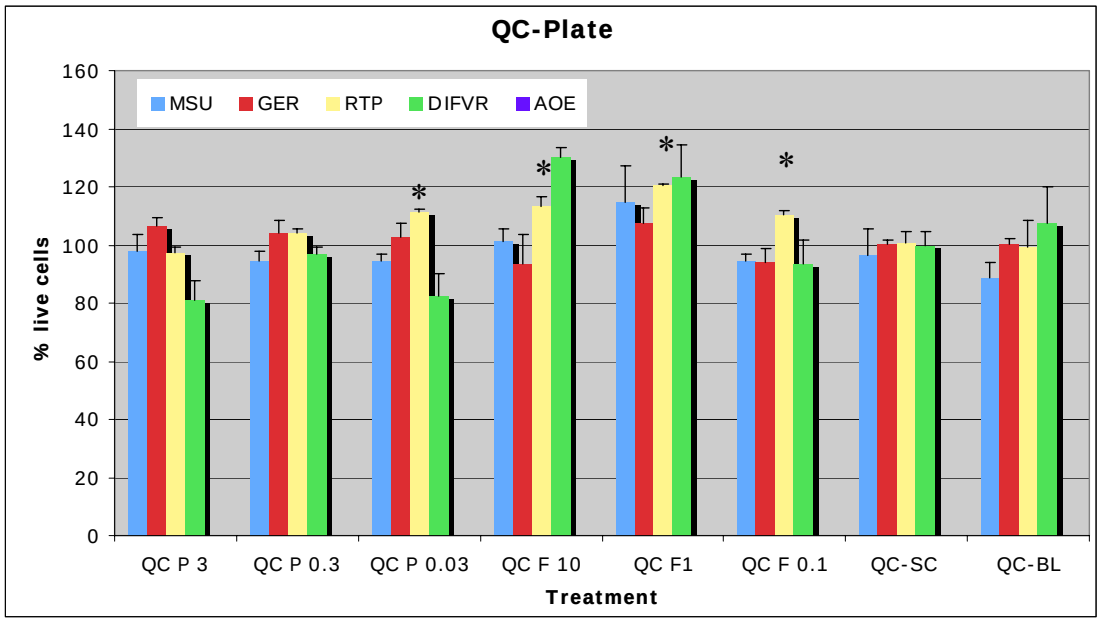

Figure 9.2: Mean % of live cells relative to solvent control (SC) in the QC-Plates. Error bar = Standard Error (SEM). BL = blank; QC F = Forskolin; QC P = Prochloraz.

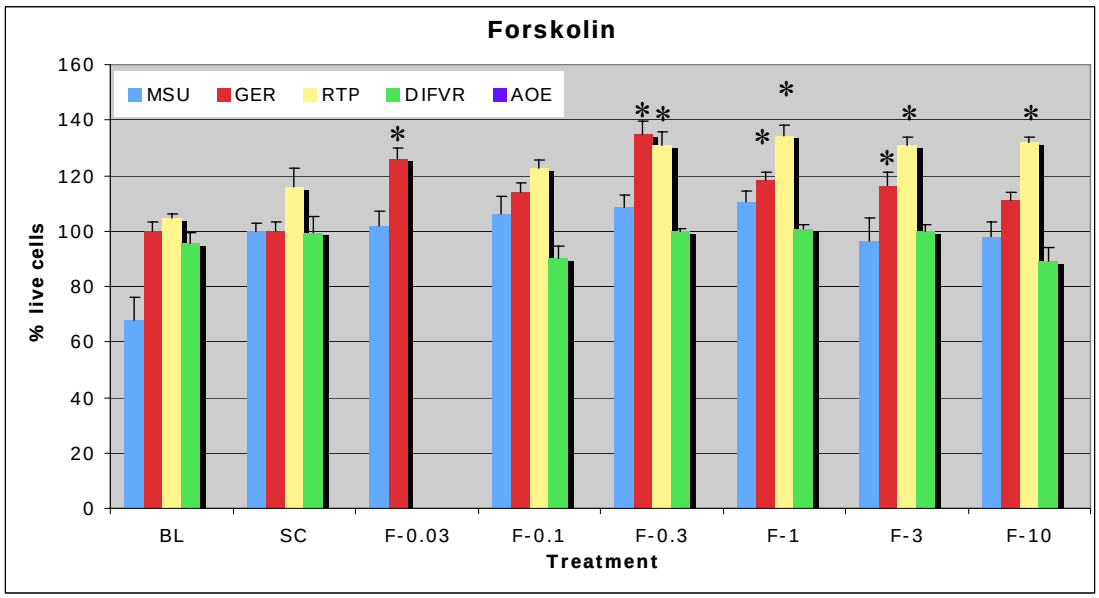

Figure 9.3: Mean % of live cells relative to solvent control (SC) in the forskolin exposure plates. Error bar = Standard Error (SEM). BL = blank. * = $p < 0.05$.

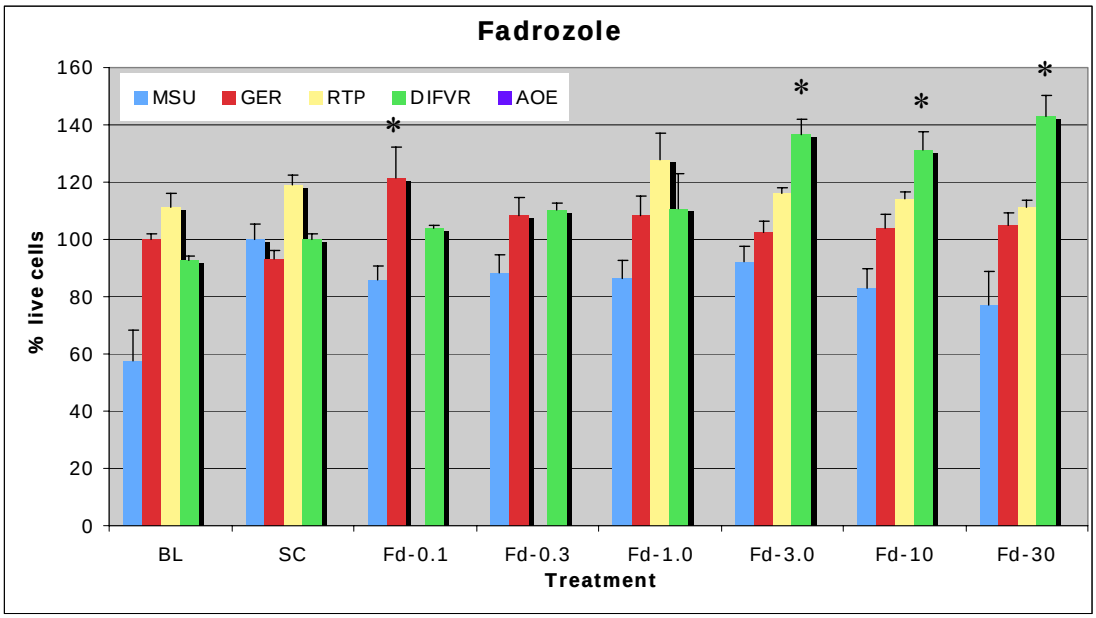

Figure 9.4: Mean % of live cells relative to solvent control (SC) in the fadrozole exposure plates. Error bar = Standard Error (SEM). BL = blank. * = $p < 0.05$.

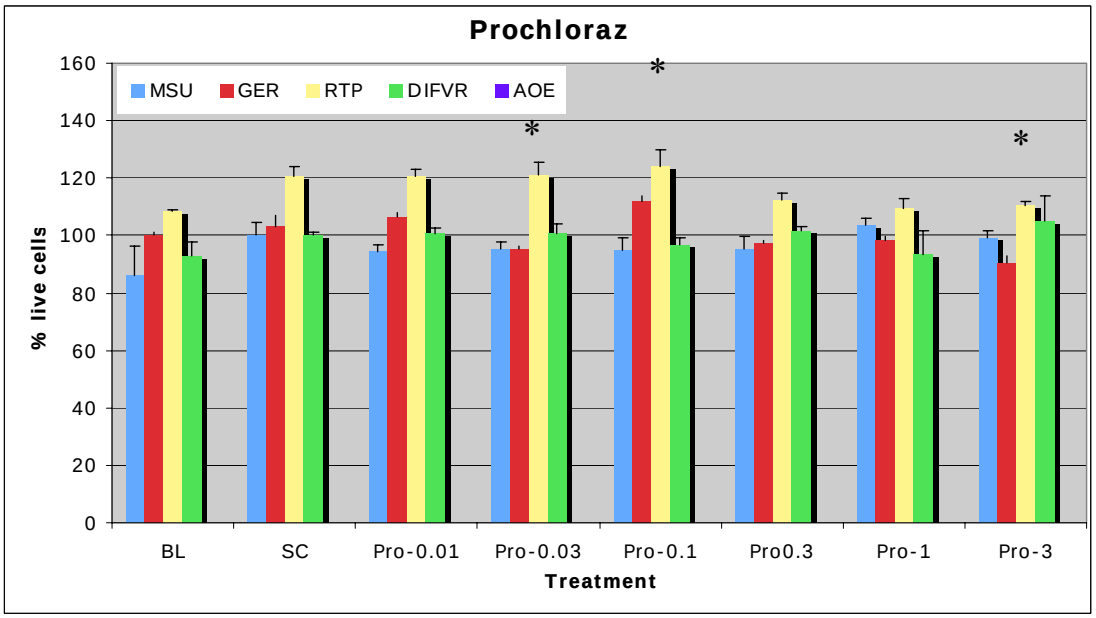

Figure 9.5: Mean % of live cells relative to solvent control (SC) in the prochloraz exposure plates. Error bar = Standard Error (SEM). BL = blank; QC F = Forskolin; QC P = Prochloraz. * = $p < 0.05$.

9.2.2 Hormone Production

To account for potential variations in E2 production as a function of cell age (passage), the data was stratified by cell passage instead of by experiment. However, it is important to note that hormone data are not available from the same cell passages from all laboratories (**Table 9.2**). To adjust for some potential variation in hormone production related to the use of data from different cell passages from the participating laboratories, the analyses were only conducted on hormone data normalized to the solvent control values (SCs) and/or data that were normalized to maximum response. Since the basal production of E2 from passage 5 cells at both the MSU and GER laboratories was near the method detection limit of the E2 assay systems, it was not possible to accurately measure dose-related suppression of E2 production in H295R cells treated with fadrozole or prochloraz. As a result, these E2 data were excluded from further consideration.

Table 9.2: *Passages used for the exposure experiments at the different laboratories participating in the inter-laboratory test.*

Passage #	MSU	GER	RTP	DIFVR	AOE
5	√	√		√	√
6	√	√	√	√	√
7	√	√	√		√
8			√	√	

9.2.2.1 QC-Plates and Plate Internal QC-Wells

To control for potential variations between and within experiments, quality controls (QC) were included in each experiment as both QC-plates and QC-wells on the chemical exposure plates. While only two individual QC-wells (one inducer and one inhibitor) were included on each plate to address possible plate-to-plate variations in each experiment, a separate QC-plate was included with each experiment to identify changes as a function of cell age (passage).

