PRELIMINARY REPORT

Response of H295R Cells to the Exposure to Four Model Chemicals (Prochloraz, Aminoglutethimide, Forskolin & Vinclozolin)

Experiment #: 10 **Date:** 1/20/05-2/18/05 **Expt. Leader:** MH/MM/EH/AT

1. Title: Determination of changes in hormone production of H295R cells exposed to model compounds.

2. Question:
What are the changes in media hormone concentrations induced by exposure to different model compounds?

3. Experimental Design: H295R cells were exposed in 24 well plates to up to ten different concentrations of Prochloraz, Aminoglutethimide, Forskolin and Vinclozolin (see table below). In addition to the chemical treatments a blank and a solvent control were run on each plate. In order to determine possible effects of the solvent different solvents (DMSO, Methanol, Acetonitrile) were tested as well. Media from cells was analyzed for concentrations of T, P and E2 using the ELISA systems established under experiments 1-9.

Prochloraz	Aminoglutethimide	Forskolin	Vinclozolin
0.001uM	0.003uM	0.01uM	0.1uM
0.003uM	0.01uM	0.03uM	0.3uM
0.01uM	0.03uM	0.1uM	1uM
0.03uM	0.1uM	0.3uM	3uM
0.1uM	0.3uM	1uM	10uM
0.3uM	1uM	3uM	30uM
1uM	3uM	10uM	100uM
3uM	10uM	30uM	300uM
10uM	30uM	100uM	1000uM

4. Methods:
Cells are cultured as described in ATL-SOP Culturing of the H295R human adrenocortical carcinoma cell line (ATCC CLR-2128). All test chemicals will be dosed in triplicates in each 24 well plate using either 1% or 0.1% solvent per well. Samples are ether extracted (MSU-ATL SOP 253) and reconstituted in 250 ul EIA buffer or manufacturer-provided buffer. Make dilutions in the assay buffer provided by the manufacturer. For initial screening, samples are diluted 1:50 and 1:100 for P and T, and 1:2 and 1:5 for E2. Media extracts are run on the kit plate in quadruplicate. These dilutions will be adjusted – if necessary – in subsequent measures to match the linear part of the standard curve for each sample. Follow kit instructions with regards to blank, non-specific binding and total binding wells.
Cytotoxicity will be determined as % viable cells using the "Procedure for determination of cell viability/cytotoxicity using the MTT bioassay" (APPENDIX I).

5. Statistics:
Descriptive stats (means +/- SD, CV's); compare the average concentrations measured for the different samples using basic parametric tests (t-test).

6. Expected Outcome:
Based on the previously determined changes in gene expression for cells exposed to the above chemicals as well as the preliminary results on hormone concentrations in Forskolin exposed cells the following alterations are expected for hormone production:

- Treatment with Forskolin will result in a dose-dependent increase in all three hormones.
- Based on the results derived from the experiments on changes in gene expression (Prochloraz exposure is likely to result in an increased formation of P while no effects on T and E2 genesis would be expected.
- Exposure to Aminoglutethimide did not cause any significant changes for any of the genes except from CYP11B1. Thus, for the hormones measured no changes can be predicted at this time.
- Cells treated with vinclozolin had a massive increase in CYP11B2 gene expression which could result in an increased depletion of Progesterone. No effects were observed for genes encoding for enzymes involved in T or E2 formation, and therefore, no changes or decreases as a consequence of the increased depletion of the precursor P for both hormones would be expected.

Given the results from the previous gene expression experiments cytotoxicity is expected to occur at the highest doses only (if at al).

7. Summary:
7.1. Cytrotoxicity
The 1% DMSO solvent control (SC1) had approximately 50% less viable cells as indicated by the MTT cell viability kit (t-test: $p<0.05$) (Figure 1). No significant less viable cells were observed for the 0.1% solvent control (SC2). Neither Aminglutethimide nor Forskolin affected cell viability at any of the concentrations tested. Exposure to Prochloraz significantly decreased cell viability in a dose dependent manner with only 1% viable cells in the highest treatment (30uM). A Prochloraz concentration of 3uM was the lowest concentration that was not significantly different from the 0.1% SC with 77% viable cells left per well. The two highest Vinclozolin concentrations tested resulted in a significant decrease of viable cells (t-test: $p<0.05$) compared to the 0.1% SC. However, no dose-response relationship was observed for increasing exposures to this chemical.

