Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis: Hormone production

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Abstract

To identify and prioritize chemicals that may alter steroidogenesis, an in vitro screening assay based on measuring alterations in hormone production was developed using the H295R human adrenocortical carcinoma cell line. Previous studies indicated that this cell line was useful to screen for effects on gene expression of steroidogenic enzymes. This study extended that work to measure the integrated response on production of testosterone (T), estradiol (E2), and progesterone/pregnenolone (P) using an ELISA. Under optimized culture and experimental conditions, the basal release of P, T and E2 into the medium was 7.0±1.2 ng/ml, 1.6±0.4 ng/ml, and 0.51±0.13 ng/ml, respectively. Model chemicals with different modes of action on steroidogenic systems were tested. Exposure to forskolin resulted in dose-dependent increases in all three hormones with the greatest relative increase being observed for E2. This differed from cells exposed to prochloraz or ketoconazole where P concentrations increased while T and E2 concentrations decreased in a dose-dependent manner. In cells exposed to fadrozole, E2 decreased in a dose-dependent manner while T and P only decreased at the greatest dose tested. Aminoglutethimide decreased P and E2 concentrations but increased T concentrations. Vinclozolin reduced both P and T but resulted in a slight increase in E2. The alteration in the patterns of hormone production in the H295R assay was consistent with the modes of action of the chemicals and was also consistent with observed effects of these chemicals in animal models. Based on these results, the H295R in vitro system has potential for high throughput screening to not only characterize the effects of chemicals on endocrine systems but also to prioritize chemicals for additional testing.

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Introduction

Recently, studies have suggested links between the exposure to natural and man-made substances in the environment and alterations in endocrine and reproductive systems in wildlife and humans (Kavlock et al., 1996; Ankley et al., 1998). To address emerging concerns that such substances may alter endocrine system function, the US EPA has developed and implemented an endocrine disruptor-screening program (EDSP) as mandated by the Food Quality Protection Act of 1996. The focus of this multi-tiered program has been first to develop in vitro and in vivo assays to identify and classify substances relative to their potential interaction with endocrine systems (Tier I) and then to develop concentration–response relationships in animal models (Tier II) (EDSTAC, 1998).

Much of the effort to establish Tier I assays has been focused on the development and validation of in vitro estrogen receptor (ER) and androgen receptor (AR) binding assays (Villeneuve et al., 1998) or on the development and characterization of transcriptional activation assays with ER and AR in stably transfected cell lines (Wilson et al., 2002, 2004). However, there are a number of...
non-receptor-mediated processes that may also alter endocrine function (Sanderson et al., 2002). For example, compounds can modulate the enzymes involved in the production, transformation, or elimination of steroid hormones, and thus may alter the absolute and relative concentrations of hormones in blood and tissues (Ohno et al., 2002; Hilscherova et al., 2004). These non-receptor-mediated effects are often exerted indirectly via alterations of common signal transduction pathways (Rainey et al., 1993; Zhoa et al., 1998). Currently there are additional efforts underway to establish and validate a series of in vitro and in vivo test systems such as microsomal aromatase assays, pubertal male and female rat assays, the fish reproductive screen, and the frog thyroid assay that attempt to address some of the issues arising from non-receptor-mediated endocrine disruption. In this context, a rodent minced-testis assay was proposed as the Tier I screen to detect chemicals with potential to disrupt steroid hormone production (EDSTAC, 1998). However, questions have been raised about the utility of gonad explant assays. In particular, rodent-based testis and ovary explant assays were found to yield high rates of false positive/negative responses (Powlin et al., 1998). Also, in testis explant assays, it is not possible to discriminate between cytotoxicity of hormone-producing cells and other cell types. Finally, there has been increasing criticism regarding the use of animals in Tier I screening assays. As a consequence, there is need for less variable and more reliable in vitro test systems as alternatives to these tissue explant assays. One cell line that has been shown to be a useful in vitro model for studying steroidogenic pathways and processes is the human H295R adrenocarcinoma cell line (Hilscherova et al., 2004; Zhang et al., 2005).

The H295R cell line has been shown to express all the key enzymes of steroidogenesis (Gazdar et al., 1990; Rainey et al., 1993; Staels et al., 1993). H295R cells have physiological characteristics of zonally undifferentiated human fetal adrenal cells and have the ability to produce the steroid hormones found in the adult adrenal cortex (Gazdar et al., 1990; Staels et al., 1993; Harvey and Everett, 2003). As well as permitting the measurement of hormone production, another advantage of the H295R cell bioassay is that it can be used to evaluate effects of chemicals on gene expression and the enzymatic activities of steroidogenic genes (Hilscherova et al., 2004; Sanderson et al., 2000; Zhang et al., 2005). Thus, the H295R cell line holds promise as a substitute for gonad explant assays within the tiered endocrine disruptor-screening and testing framework, and as an alternative to explant assays for research applications.