For the QC-plates, the effects of increasing exposure concentrations of forskolin and prochloraz on the production of T were comparable across all laboratories. In these experiments hormone concentrations increased with increasing forskolin concentrations while increasing prochloraz concentrations resulted in a concurrent decrease in hormone concentrations (**Figures 9 .6** & **9.7**). With the exception of cells treated with 0.1 μM forskolin in studies conducted at the DIFVR and AOE laboratories, T concentrations significantly differed from their respective solvent controls at all concentrations tested with both chemicals ($p < 0.05$). The only observable differences among laboratories were that at the RTP laboratory there was a slightly greater increase in T concentrations, and at the AOE laboratory there was a slightly lesser decrease in T production in cells

exposed to forskolin and prochloraz, respectively. This difference was not apparent when comparing dose-response curves that were calculated relative to the maximum response among the laboratories.

The trends in E2 production were similar in cells exposed to either forskolin or prochloraz across all laboratories, as was also observed for T production (**Figures 9.8 & 9.9**). There was a significant increase in mean E2 concentrations ($p < 0.05$) at 1 μM forskolin doses at all laboratories with the exception of the RTP group for which significant effects were observed at a forskolin concentration of 0.1 μM. In studies conducted at MSU, GER, DIFVR, and AOE, exposure to prochloraz concentrations of 0.3 μM and greater resulted in significantly reduced E2 production relative to the solvent controls. At RTP a significant decrease was observed at all doses tested including 0.03 μM prochloraz. While the trend in E2 production for cells exposed to forskolin and prochloraz was comparable between laboratories, the fold-changes in E2 relative to the SCs varied considerably at the two greatest chemical concentrations among laboratories (**Figure 9.9**). It was hypothesized that this result was mainly due to the differences in method detection limits (MDLs) for the hormone assays utilized by the various laboratories. This may have been especially true for both MSU and GER where E2 production in the solvent controls was close to the MDL. This is further confirmed by the fact that the differences observed between laboratories for data expressed as fold-change relative to the SC were not apparent when compared to the results that were analyzed when the data expressed as %-maximum response.

Across-laboratory variations in the hormone production on the QC-plates varied between 29 and 57% for changes relative to SC and from 8 and 48% for changes relative to %-maximum effect (**Table 9.3**). Coefficients of variation only exceeded 50% for the relative fold-change in E2 concentrations in the QC prochloraz experiments.

Hormone concentrations in the internal control wells were within the range of the control plates indicating that there was no substantial difference in performance between the plates of one experiment (**Figures 9.10 and 9.11**). However, there was a relatively great variation among plates at some of the laboratories, which is likely to be due to the fact that there was only one replicate well run per plate. No data on the in-plate QCs is available from the DIFVR laboratory.

***Table 9.3**: Among-laboratory coefficients of variation (CV) of T and E2 production by H295R cells exposed to forskolin (QC F) and prochloraz (QC P) in the QC-plates. CVs were calculated based on the average response measured at each laboratory for changes relative to the SCs as well as for changes relative to the maximum effect observed.*

	Fold-Change Relative to SC		%-Maximum Effect	
	QC F	QC P	QC F	QC P
T	29%	42%	24%	8%
E2	44%	57%	32%	48%

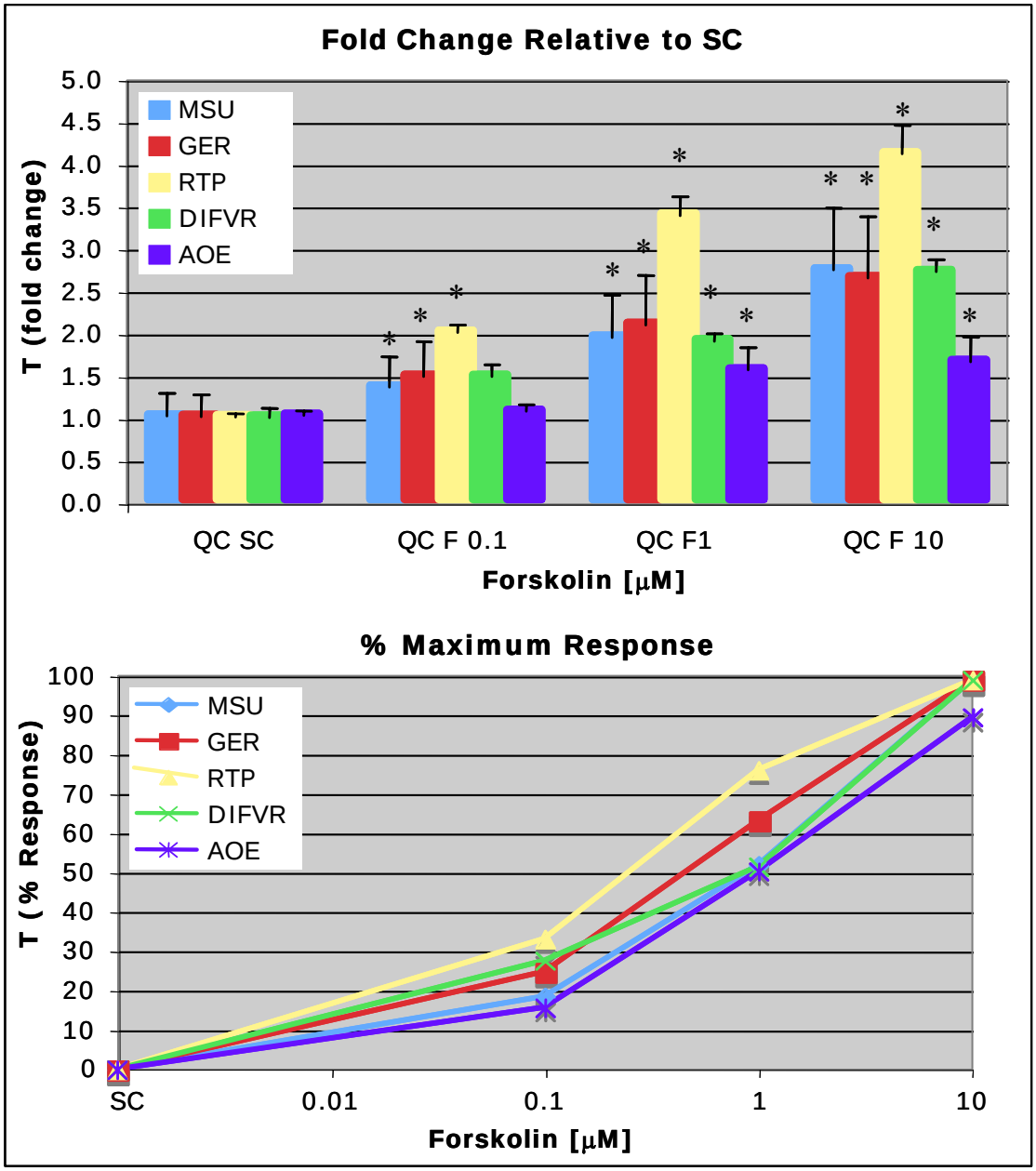

Figure 9.6: Changes in T production by H295R cells in the QC plates expressed as relative changes compared to the SC (upper graph) and percent of the maximum hormone concentration measured across all doses (maximum induction = 100%; lower graph) observed after 48 h exposure to forskolin in the QC-plates. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

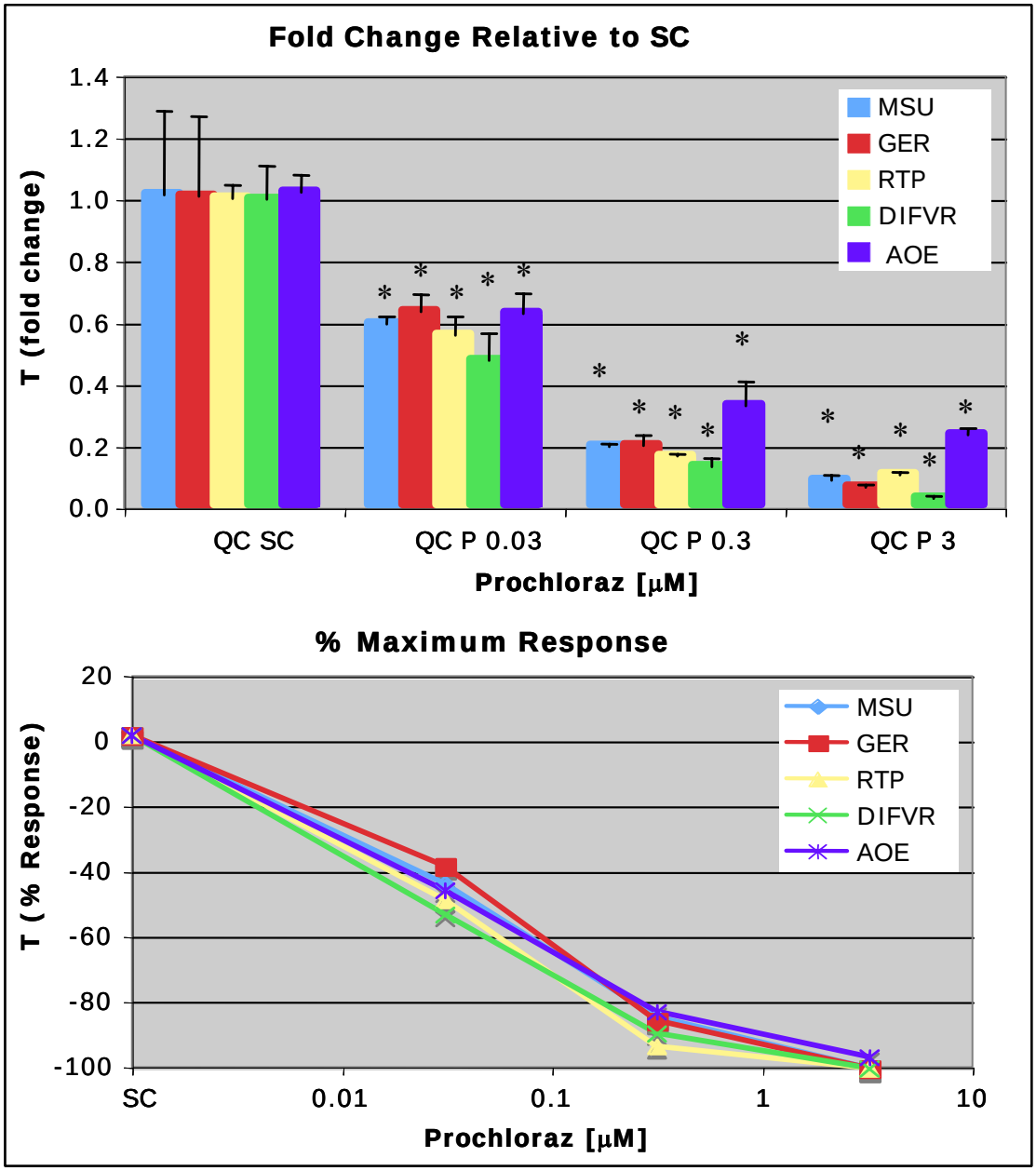

Figure 9.7: Changes in T production by H295R cells in the QC plates expressed as relative changes compared to the SC (upper graph) and percent of the least hormone concentrations measured across all doses (maximum suppression = -100%; lower graph) observed after exposure to prochloraz in the QC-plates for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