In conclusion it appears that 1% DMSO as the carrier for the tested chemicals has a significant impact on cell viability. Therefore, for future experiments we recommend to use a carrier solvent concentration of 0.1% DMSO which did not have any significant impact on viability if the H295R cells. Furthermore, we conclude that the highest three concentrations of Prochloraz also affect the health status in a way that makes it impossible to interpret effects on steroid concentrations. Although Vinclozolin affected cell viability significantly at all concentrations tested no dose-response was observed here, indicating that this response may not be due to cytotoxicity but other yet unknown factors. To address this

issue, additional measures of cytotoxicity will be conducted using different tests such as LDH.

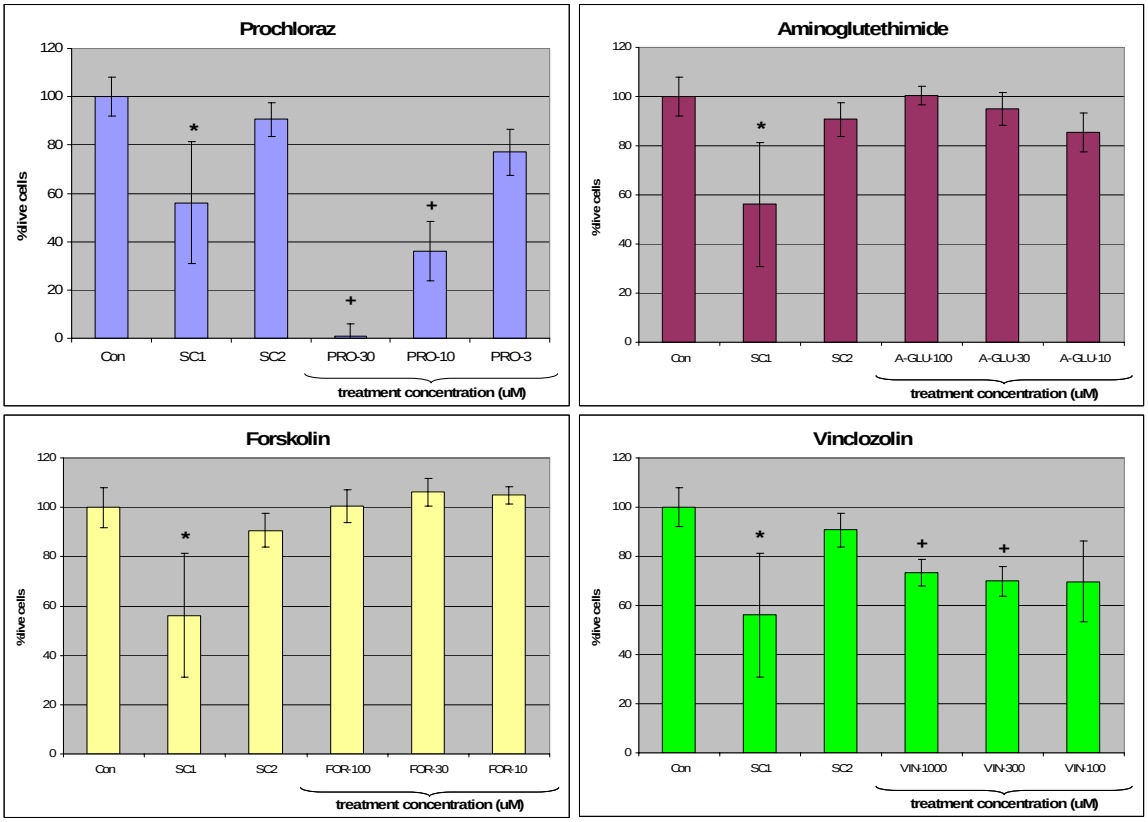

*Figure 1: Effects of different solvent (DMSO) concentrations and exposure to selected concentrations of model compounds on H295R cell viability. Bars represent means. Error bars are standard error of the mean. * indicate significant differences to the solvent control. + indicate significant differences to the blank. Significance level is $\alpha<0.05$.*

7.2. Responses to Model Compounds:

Regardless of the concentration of the DMSO solvent carrier model compound specific response was observed for media hormone concentrations (Table 1, Figures 2,3,4 & 5). When using a final concentration of 1% DMSO per well media E2 concentrations in the blanks were significant lower than those in the SC of the Aminoglutethimide, Forskolin and Vinclozolin treatments. For the 0.1% DMSO solvent experiment significant differences between the SC and the blanks only occurred for T and P in the Vinclozolin exposure. The most potent compounds were Prochloraz and Forskolin that caused significant changes in media hormone concentrations at concentration in the upper nM range (Table 1).