In order to validate the capacities of the H295R cells to identify effects on sex steroid production, the cells were exposed to six model chemicals, chosen based on their known effects on steroid metabolism and steroidogenic gene expression. The chemicals tested were prochloraz, ketoconazole, fadrozole, aminogluthetimide, forskolin, and vinclozolin. Prochloraz is an agricultural imidazol fungicide that inhibits a CYP enzyme involved in ergosterol synthesis, but has also been reported to inhibit other CYP enzymes, and to act as a potent aromatase inhibitor (Mason et al., 1987; Laignel et al., 1989; Troesken et al., 2006). Ketoconazole is a pharmaceutical imidazole fungicide that has been shown to alter the activity of several cytochrome P450s involved in steroid synthesis (e.g. CYP11A, CYP17, and CYP19) (Kan et al., 1985; Johansson et al., 2002; Hilscherova et al., 2004). Fadrozole is a non-steroidal reversible and competitive inhibitor of aromatase as well as of CYP11B-associated enzymatic activities (Steele et al., 1987; Muller-Vieira et al., 2005). Aminogluthetimide is an aromatase inhibitor that was also reported to interact with several other steroidogenic enzymes such as StAR and 17β-HSD (Johansson et al., 2002; Hilscherova et al., 2004). Forskolin is a general inducer of steroidogenesis that acts via the activation of cAMP pathways (Hilscherova et al., 2004). Vinclozolin is a chlorinated fungicide that has been shown to act as an antagonist of the androgen receptor (Dai et al., 2001; Kelce et al., 1997; Sanderson et al., 2000).

The aim of the current study was to develop and standardize an in vitro Tier 1 screening assay using the H295R cell line to prioritize chemicals that act to alter hormone production. Specifically, the goals of this study were to: (1) optimize culture and experimental assay conditions; (2) characterize the basal production of testosterone, estradiol and progesterone; (3) develop dose–response relationships for six model chemicals; and (4) compare possible effects of these model chemicals to available in vivo data.

Materials and methods

Test chemicals. Forskolin (FOR), vinclozolin (VIN), ketoconazole (KET), and aminogluthetimide (AMG) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Prochloraz (PRO) was purchased from Aldrich (St. Louis, MO, USA). Fadrazole (FAD) was obtained from Novartis Pharma AG (Basel, CH).

Cell culture. The H295R human adrenocortical carcinoma cell line was obtained from the American Type Culture Collection (ATCC CRL-2128; ATCC, Manassas, VA, USA) and was grown in 100 × 20 mm Petri dishes with 12.5 ml of supplemented medium at 37 °C with a 5% CO₂ atmosphere as described previously (Hilscherova et al., 2004). Briefly, the cells were grown in a 1:1 mixture of Dulbecco’s Modified Eagle’s Medium and Ham’s F-12 Nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma, St. Louis, MO, USA) supplemented with 1.2 g/l Na₂CO₃, 5 ml/l of ITS+ Premix (BD Bioscience; 354352), and 12.5 ml/l of BD Nu-Serum (BD Bioscience; 355100) unless otherwise specified.

Experimental design. All experiments were conducted in 24-well cell culture plates (COSTAR, Bucks, UK). One ml of cell suspension was added to each well and the cells were allowed to attach for 24 h. After the attachment period, the medium was changed and the experiment was initiated. The effect of cell confluency on steroid hormone production was evaluated at two different seeding densities, 30% (~200,000 cells/ml) and 70% (~470,000 cells/ml) confluency. To examine the possible influence of hormones and other substances in the cell culture medium on hormone production, the medium was stripped with dextran-coated charcoal prior to test initiation and the results compared to normal supplemented medium. In the time course experiment, H295R cells were cultured under standard conditions and basal hormone production was determined in medium collected at 3, 6, 12, 24, 48, and 72 h.

For chemical tests, cells were exposed for 48 h in 24-well plates. DMSO was used as carrier solvent and did not exceed 0.1% v/v. Test plates included six chemical concentrations, a solvent control (SC), and a blank control (CTR), in triplicate. At the end of each experiment, the culture medium was transferred to an Eppendorf tube and stored at ~80 °C prior to analysis. Four separate tests were conducted for each model chemical.