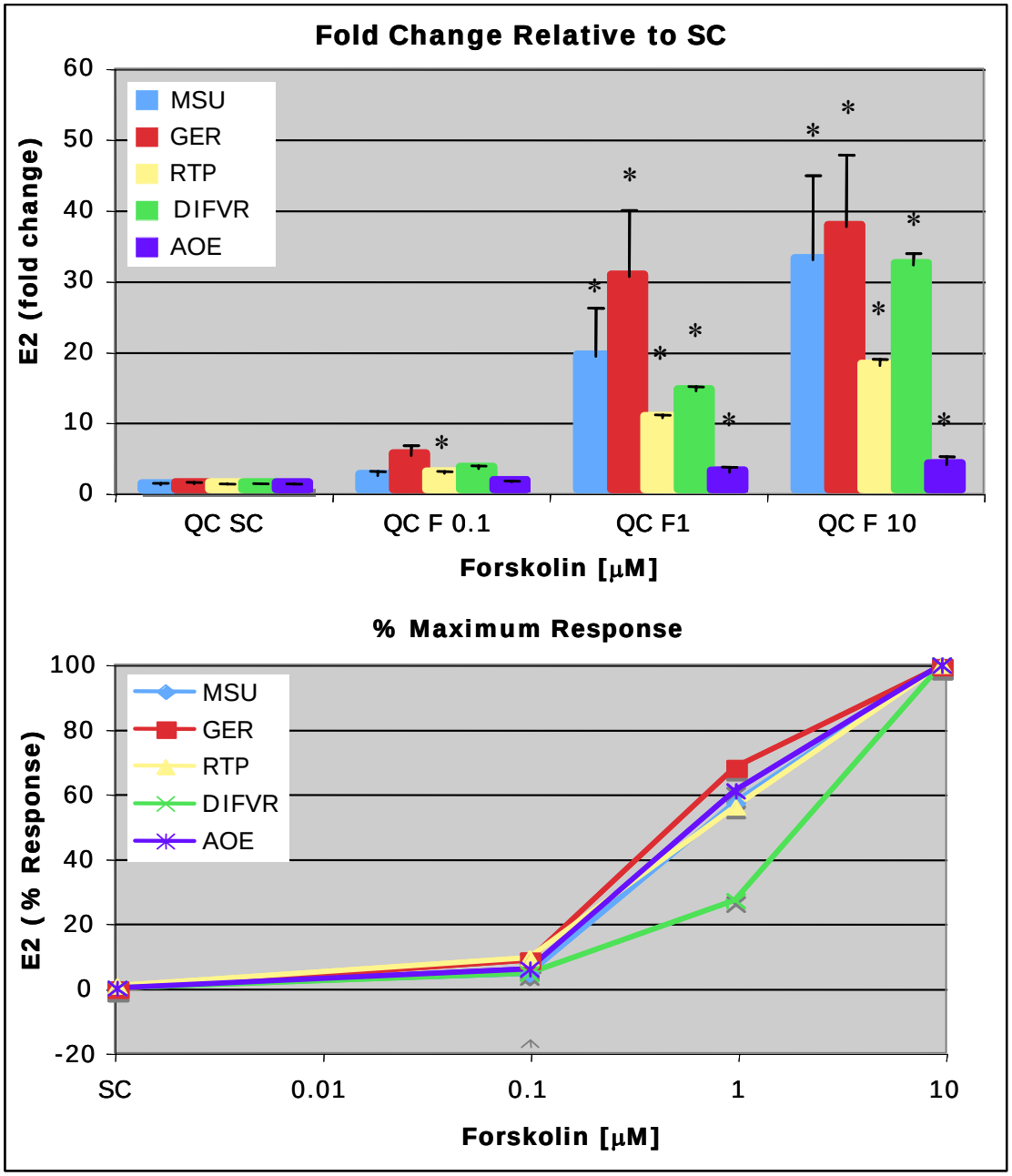

Figure 9.8: Changes in E2 production by H295R cells expressed as relative changes compared to the SC (upper graph) and percent of the maximum hormone concentration measured across all doses (maximum induction = 100%; lower graph) observed after exposure to forskolin in the QC-plates for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

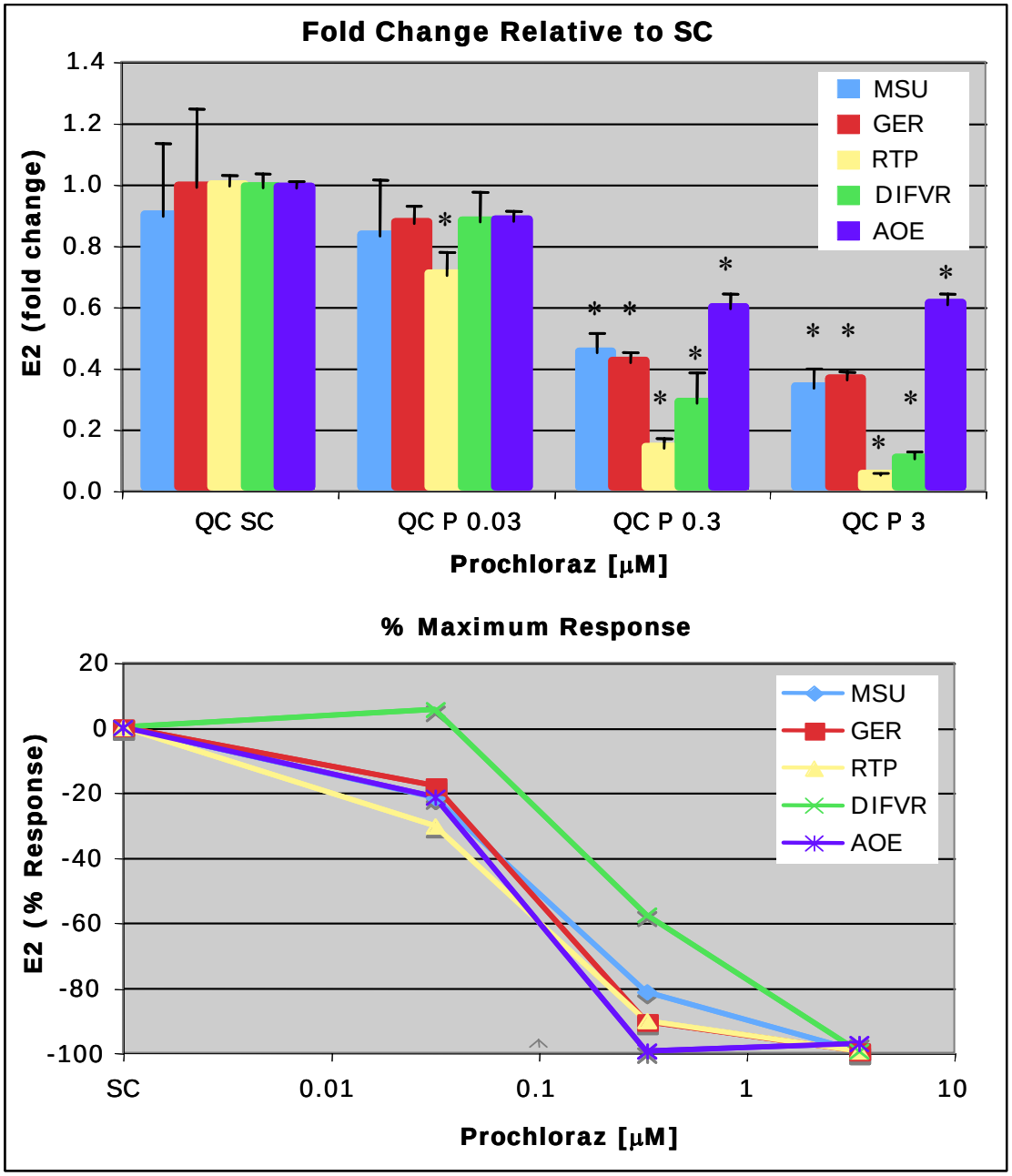

Figure 9.9: Changes in E2 production by H295R cells expressed as relative changes compared to the SC (upper graph) and percent of the least hormone concentrations measured across all doses (maximum suppression = -100%; lower graph) observed after exposure to prochloraz in the QC-plates for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