Exposure to Prochloraz resulted in a dose-dependent increase of media P concentrations starting at 0.1uM and reaching a maximum at 1uM. This is in agreement with earlier finding from a study on steroidogenic gene expression that found a highly significant increase of 3bHSD mRNA after exposures up to 1uM Prochloraz, the gene that encodes for the enzyme responsible for the synthesis of P. Media T and E2 concentrations started to decrease at a Prochloraz concentration of 0.1uM and were approximately 3- to 5-

fold less than the SC at 3uM. The decrease in all three hormones observed for the 10 and 30uM exposures are likely to be due to the cytotoxicity that occurred at these doses (see 7.1.).

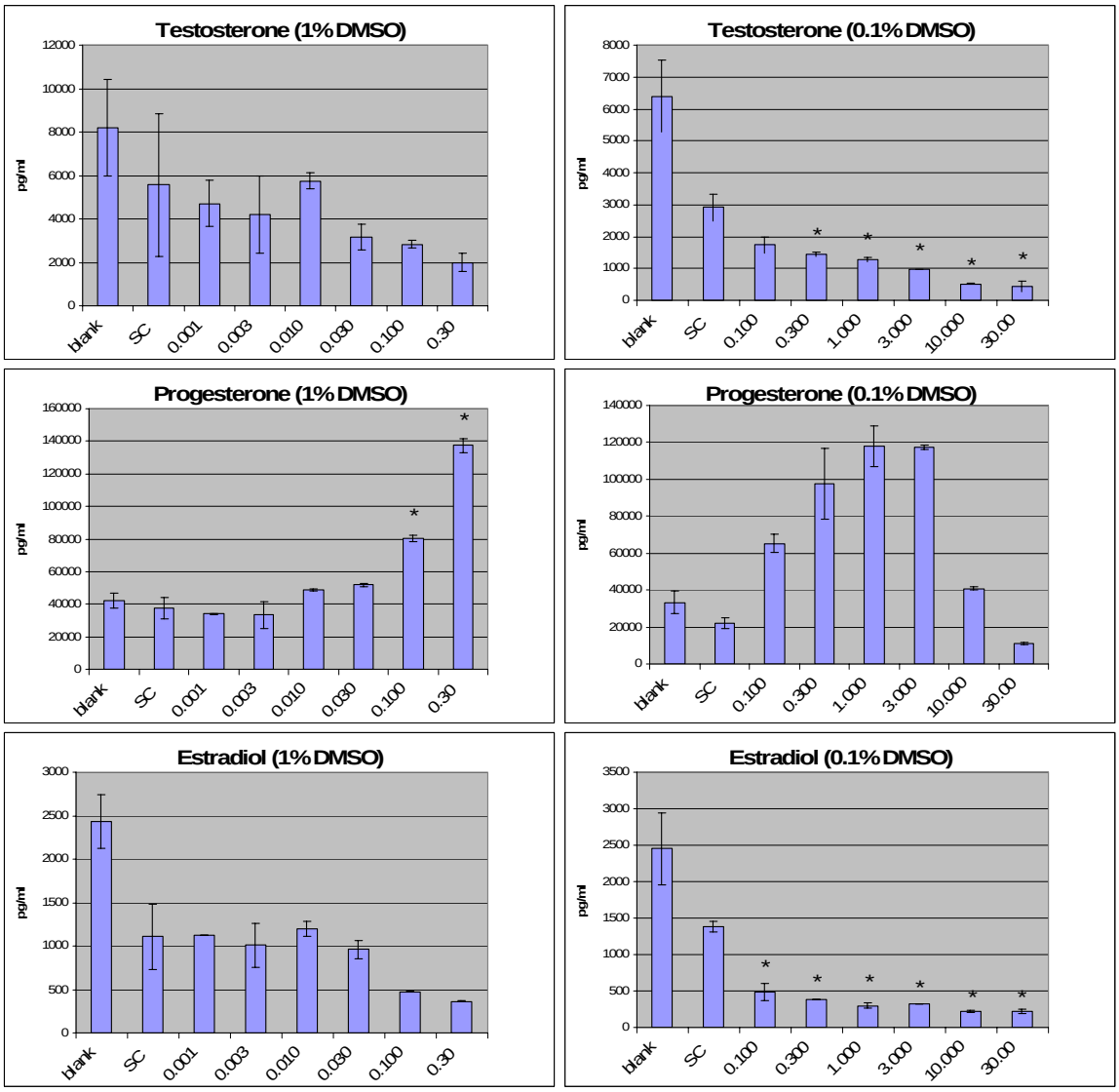

*Figure 2: Hormone concentrations in media from cells exposed to Prochloraz. Left: 1% DMSO per well was used as solvent carrier. Right: 0.1% DMSO was used as solvent carrier. Bars represent means. Error bars are standard error of the mean. * indicate significant differences to the solvent control. Significance level is $\alpha<0.05$.*

Although there appeared to be a dose dependent increase of media T in the Aminoglutethimide group this was not significant for any of the concentrations tested. This is likely to be due to the relatively high variability observed among the replicates for each treatment. Otherwise Aminoglutethimide suppressed media P and E2 at concentrations below 10uM. The only significant effect on gene expression was an increase of CYP11B1 which encodes for an enzyme downstream of P, and thus may explain the depletion of P.