Prior to measuring hormone concentrations, cell viability was evaluated in CTR, SC, and the three greatest exposure test chemical concentrations of each chemical with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) bioassay (Mosman, 1983). Cytotoxic chemical concentrations were not included in the hormone concentration measurements.
Table 1

Quality performance criteria for determining progesterone (P), testosterone (T), and estradiol (E2) in the medium of H295R cells

<table>
<thead>
<tr>
<th>Detection limit (pg/ml)</th>
<th>Linearity within medium (mean±SD)</th>
<th>Accuracy&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay&lt;sup&gt;d&lt;/sup&gt;</td>
<td>S-LOQ&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P</td>
<td>7.8</td>
<td>&lt;100</td>
</tr>
<tr>
<td>T</td>
<td>3.9</td>
<td>&lt;50</td>
</tr>
<tr>
<td>E2</td>
<td>7.8</td>
<td>&lt;8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated based on triplicate measures for each sample.
<sup>b</sup> Same sample measured on different ELISA plates; total number of plates=11.
<sup>c</sup> Liner regression between nominal and measured spike concentrations. Spike concentrations were as follows: P, 0, 250, 500, 1000, 500 pg/ml; T, 0, 100, 250, 500, and 3000 pg/ml; E2, 0, 100, 1000 pg/ml.
<sup>d</sup> Detection limit of the assay represents the lowest detectable amount of hormone per milliliter standard.
<sup>e</sup> S-LOQ: Sample limit of quantification. Lowest detectable concentration of each hormone in medium after 48 h and at a cell density of 200,000 cells per milliliter medium within the tested linear range of dilutions.

Hormone measurements. Frozen medium was thawed on ice, the hormones were extracted twice with diethyl ether (5 ml) in glass tubes, and phase separation was achieved by centrifugation at 2000 x g for 10 min. Solvent was evaporated under a stream of nitrogen, and the residue was dissolved in ELISA assay buffer and was either immediately measured or frozen at −80 °C for later analysis. Hormones in culture medium were measured by competitive ELISA using the manufacturer’s recommendations (Cayman Chemical Company, Ann Arbor, MI; Progesterone [Cat # 582601], Testosterone [Cat # 582701], 17β-Estradiol [Cat # 582251]). However, since there is a 61% cross-reactivity of the progesterone antibody with pregnenolone, progesterone concentrations are expressed as total progesterone+pregnenolone (P). Extracts of culture medium were diluted 1:2, 1:5, and 1:10 for estradiol, and 1:50, 1:100, and 1:150 for testosterone and P prior to use in the ELISAs.

Statistical analyses. Statistical analyses of hormone data were conducted using SYSTAT 11 (SYSTAT Software Inc., Point Richmond, CA). All data are expressed as means and standard deviations. Student’s t test was used to evaluate differences in hormone production between high and low confluency cells and to examine differences between cells cultured in regular and charcoal-stripped (DCC) medium. Analysis of variance (ANOVA) was conducted to test for differences among all treatments. Differences between controls and cells exposed to model compounds were evaluated using Dunnett’s test applied to recovery, as monitored by the addition of a tracer amount of 3H-labelled T to each sample before extraction, was similar between non-extracted and extracted samples (results not shown).

Results

Hormone measurement

All ELISA systems used in this study to measure hormones were validated in terms of sensitivity, accuracy, precision, linearity, and parallelism to the standard curves (Table 1). To evaluate the need for extraction, hormone concentrations in ether-extracted medium were compared to non-extracted medium. While extraction did not have a significant effect on T concentrations, the reported concentrations of both P and E2 concentrations were approximately 3-fold greater in the non-extracted medium. The apparent concentrations of P and E2 in the extracted samples were 8.6±1.2 ng/ml and 0.34±0.01 ng/ml, respectively, while in the non-extracted samples concentrations were 25±7 ng/ml and 0.97±0.22 ng/ml, respectively. This difference was not due to poor extraction efficiency since the converted amount of 3H-labelled T to each sample before extraction, was similar between non-extracted and extracted samples (results not shown).

Basal hormone production

In regular supplemented medium, concentrations of T and E2 in cells seeded to 70% confluence were approximately double those measured in wells seeded at 30% confluence while

Table 2

Concentrations of progesterone, testosterone and estradiol in the medium of H295R cells treated with either DMSO or with prochloraz (PRO) for 48 h<sup>b</sup>