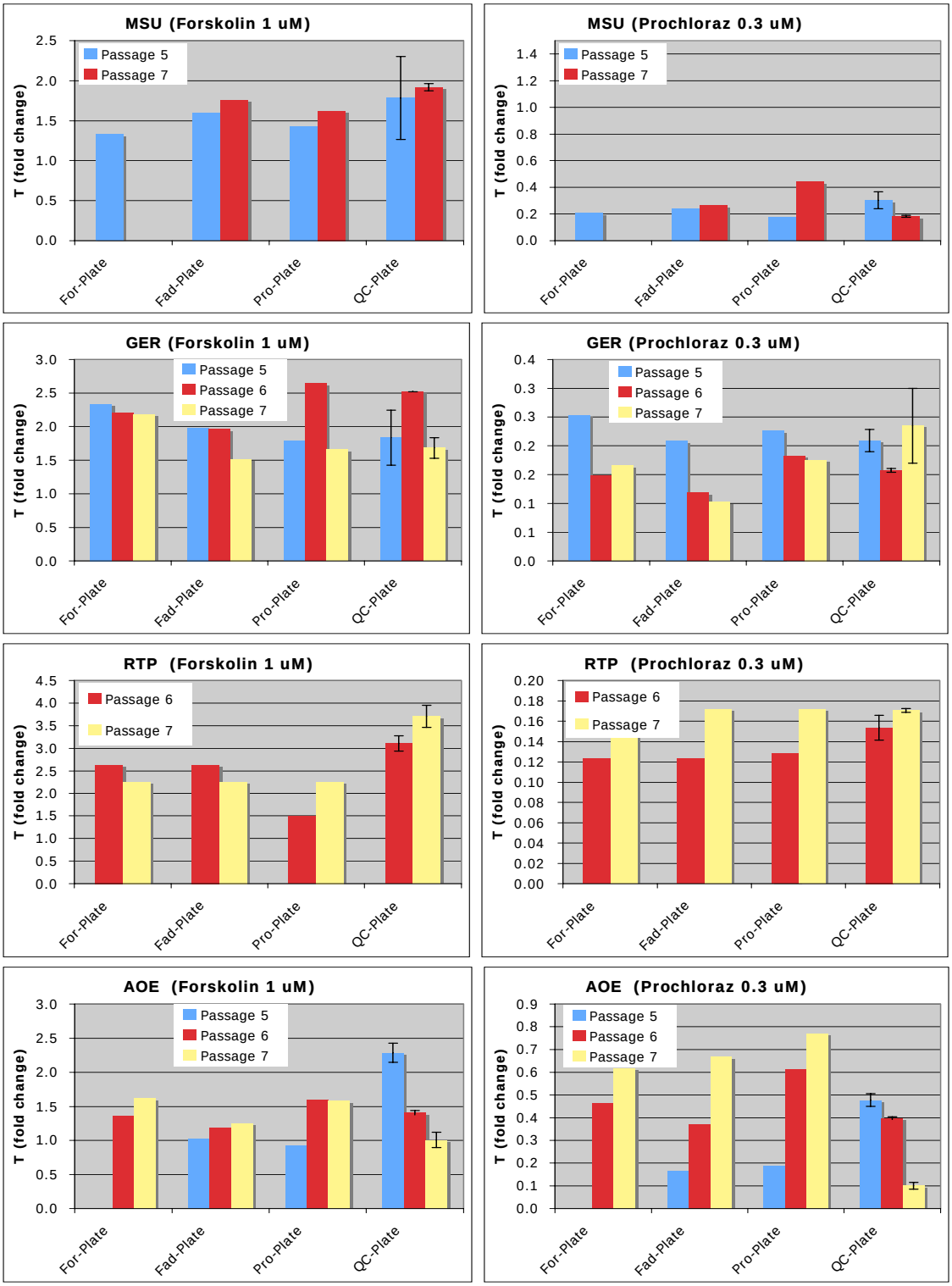

Figure 9.10: Changes in T production by H295R cells expressed as relative changes compared to the SC (SC = 1.0) observed after exposure to forskolin (left figures) and prochloraz (right figures) in the QC- and exposure plates for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard deviation.

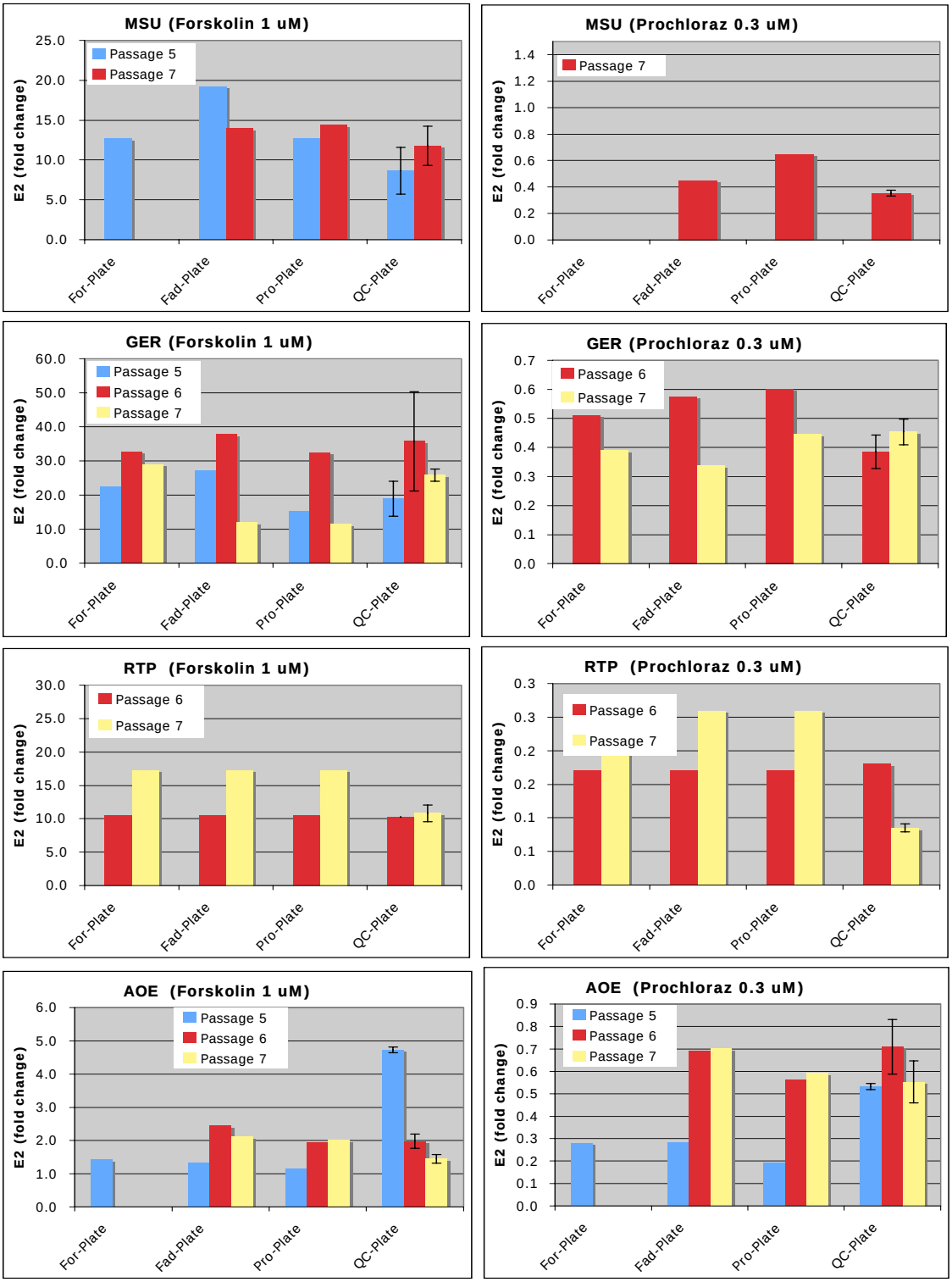

Figure 9.11: Changes in E2 production by H295R cells expressed as relative changes compared to the SC (SC = 1.0) observed after exposure to forskolin (left figures) and prochloraz (right figures) in the QC- and exposure plates for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard deviation.

9.2.2.2 Chemical Exposure Experiments

Exposure of H295R cells to the three model compounds resulted in marked dose-response changes in the profiles of T and E2. Overall, the response patterns in hormone production were similar among all laboratories with the only difference being observed in the magnitude of the effects among laboratories. In general, variation among laboratories was greater for E2 than for T. This was most likely due to the lower basal E2 concentrations that approximated the method detection limit at three of the five laboratories. The greatest difference in E2 concentrations, when compared across all laboratories, was observed at the AOE laboratory for which the least efficacies occurred. In almost all cases the differences between laboratories were not apparent when data was expressed as %-change relative to the maximum response. The exception was T measured in the forskolin exposure experiments conducted at the AOE laboratory, where the threshold for effects in the dose-response curve was shifted towards greater chemical concentrations by approximately one order of magnitude. In general, there was an increase in T and E2 production in H295R cells that were exposed to increasing concentrations of forskolin. In cells exposed to either fadrozole or prochloraz there was a concentration-dependent decrease in these hormones (**Figures 9.12 – 9.17**).

Forskolin: Significant increases in T production occurred at forskolin concentrations greater or equal to 0.1 μM in studies conducted at MSU, RTP and DIFVR **(Figure 9.12)**. At the GER and AOE, significant increases of T production were observed at concentrations greater or equal to 0.03 and 1.0 μM, respectively. Estradiol production by H295R cells was significantly increased at all laboratories at concentrations greater or equal to 0.3 μM with the exception of the GER and AOE **(Figure 9.13)**. At these laboratories, significant alterations in T production were observed at 0.1 and 1 μM, respectively. While the marked differences in relative changes T that were observed between the AOE and the other laboratories persisted when expressing the data as %-maximum response, the differences among laboratories for E2 production was not nearly as apparent when the data were transformed to %-maximum response values (**Figures 9.12 and 9.13**). Forskolin doses of less than 0.1 μM were not tested at the RTP, DIFVR and AOE laboratories.

Fadrozole: With the exception of the AOE results, there was a significant decrease in T production at fadrozole exposure concentrations greater than or equal to 1.0 μM for all the other participating laboratories **(Figure 9.14)**. At AOE, there were significant decreases in T that were observed at only at concentrations greater 1.0 μM. At the least fadrozole concentration (0.1 μM) there was a slight increase in T concentrations at GER, DIFVR and AOE (only significant for DIFVR data; $p < 0.05$). Changes relative to both SCs and maximum response were comparable among laboratories and the only significant differences observed were slightly greater changes relative to the SCs at doses greater than 1 μM at the AOE laboratory. Production of E2 was significantly reduced compared to the SCs at all doses tested for all laboratories **(Figure 9.15)**. Also, there was no clear dose-response pattern observable as reported for T, and it appeared that at most of the laboratories the maximum observable effect already occurred at the lowest two

fadrozole concentrations. Fadrozole doses of less than 1.0 μM were not tested at the RTP laboratory.

Prochloraz: Increasing prochloraz concentrations resulted in a marked decrease of both T and E2 regardless of laboratory and experiment **(Figures 9.16 and 9.17)**. Patterns observed for T were comparable among groups with maximum decreases at the two greatest doses tested. These decreases were significant at concentrations greater than or equal to 0.01 μM in studies conducted at MSU and DIFVR whereas significant decreases were only observed at prochloraz concentrations greater than 0.03 μM at GER, RTP and AOE. Responses of E2 to increasing concentrations of prochloraz were more variable with significant decreases in E2 occurring at 0.1 μM for MSU and AOE and at 0.3 μM for all the other laboratories when compared to the SCs. The observed differences in sensitivity in E2 productions of H295R cells among laboratories to prochloraz exposure were apparent regardless of the data being presented as changes relative to the SCs or as %- maximum response.

Data Evaluation: Data were evaluated using two separate statistical approaches. In the first approach, relative changes in hormone concentrations were compared to the SCs and tested for significance using the Dunnett's test. This approach resulted in the derivation of lowest observable effect concentrations (LOECs) and no observable effect concentrations (NOECs) for each of the chemical exposures. In the second approach, data were transformed to probit values and then were plotted as a function of log chemical dose. To identify the linear portion of the log dose - probit curves, data points from the tails were dropped from the analysis until the r^2 was greater 0.9 and then a linear regression model was fit through the remaining points. A minimum of three data points were used in each analysis and effective concentrations (ECs) were calculated from these fitted data.

With some exceptions, LOECs and NOECs among all laboratories typically ranged within 2- to 3-fold of each other and the variation never exceeded one order of magnitude (**Table 9.4**). In contrast, the data of the AOE laboratory was generally characterized by a greater variation when compared to the other groups and resulted in a NOEC and LOEC for forskolin that was greater than 5-fold different from the other laboratories. At each laboratory, both LOECs and NOECs generally did not exceed a 2-fold range regardless of hormone or chemical analyzed.