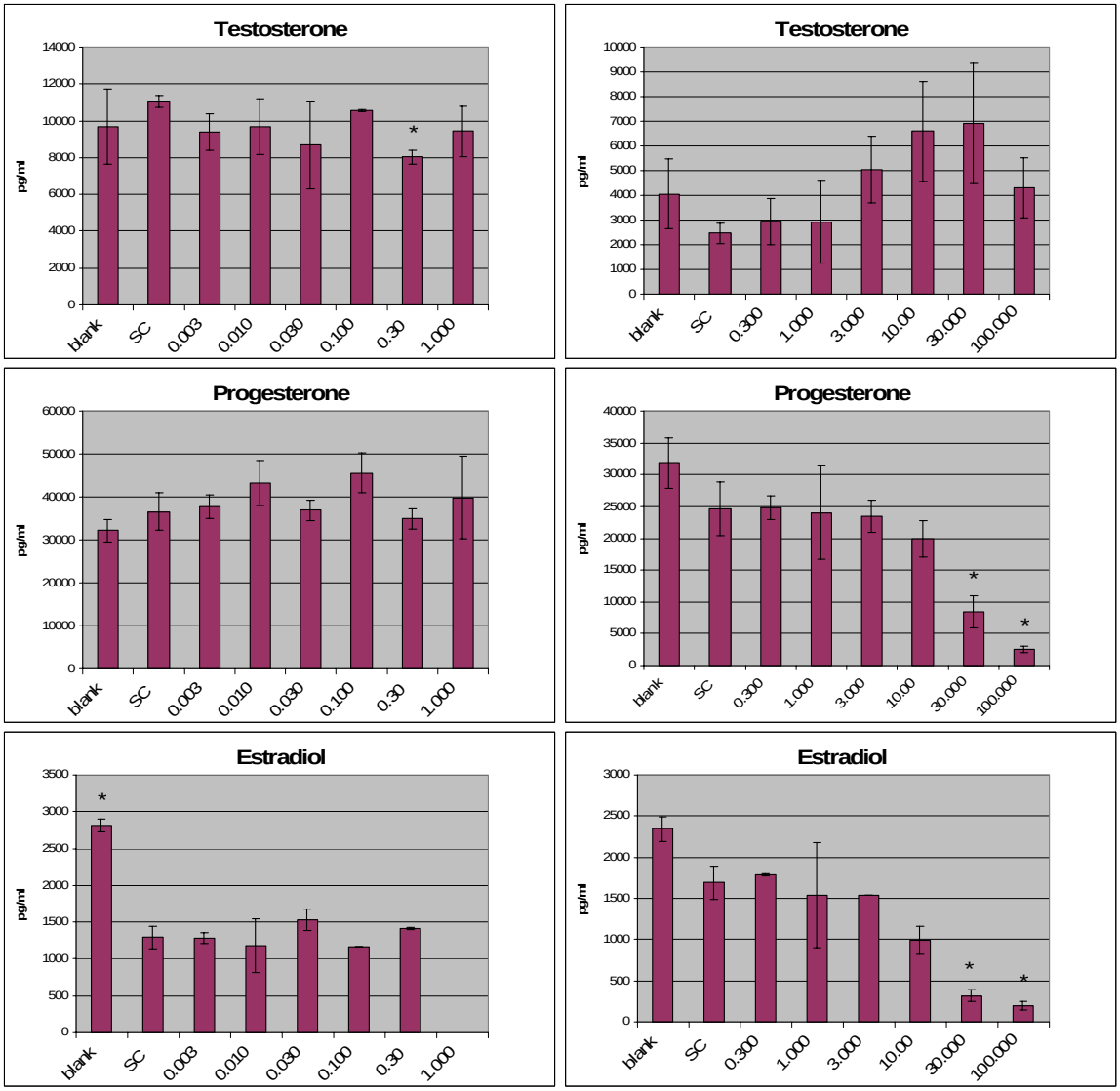

*Figure 3: Hormone concentrations in media from cells exposed to Aminoglutethimide. Left: 1% DMSO per well was used as solvent carrier. Right: 0.1% DMSO was used as solvent carrier. Bars represent means. Error bars are standard error of the mean. * indicate significant differences to the solvent control. Significance level is $\alpha<0.05$.*

Forskolin resulted in a dose-dependent increase of P, T and E2 concentrations in media. T was significantly affected at the lowest concentration tested (0.03uM), while an increase of P and E2 occurred at slightly higher concentrations of 0.03 and 0.3uM Forskolin, respectively. Again, this is in accordance with results on gene expression that found a general increase in most steroidogenic genes.

The only steroid hormones that were affected by Vinclozolin were P & E2. Here a significant increase in media E2 and a decrease in media P occurred at concentrations below 30uM. When related to gene expression data the decrease in media P could be explained by the dramatic increase in CYP11B2 that was observed at the same concentration. CYP11B2 encodes for an enzyme downstream of Progesterone that might have resulted in an increased depletion of P. Currently, there is no explanation available for the increase in media E2.

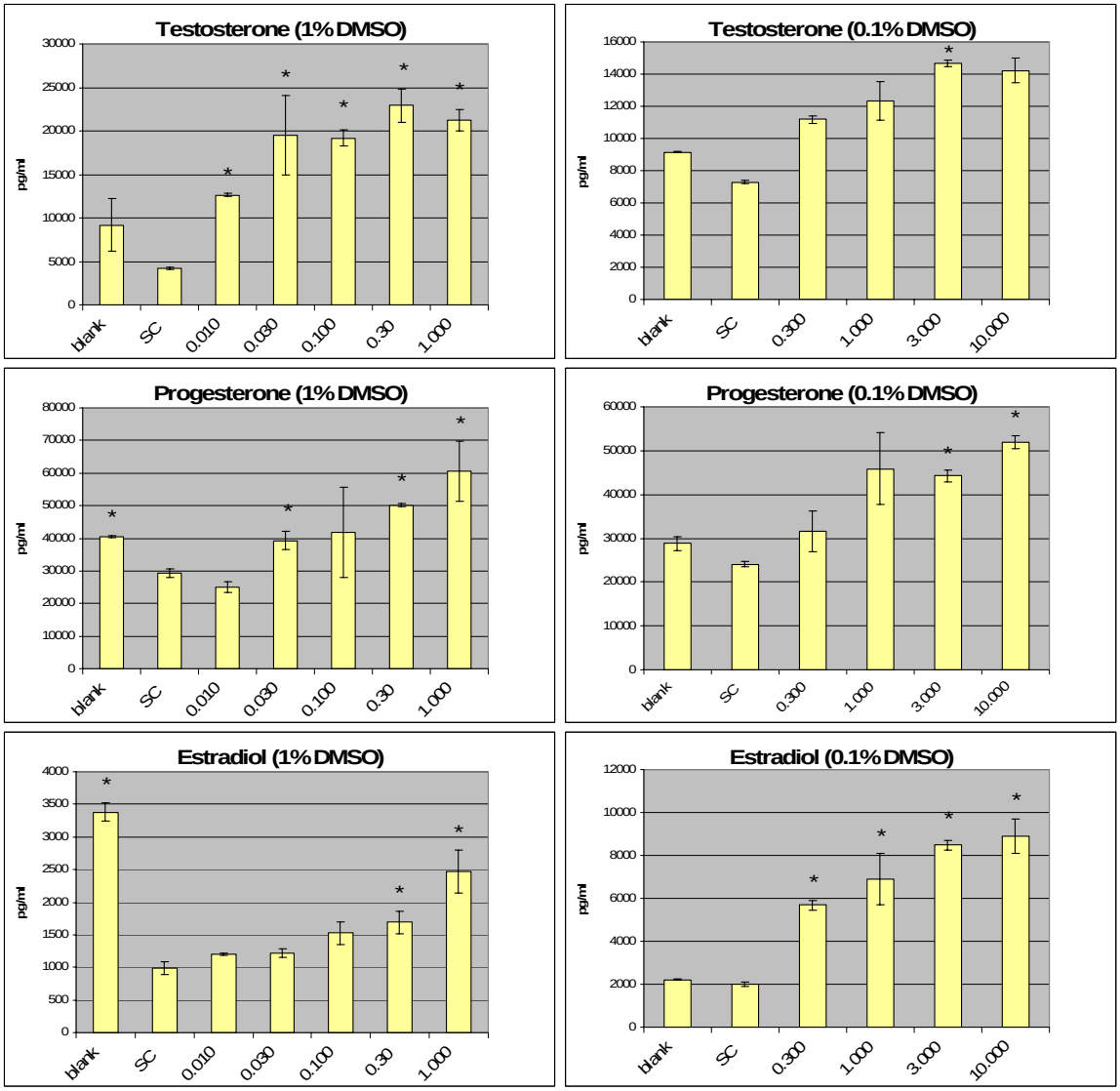

*Figure 4: Hormone concentrations in media from cells exposed to Forskolin. Left: 1% DMSO per well was used as solvent carrier. Right: 0.1% DMSO was used as solvent carrier. Bars represent means. Error bars are standard error of the mean. * indicate significant differences to the solvent control. Significance level is $\alpha<0.05$.*