Effective concentrations for both T and E2 were generally within a 2-fold range regardless of laboratory and chemical tested (**Table 9.5**). EC_{20}s were more variable (among laboratory CV range: 55 – 147%) than EC_{50}s (among laboratory CV range: 33 – 127%). No EC values for E2 could be calculated for the fadrozole treatment. This was due to the fact that the maximum decrease in E2 production had already occurred at the two lowest concentrations and no clear dose-response relationships could be derived. EC values were comparable across laboratories and chemicals with the exception of T in the forkolin experiment conducted at the AOE laboratory. Here EC_{20}s were approximately 10-fold greater than the those reported by the other laboratories. Hormone production by H295R cells at all laboratories was most sensitive to the exposure with prochloraz.

Table 9.4: *No observable effect concentrations (NOEC) and mean lowest observable effect concentrations (LOEC) calculated based on the lowest significant change relative to the SCs (Dunnett's test) that was observed for T and E2 at each of the laboratories. Data is expressed as mean of all passages +/- standard deviation (numbers in brackets). Lab = laboratoriesFor = forskolin; Fad = fadrozole; Pro = prochloraz.*

Lab	Steroid	For		Fad		Pro	
		NOEC (μM)	LOEC (μM)	NOEC (μM)	LOEC (μM)	NOEC (μM)	LOEC (μM)
MSU	T	0.17 (0.12)	0.53 (0.40)	0.21 (0.16)	0.70 (0.52)	0.003 (0.000)	0.010 (0.000)
	E2	0.17 (0.12)	0.53 (0.40)	n.d.	0.10 (0.00)	0.017 (0.012)	0.053 (0.040)
GER	T	0.03 (0.00)	0.10 (0.00)	0.21 (0.16)	1.00 (0.0)	0.017 (0.012)	0.053 (0.040)
	E2	0.03 (0.00)	0.10 (0.00)	n.d.	0.10 (0.00)	0.063 (0.050)	0.20 (0.040)
RTP	T	0.17 (0.19)	0.55 (0.64)	0.30 (0.00)	1.00 (0.00)	0.007 (0.0050)	0.020 (0.014)
	E2	0.20 (0.14)	0.65 (0.50)	n.d.	1.00 (0.00)	0.020 (0.014)	0.052 (0.069)
DIFVR	T	0.07 (0.05)	0.20 (0.14)	0.65 (0.50)	2.00 (1.40)	0.010 (0.00)	0.030 (0.000)
	E2	0.10 (0.00)	0.30 (0.00)	n.d.	0.30 (0.00)	0.100 (0.000)	0.300 (0.000)
AOE	T	4.70 (4.70)	14.00 (14.00)	1.70 (1.20)	5.30 (4.00)	0.040 (0.052)	0.120 (0.160)
	E2	0.44 (0.50)	1.4 (1.5)	n.d.	0.10 (0.00)	0.340 (0.570)	1.000 (1.700)
All Labs	T	0.11[a] (0.07)	0.35[a] (0.23)	0.61 (0.62)	2.00 (1.90)	0.015 (0.015)	0.047 (0.044)
	E2	0.12 (0.08)	0.40 (0.25)	n.d.	0.28 (0.40)	0.120 (0.130)	0.350 (0.390)

n.d. = NOEC was not determinable due to lack of a dose-response pattern (lowest dose = LOEC).

[a] AOE data was not considered when calculating this value because the response to forskolin at this lab was extremely great compared to all other laboratories.

Table 9.5: *Effective concentrations (EC_{20} and EC_{50}) calculated based on dose-response curves of T and E2 after increasing exposure concentrations of forskolin (FOR), fadrozole (FAD) and prochloraz (PRO) at all laboratories. Data is expressed as mean of all passages +/- standard deviation (number in brackets). Lab = laboratories. CV = coefficient of variation.*

		FOR		FAD		PRO	
		T (μM)	E2 (μM)	T (μM)	E2 (μM)	T (μM)	E2 (μM)
MSU	EC20	0.058 (0.040)	0.12 (0.032)	0.25 (0.16)	n.d.	0.003 (0.001)	0.022 (0.009)
	EC50	0.26 (0.16)	0.44 (0.11)	1.2 (0.503	n.d.	0.019 (0.005)	0.11 (0.052)
GER	EC20	0.027 (0.02)	0.072 (0.017)	0.46 (0.21)	n.d.	0.018 (0.008)	0.070 (0.074)
	EC50	0.26 (0.12)	0.26 (0.054)	1.5 (0.38)	n.d.	0.055 (0.013)	0.12 (0.082)
RTP	EC20	0.095 (0.038)	0.19 (0.010)	0.83 (0.24)	n.d.	0.014 (0.014)	0.033 (0.042)
	EC50	0.53 (0.18)	0.87 (0.30)	2.2 (0.31)	n.d.	0.039 (0.043)	0.076 (0.096)
DIFVR	EC20	0.064 (0.033)	0.28 (0.030)	1.5 (1.5)	n.d.	0.013 (0.002)	0.079 (0.040)
	EC50	0.19 (0.067)	0.78 (0.005)	3.0 (1.9)	n.d.	0.052 (0.004)	0.20 (0.052)
AOE	EC20	0.69 (0.20)	0.077 (0.11)	0.67 (0.031)	n.d.	0.008 (0.010)	0.017 (0.023)
	EC50	2.3 (0.26)	0.47 (0.50)	2.56 (0.434)	n.d.	0.043 (0.024)	0.058 (0.062)
Mean All Labs	EC20	0.19 (0.28)	0.15 (0.088)	0.74 (0.48)	n.d.	0.011 (0.006)	0.038 (0.028)
	EC50	0.71 (0.90)	0.56 (0.25)	2.1 (0.74)	n.d.	0.042 (0.014)	0.11 (0.055)

n.d. = Effective concentrations were not determinable due to lack in dose-response pattern (maximum response occurred at one of lowest two doses.

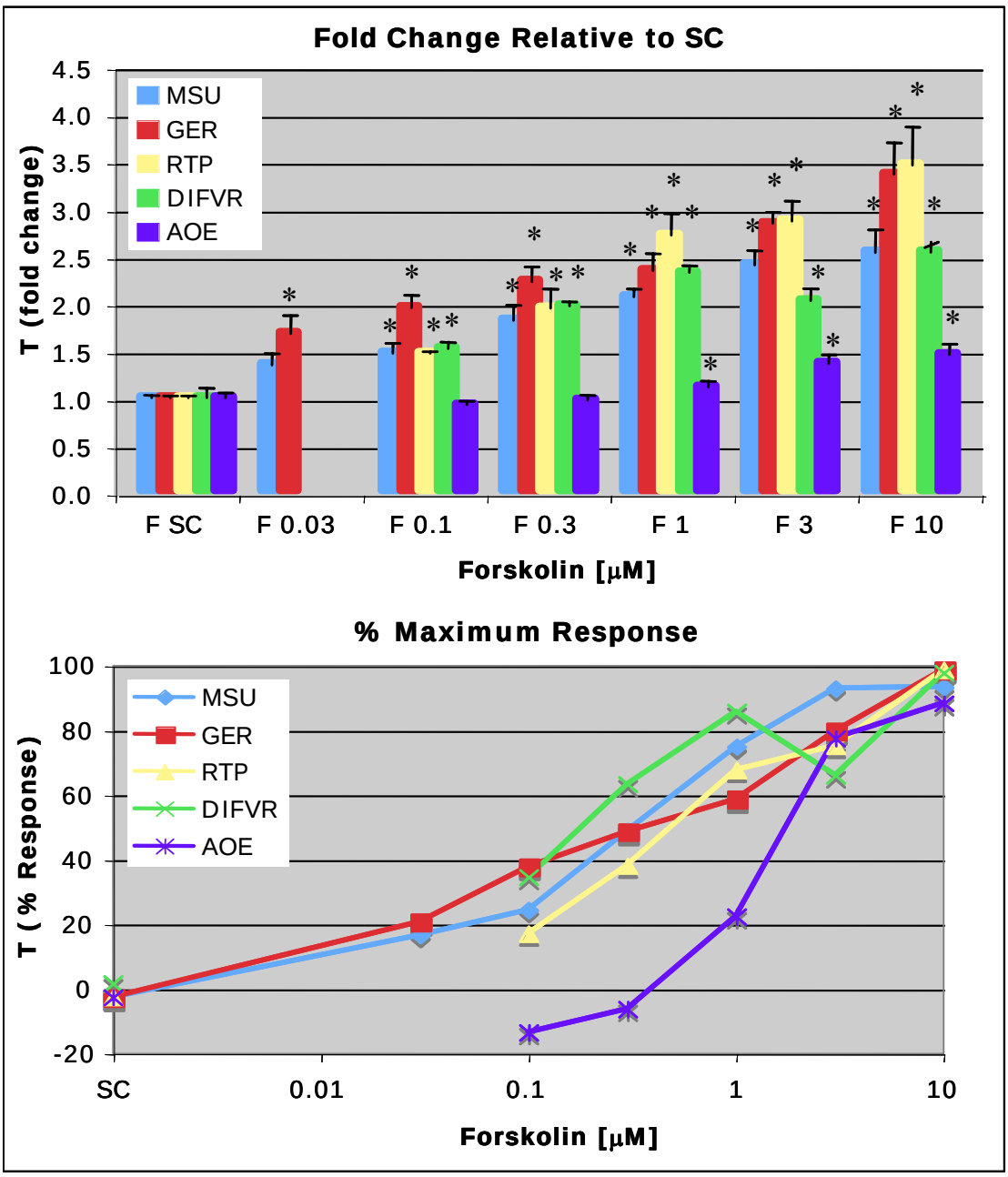

Figure 9.12: Changes in T production by H295R cells expressed as relative changes compared to the SC (upper graph) and percent of the maximum hormone concentration measured across all doses (maximum induction = 100%; lower graph) observed after exposure to forskolin for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