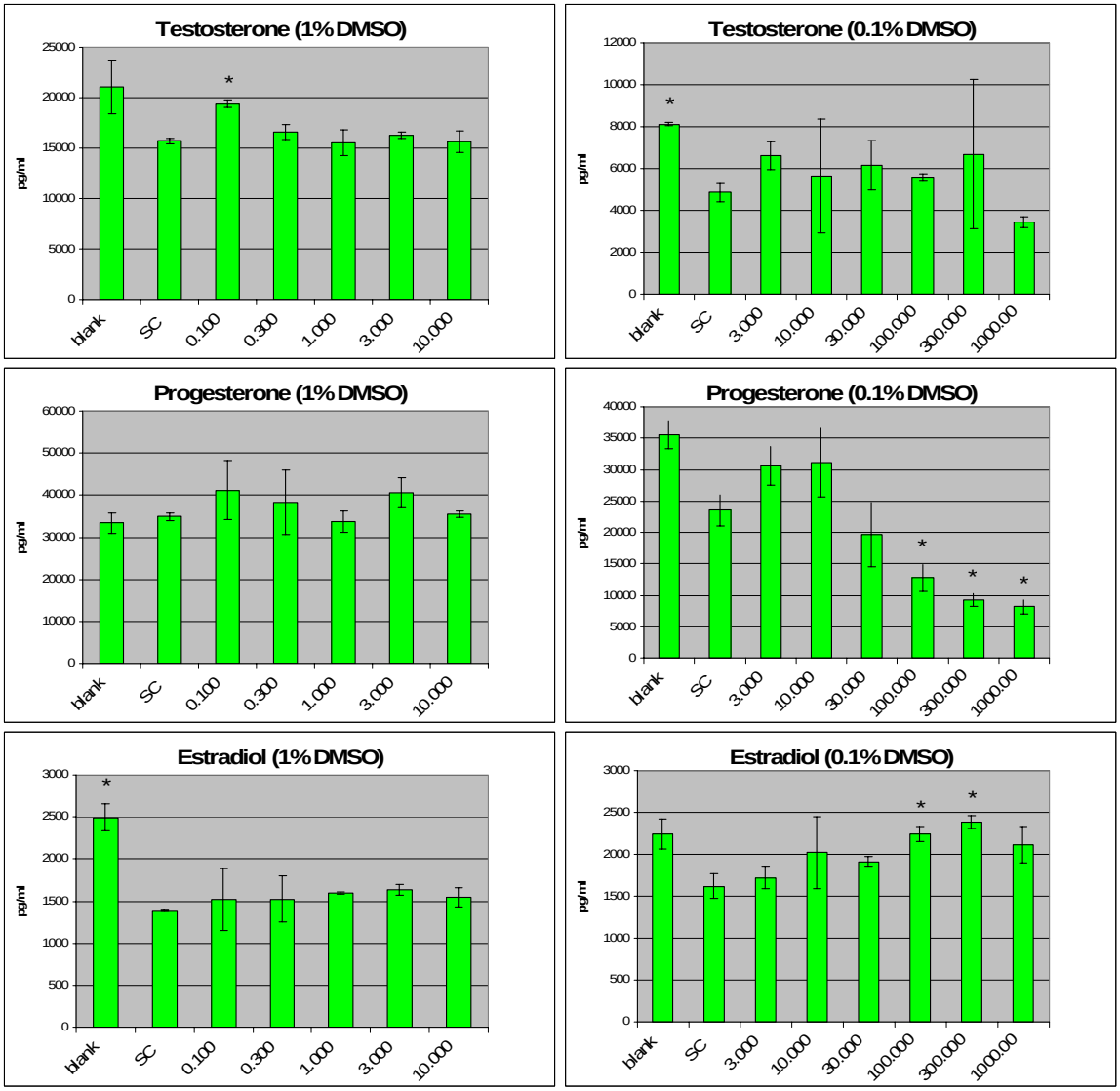

*Figure 5: Hormone concentrations in media from cells exposed to Vinclozolin. Left: 1% DMSO per well was used as solvent carrier. Right: 0.1% DMSO was used as solvent carrier. Bars represent means. Error bars are standard error of the mean. * indicate significant differences to the solvent control. Significance level is $\alpha<0.05$.*

Table 1: T-test probabilities for individual exposure tests compared to the solvent control (SC). Significance was accepted when $p<0.05$. Significant differences are indicated by bold numbers.

		Prochloraz			***Aminoglutethimide***			***Forskolin***			***Vinclozolin***		
%DMSO	***Treatment***	**T**	**P**	**E2**	**T**	**P**	**E2**	**T**	**P**	**E2**	**T**	**P**	**E2**
1	Blank	0.659	0.647	0.783	0.448	0.349	**0.006**	0.146	**0.006**	**0.003**	0.104	0.483	**0.010**
	0.001	0.761	0.557										
	0.003	0.941	0.139	0.779	0.389	0.847	0.954						
	0.01	0.941	0.139	0.779	0.343	0.307	0.718	**0.000**	0.107	0.102			
	0.03	0.418	0.093	0.640	0.293	0.934	0.244	**0.042**	**0.043**	0.113			
	0.1	0.364	**0.013**	0.139	0.152	0.181	0.376	**0.002**	0.332	0.063	**0.008**	0.332	0.657
	0.3	0.268	**0.003**	0.106	**0.013**	0.684	0.385	**0.005**	**0.002**	**0.037**	0.250	0.608	0.545
	1				0.238	0.706		**0.003**	**0.041**	**0.026**	0.207	0.162	**0.035**
	3										0.207	0.162	**0.035**
	10										0.919	0.535	0.192
0.1	Blank	0.055	0.143	0.092	0.263	0.224	0.068	0.416	0.060	0.127	**0.009**	**0.036**	0.064
	0.1	0.077	**0.008**										
	0.3	**0.039**	**0.031**	**0.003**	0.670	0.975	0.729						
	1	**0.032**	**0.007**	**0.003**	0.737	0.925	0.775	0.254	0.066	**0.029**			
	3	**0.023**	**0.001**	**0.002**	0.051	0.759	0.392	**0.038**	**0.003**	**0.001**			
	10	**0.015**	**0.013**	**0.002**	0.127	0.323	0.064	0.074	**0.001**	**0.007**	0.726	0.214	0.333
	30	**0.016**	**0.032**	**0.002**	0.179	**0.044**	**0.012**				0.276	0.438	0.115
	100				0.528	**0.018**					0.149	**0.043**	**0.036**
	300										0.544	**0.017**	**0.022**
	1000										0.054	**0.015**	0.117