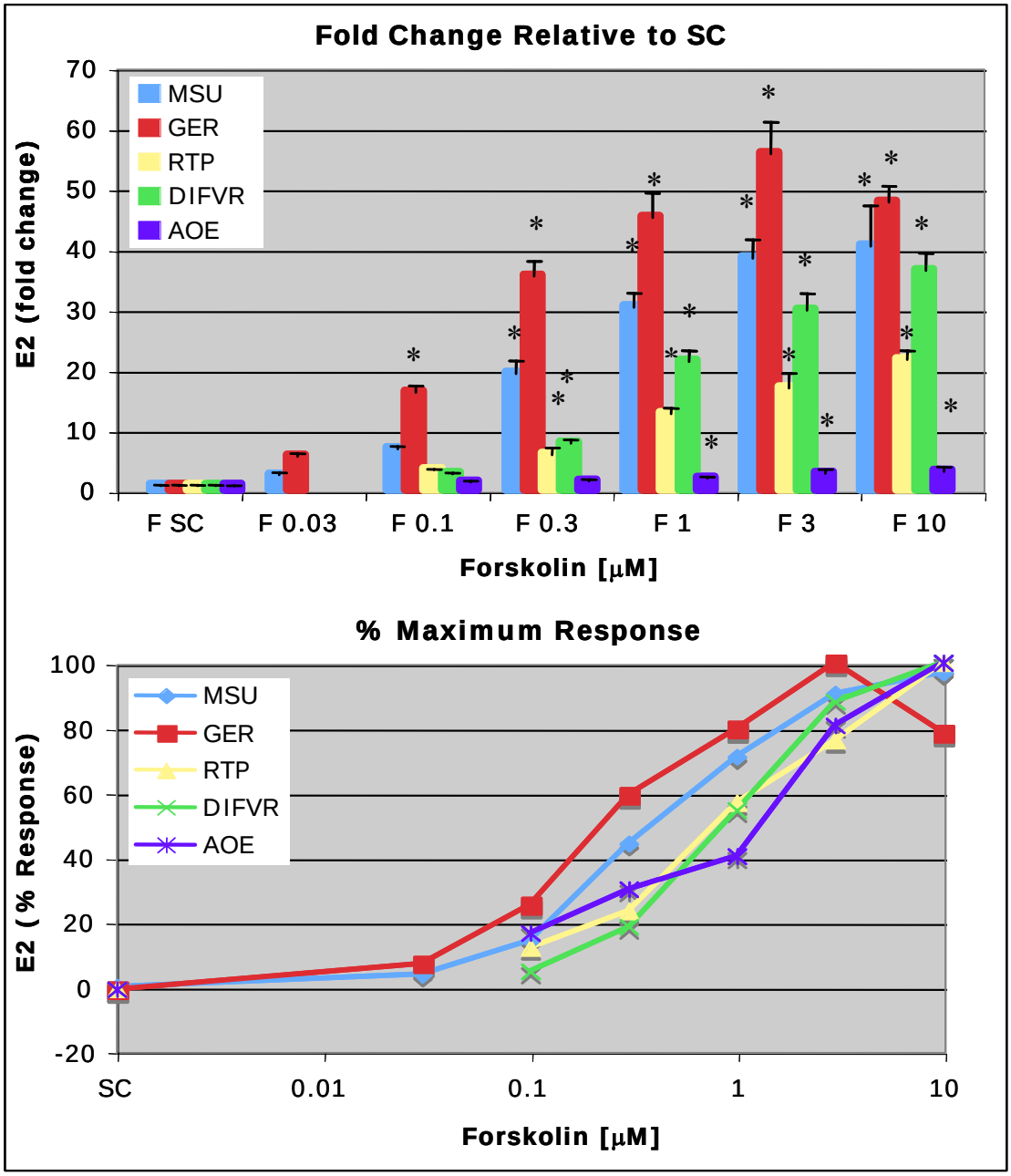

Figure 9.13: Changes in E2 production by H295R cells expressed as relative changes compared to the SC (upper graph) and percent of the maximum hormone concentration measured across all doses (maximum induction = 100%; lower graph) observed after exposure to forskolin for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). *

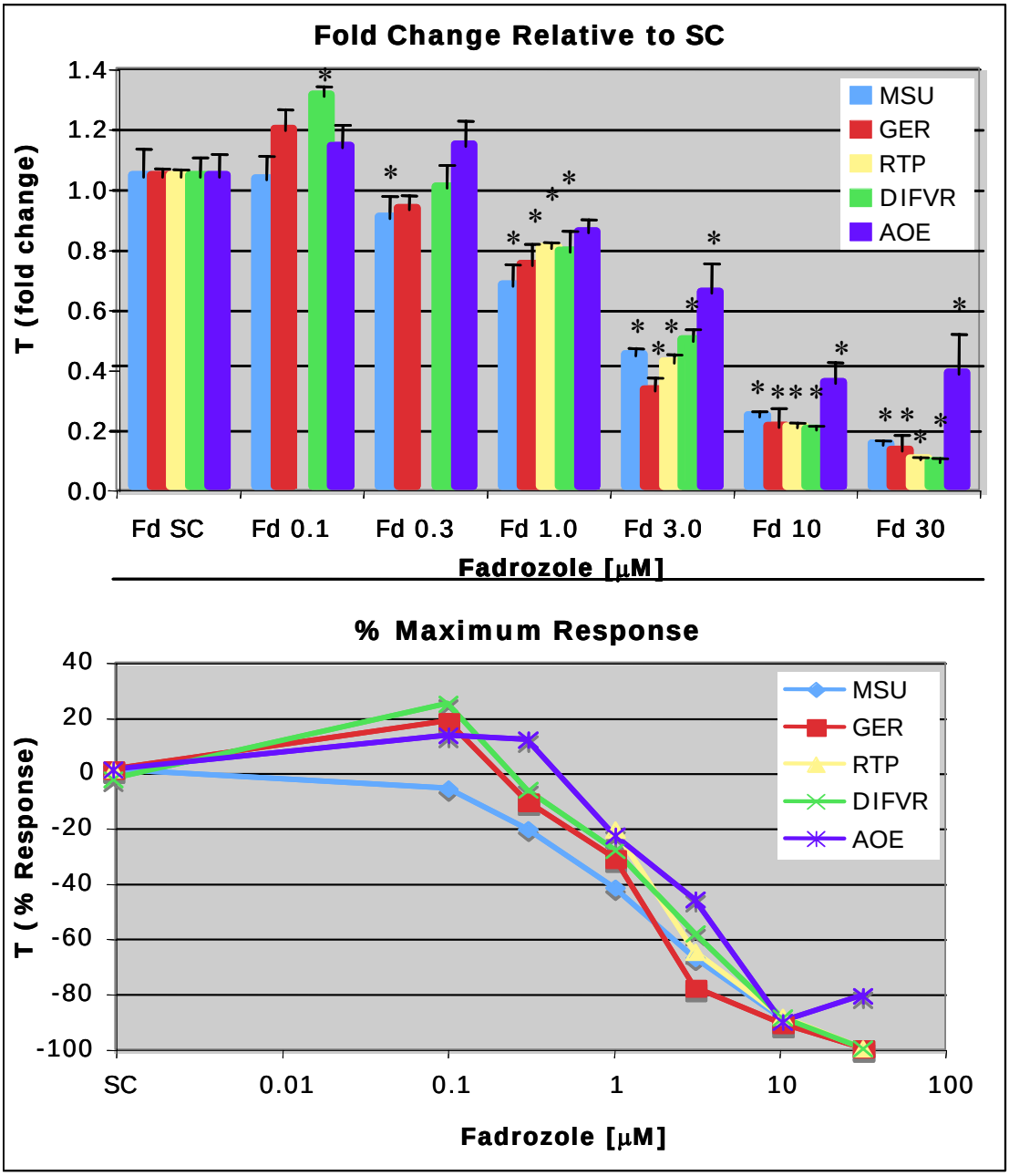

Figure 9.14: Changes in T production by H295R cells expressed as relative changes compared to the SC (upper graph) and percent of the least hormone concentration measured across all doses (maximum suppression = -100%; lower graph) observed after exposure to fadrozole for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

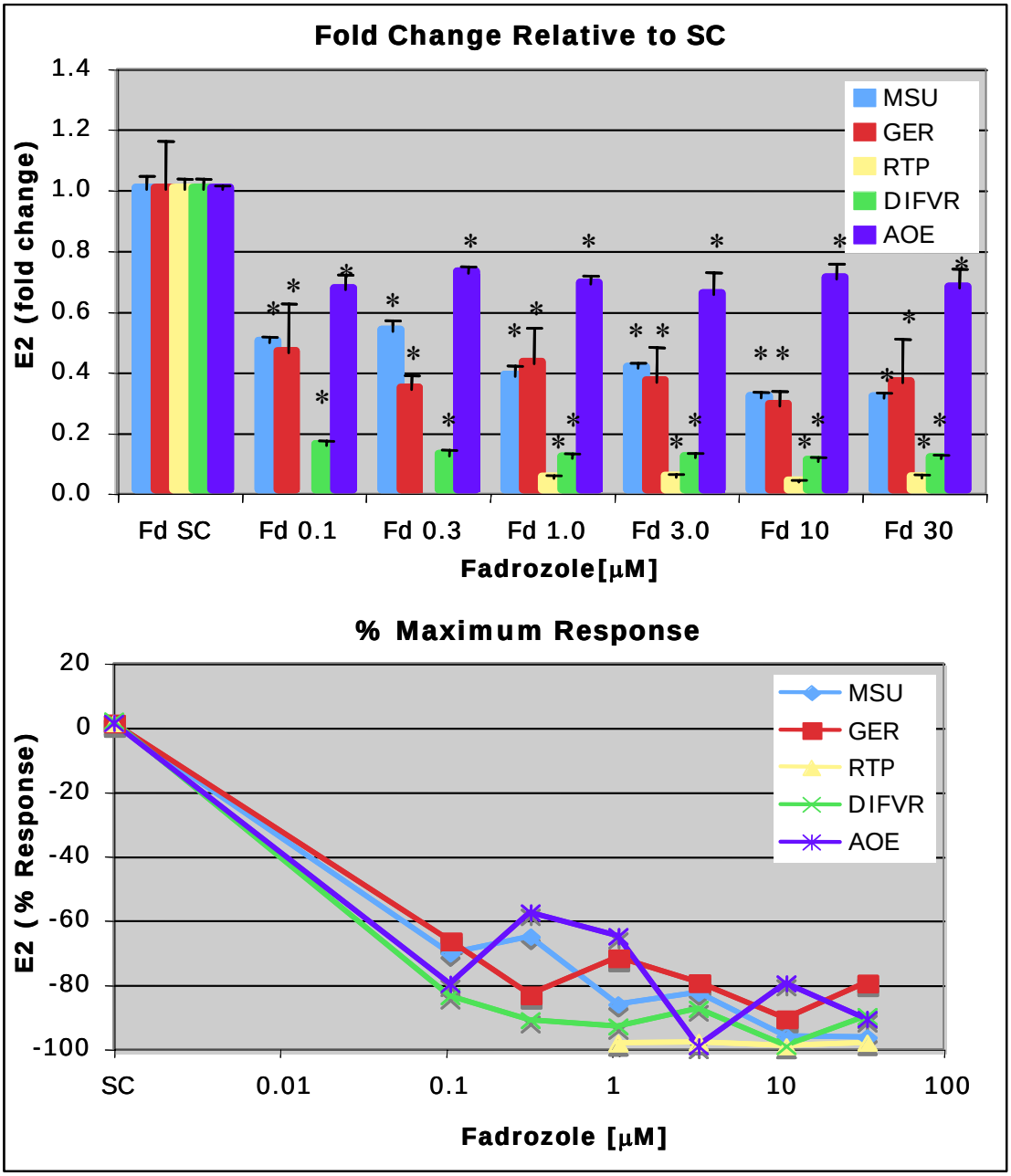

Figure 9.15: Changes in E2 production by H295R cells expressed as relative changes compared to the SC (upper graph) and percent of the least hormone concentration measured across all doses (maximum suppression = -100%; lower graph) observed after exposure to fadrozole for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

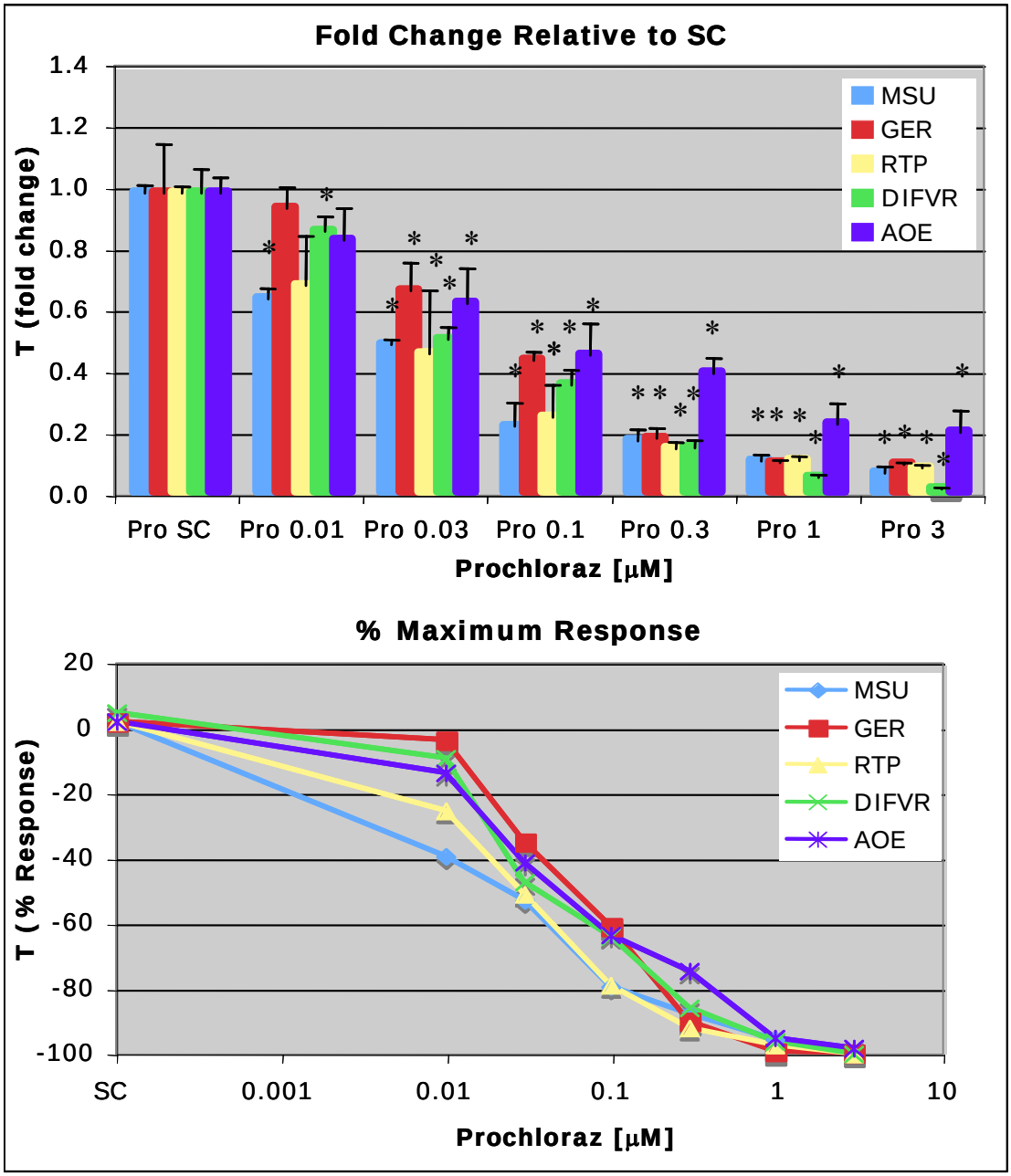

Figure 9.16: Changes in T production by H295R cells expressed as relative changes compared to the SC (upper graph) and percent of the least hormone concentration measured across all doses (maximum suppression = -100%; lower graph) observed after exposure to prochloraz for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

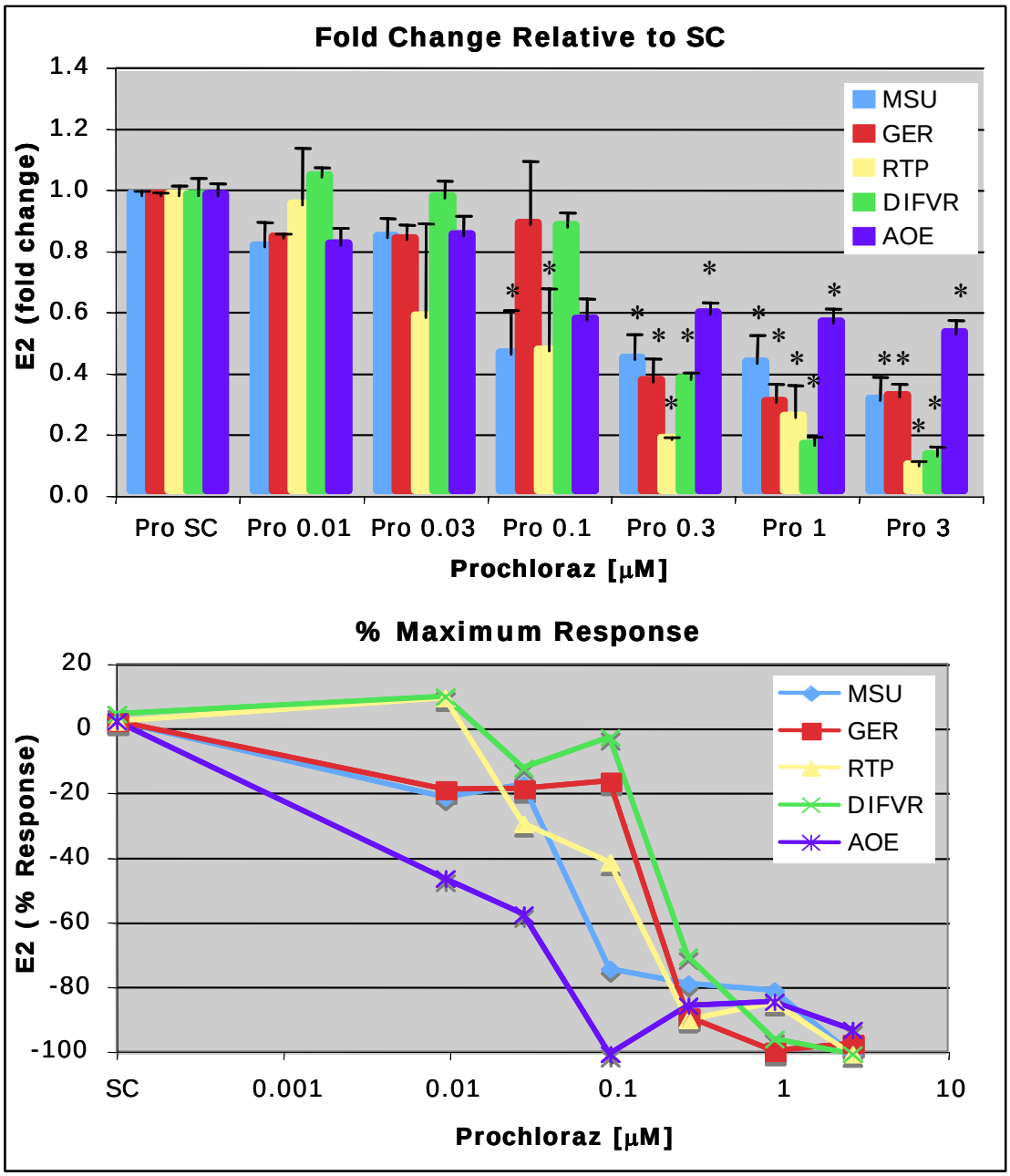

Figure 9.17: Changes in E2 production by H295R cells expressed as relative changes compared to the SC (upper graph) and percent of the least hormone concentration measured across all doses (maximum suppression = -100%; lower graph) observed after exposure to prochloraz for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

10 CONCLUSIONS AND RECOMMENDATIONS

10.1 Quality Controls

The use of both QC plates and QC wells demonstrated good reproducibility between both experiments and plates. Furthermore, the use of a separate QC plate with each experiment eliminates the need to run a full set of internal controls (Live/Dead® 100% mortality controls, different positive and negative control doses, etc.) on each individual exposure plate. This will allow for inclusion of a wider dose-range (total = 6 doses) per exposure plate in future studies. However, there was some variation observed between the some internal positive (forskolin, 1 μM) and negative (prochloraz, 0.3 μM) control wells across plates and experiments. Most likely this variability was due to presence of only one QC well per plate was used for each control and could have increased the likelihood of extreme values that are not representative of the average response. To control for this, it is recommended that at least one true replicate well per positive control be included in each plate for future experiments. Given the similarities between blanks and SCs the additional positive control wells could be included by omitting the blank wells. The parallel inclusion of blank and SC wells on the QC plate will control for any potential effects of the solvent used for any given cell batch and/or passage.

10.2 Exposure Studies

10.2.1 Cell Viability/Cytotoxicity

There were no marked decreases observed in cell viability for any of the compounds tested at any of the laboratories. However, at several laboratories there was a slight increase in the percent viable cells that occurred in the forskolin exposures. While this could indicate that this compound may have stimulated cell proliferation in these treatment, this particular effect was not further investigated in this study. Since the approach used in the current study measured cell viability in every exposure well and as a result, these viability data could potentially be used to correct for differences in the number of viable cells between wells. However, it was decided not to pursue this option due to the incompleteness of the cell viability/cytotoxicity data sets at some of the laboratories. This problem was primarily related to the fact that the methods required the complete removal of medium from all wells before the cell viability test buffer was added to the cells. As a result, some drying out of

the wells in some of the initial experiments occurred, and which affected the results from these assays. If the drying problem is accounted for when sampling culture medium in future studies, it is recommended that laboratories evaluate the normalization of the data as a function of cell viability to account for potential variation in cell growth between wells. The use of optical density measurements (e.g. by using a photometric microtiter plate-reader) to estimate cell densities per well should be explored as an alternative.

10.2.2 Hormone Dose-Responses

Overall, the results in the current study represent a dramatic improvement compared to results from the first series of inter-laboratory pre-validation studies (see report on "Inter-Laboratory Comparison and Validation of the H295R Cell System to Test for Effects of Chemicals on Steroid Hormone Synthesis" submitted to EPA in February 2006). The effects of all three model compounds on production of T and E2 by H295R cells into medium were very comparable among all laboratories. Hormone dose-response curves followed similar patterns and both LOECs and EC values were within a narrow range when compared among laboratories with the exception of the LOECs measured at the AOE laboratory. Variation was slightly greater for E2 than for T in most of the experiments, which was potentially due to the fact that basal E2 production was relatively low in some of the experiments. This was especially true for the earlier passages (5 and 6) for which E2 concentrations in medium were within twice the method detection limit of the hormone ELISA used at the GER, AOE and MSU laboratories. Therefore, effects were limited to a detection of maximum inhibitions of approximately 40% of the SCs levels. However, when the data was transformed to values relative to the maximum response in each dose-response experiment, these differences were not apparent.

Given the limitations due to the relatively low amounts of E2 produced by the earlier passages at some of the laboratories in terms of detecting decreases, we recommend to further investigate alternative options in growing and preparing the H295R cell culture for experiments. Studies are currently underway that evaluate such alternative methods that not only would provide cells with greater basal E2 production but also facilitate the preparation of cell cultures for use in experiments (see section on Uncertainties and Future Recommendation below).

10.2.2.1Data Evaluation

We recommend utilizing a combination of the two data evaluation approaches that were featured in this study. In a first approach, the fold-change in hormone concentrations relative to the SCs should be used to assess the magnitude of the change observed across treatments. However, the magnitude of change in hormone production that is considered to be biologically relevant still needs to decided, especially in terms of identifying chemicals for further consideration as endocrine disrupters. Considering the endpoints measured in the current study as well as the variation observed both among and within laboratories, we would recommend a 2-fold or greater change in hormone production as an alteration of potential toxicological relevance. Although results expressed as increases or decreases relative to the SCs were comparable across laboratories, there was still a certain degree of variation observed. We could demonstrate, however, that when transforming data to changes relative to the maximum response (SC = 0%; Maximum response = 100%) this variability was not apparent. The similarities in EC values calculated based on this type of transformation demonstrates both the strength of this analysis for this type of data and the reliability of the H295R test system concerning the predictivity of chemical effects on hormone synthesis. Although the prediction of effect thresholds based on EC_{20} values consistently was more sensitive when compared to LOECs, both EC_{20}s and LOECs were always within the range of one order of magnitude with the exception of the AOE laboratory (**Table 9.5**). Considering the 3-fold dose-increments of the experiments this would still allow for a relatively precise prediction of effects regardless of the evaluation system utilized. Interestingly, the differences between EC_{20}s and LOECs observed at the AOE laboratory were much greater (up to 500-fold) than that observed at the other laboratories. At this time, it is unclear as to the underlying causes for these differences but this issue should be evaluated further. It also has to be considered that this transformation does not allow testing for the magnitude of the response observed. This is a key aspect in evaluating the potential relevance of a compound in terms of its potential toxicity, and therefore, we recommend using this approach in combination with data expressed as fold-change relative to the SCs.

Alternative approaches in data assessment would include the use of a relative potency model. We are currently in the process of evaluating the extensive data sets on the hormone inducers and inhibitors forskolin and prochloraz, respectively, as potential reference chemicals for such an approach.

Table 9.5: Comparison of EC_{20}s and LOECs measured at the different laboratories. FD = fold-difference between EC_{20} and LOEC. n.d. = not detectable due to lack of dose-response.

		Forskolin				Fadrozole				Prochloraz			
		T		E2		T		E2		T		E2	
		µM	FD	µM	FD	µM	FD	µM	FD	µM	FD	µM	FD
MSU	EC_{20}	0.058	9	0.12	4	0.25	3	n.d.		0.003	3	0.022	2
	LOEC	0.53		0.53		0.70		n.d.		0.010		0.053	
GER	EC_{20}	0.027	4	0.072	1	0.46	3	n.d.		0.018	3	0.073	3
	LOEC	0.10		0.10		1.0		n.d.		0.053		0.20	
RTP	EC_{20}	0.095	6	0.19	3	0.83	1	n.d.		0.014	1	0.033	2
	LOEC	0.55		0.65		1.0		n.d.		0.020		0.052	
DIFVR	EC_{20}	0.064	3	0.28	1	1.5	1	n.d.		0.013	2	0.079	4
	LOEC	0.20		0.30		2.0		n.d.		0.030		0.30	
AOE	EC_{20}	0.045	310	0.081	17	0.43	12	n.d.		0.003	40	0.002	500
	LOEC	14		1.4		5.3		n.d.		0.12		1.0	
All labs	EC_{20}	0.055	6	0.15	3	0.63	3	n.d.		0.009	5	0.051	7
	LOEC	0.35		0.40		2.0		n.d.		0.047		0.35	

10.3 Uncertainties and Future Studies

Due to the observed "evolving" nature of the cells relative to the production of E2, the question as to whether this could be due to changes in estradiol-metabolizing capacities of the cells that may occur with increasing passage number and/or freeze thaw cycles (e.g. by enzymes such as estradiol-sulfotransferases [ESTs]) is still unanswered. Furthermore, the information available regarding the chemical-metabolizing capacities of H295R cells is limited and not well characterized. Therefore, the metabolizing "machinery" of H295R cells should be further described in such a manner that would allow for the evaluation of whether the effect of a chemical on hormone production is due to the original chemical or its metabolite(s). Finally, it is unclear what types of receptors (e.g. CAR, AR, ER, etc.) may

be expressed by the H295R cells as well as what their functionality is regarding the regulation of the steroidogenic pathway.

Regardless of uncertainties outlined in the above section, the H295R Steroidogenesis Assay performed excellently in terms of transferability, reproducibility, sensitivity and precision. Thus, it is our option that this assay would be ready for validation as soon as the following two objectives are accomplished:

1. The H295R Steroidogenesis protocol has been finalized, and accepted after peer review by an internal OECD VMG NA advisory group. The finalized protocol would include a more economical method that would make the cells more readily available for exposure experiments. This method is currently evaluated by our laboratory, and should be available by the end of March/beginning of April;
2. Selection and pre-validation of model chemicals for validation studies has been completed. Chemicals should be selected based on their known mechanism of action on steroidogenic pathways. Furthermore, the test set of chemicals should be harmonized with those used in other currently developed *in vitro* steroidogenesis assays such as those in the framework of the European REACH program (e.g. ovarian follicle assay, Sertoli- and Leydig-cell assays).

Some of the studies proposed below are important in the context of assessing the relevance of the findings of the H295R steroidogenesis assay. This is especially true regarding the metabolizing capacities of the cells. However, the additional system characterization studies, the investigation of steroid and other receptors as well as the identification of hormone mass balance shift are not required prerequisites for the initiation of the inter-laboratory validation studies.

10.4 Proposed Future Studies

In the following sections, additional studies have been propose that are designed to address various uncertainties and undefined variabilities that still exist in the assay.

10.4.1 Additional System Characterization (Metabolizing Capacities of H295R Cells)

10.4.1.1 Hormone metabolizing capacities

To address the question as to whether the changes in E2 production are due to differences in the expression and/or activities of ESTs that are a function of cell age, two experiments shall be conducted. In the first experiment, medium from different cell passages and freeze/thaw batches will be split into equal sub-samples. One sub-sample should be extracted and analyzed as originally described in the study protocols. The second sub-sample should be treated with glucuronidases and sulfatases prior to extraction to cleave all conjugated steroids. This sample should also be subjected to the same extraction and hormone analysis procedures as used in the analysis of sub-sample one. In the second experiment, the steroid-metabolizing "machinery" of the cells should be investigated by identifying and characterizing the expression of the genes that are responsible for the metabolizing E2 and T. The catalytic activities for these gene products (enzymes) should also be characterized in this study.

10.4.1.2 Chemical metabolizing capacities

Numerous chemicals require metabolic activation to exert toxicity. Thus, when working with simplified *in vitro* systems such as cell lines it is important to know whether these systems still have the "machinery" to metabolize xenobiotics. To answer this question, we propose to conduct a series of experiments that will include the characterization of the suite of metabolizing enzymes using molecular techniques as well as metabolic activation studies (e.g. exposure experiments with and without hepatic S9 mix).

10.4.2 Preparation of Inter-laboratory Validation Studies on Coded Chemicals

As pointed out above, a suite of "model" chemicals needs to be selected for the final validation experiments based on their known mechanism of action on steroidogenic pathways. This set of test chemicals should comprise a total number of ≥ 30 individual

compounds including steroidogenic inhibitors and inducers of different potencies, as well as positive and negative controls. The test set of chemicals should be harmonized with those that have been used in other steroidogenesis assays that are currently under development or in validation (currently discussions are underway with Miriam Jacobs from the IHCP to receive the list of chemicals tested within the frame of the REACH program). A series of preliminary experiments needs to be conducted to characterize the properties of the selected chemicals regarding their abilities to alter the production of T and E2. Based on these findings a final selection of test chemicals for the validation studies can be made, and dose-ranges and QC-requirements can be established for each compound. Further discussions of dose-selection criteria are required to address potential issues resulting from the selection of inappropriate dose ranges that do not allow describing a full dose-response curve. For example, in the fadrozole exposure experiments the doses were such that already at one of two lowest concentrations maximum decreases of E2 occurred, indicating the need for more rigorous chemical selection criteria. A brief discussion paper that will be established in junction with an OECD VMG NA subgroup will be amended to this report at a later point discussing such approaches.

10.4.3 Revision of Protocols/Final Report

The current versions of the draft protocols should be revised and finalized based on the results the studies that have been outlined in the preceding sections and will include a simplification of the cell culture methods that will make the cells more readily available for exposure experiments. Furthermore, the protocols should include a complete list of culture and exposure quality criteria that will be used in the validation studies. A detailed description of chemical dose-selection processes will be included in the test protocols.

10.4.4 Presence of Steroid and Other Receptors

In response to questions regarding the expression and functionality of various receptors (e.g. CAR, AR, ER, etc.) that may be present in H295R cells, we propose to conduct a series of studies to characterize the suite of receptors that are known to be involved in the steroidogenic pathways (adrenal and gonadal) using molecular techniques. While these studies are important in the context of the general characterization of H295R cells, they were not deemed essential with regard to the utilization of the cells to identify chemical effects on hormone production. As a result, these studies are considered to be a secondary

priority in the validation of the H295R Steroidogenesis Assay, and could be addressed at a later time.

10.4.5 Hormone mass balance characterization

It has been previously demonstrated that exposure to certain chemicals (e.g. cAMP) can result in a shift of steroidogenic pathways from corticoid to sex steroid production. Understanding these possible shifts in hormone production pathways will provide useful information for the interpretation of causalities for changes in hormone profiles after exposure to certain chemicals. Therefore, characterization efforts should be undertaken to describe hormone mass balances including intermediate substrates and additional products such as aldosterone and cortisol.

11 APPENDICES

APPENDIX I – H295R Culture Protocol

APPENDIX II – H295R Exposure Protocol

APPENDIX III – Raw Data Cell Viability / Cytotoxicity

APPENDIX IV – Raw Data Testosterone

APPENDIX V – Raw Data Estradiol

APPENDIX VI – Statistical Data