<table>
<thead>
<tr>
<th></th>
<th>0.1% DMSO</th>
<th>PRO (1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30% Nu</td>
<td>30% DCC</td>
</tr>
<tr>
<td>P (ng/ml)</td>
<td>8.0±2.0</td>
<td>6.6±0.8</td>
</tr>
<tr>
<td>T (ng/ml)</td>
<td>2.3±0.4</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>E2 (ng/ml)</td>
<td>0.41±0.05</td>
<td>0.14±0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> P=progesterone/pregnenolone; T=testosterone; E2=17β-estradiol. Concentrations are in units of ng/ml medium and are given as means and standard deviations.
<sup>b</sup> H295R cells were seeded at 30% confluence or 70% confluence. “Nu” indicates regular cell culture medium with Nu serum while DCC indicates medium that has been stripped prior to seeding of cells.
concentrations of P were unaffected (Table 2). When comparing regular and DCC medium, E2 concentrations were greater in regular medium in both 30% (t-test: \( p<0.001 \)) and 70% (t-test: \( p<0.001 \)) confluent wells when compared to cells grown in DCC medium. In contrast, P concentrations in the 70% confluent wells (t-test: \( p<0.05 \)) were greater in DCC medium than in regular medium while P did not differ from the 30% confluent wells. In cells treated with prochloraz (PRO) E2 concentrations were significantly less in regular medium (t-test; \( p<0.05 \)) than in DCC medium.

In the time course experiment, all three steroids showed increases in concentration overtime with maximum P and E2 concentrations being measured at 48 h whereas T increased throughout the experiment (Fig. 1). Consequently, all further experiments were conducted with regular medium using cells seeded at approximately 30% confluency (200,000 cells per ml) for 48 h since these conditions were deemed optimal for monitoring both positive and negative changes in hormone production.

In the CTRs, concentrations of all three steroids showed considerable variability among experiments (\( n=5 \)) conducted on different days and with different batches of cells. P concentrations from the different experiments ranged from 3.0±0.6 (mean of all plates from one experiment±SEM) to 9.2±0.7 ng/ml in culture medium with an overall average of 7.0±1.2 ng/ml. Mean concentrations of T ranged from 0.6±0.2 to 2.3±0.1 ng/ml medium (overall mean 1.6±0.4 ng/ml). E2 concentrations ranged from 0.21±0.05 to 0.81±0.24 ng/ml (overall mean of 0.51±0.13 ng/ml). The mean coefficients of variation (CV) for the duplicates within a plate (\( n=15 \) plates) were 9±2 (mean±SEM), 17±3, and 8±2% for P, T, and E2, respectively.

While not consistently observed, DMSO appeared to have some effect on hormone production. On average, hormone production in cells treated with 0.1% DMSO was slightly less than in the CTRs. For DMSO-treated cells, concentrations of P were approximately 80% (±12%) (mean±SEM; \( n=5 \)) of those measured in the CTR while T and E2 concentrations were 82% (±10%) and 80% (±15%) of CTR concentrations, respectively. Therefore, in tests with model chemicals, hormone production was compared to the SCs rather than to CTRs. To account for inter-plate variability in hormone production, concentrations for each test chemical were expressed relative to the average SC response of each plate.

Effects of model chemicals

Cytotoxicity/Cell viability

Cytotoxicity was observed in cells treated with PRO at concentrations greater than 3 \( \mu \)M (results not shown). Therefore, the prochloraz concentrations evaluated in this study ranged from 0.001 to 1 \( \mu \)M. None of the concentrations tested for any of the other chemicals affected cell viability. A summary of the cell viability results is given in the Supplementary materials.

Prochloraz (PRO)

Exposure to PRO resulted in significant changes in concentrations of P, T, and E2 (Fig. 2). P concentrations increased in a dose-dependent manner with concentrations being significantly greater than the SCs at PRO concentrations greater than 0.01 \( \mu \)M. Testosterone and E2 decreased in a dose-dependent manner at PRO concentrations greater than 0.03 and 0.003 \( \mu \)M, respectively. Maximal changes in hormone concentrations were observed at the greatest non-cytotoxic dose of PRO (1 \( \mu \)M). At this dose, P concentrations were 5-fold greater than the SC while E2 and T concentrations were 5.5- and 3-fold less, respectively, than their respective SCs.

Ketoconazole (KET)

Ketoconazole significantly inhibited E2 and T production and induced P production by H295R cells (Fig. 3). Concentrations of
KET greater than 0.03 μM significantly reduced testosterone production and the effect became more pronounced with increasing concentrations. Similarly, mean E2 production showed a steady decline with increasing KET concentration, and the effect was significant at concentrations greater than 0.3 μM. Progesterone concentrations in medium increased progressively, and were significantly greater than the SC at a KET dose of 1 μM (p<0.01). At KET concentrations greater than 1 μM, P production started to decrease again. The effect of KET on T production occurred at lesser concentrations than for E2, causing a 50% reduction compared to the solvent controls at approximately 2 μM. The least change was observed for P (∼2.5-fold).

**Fadrozole (FAD)**

A significant, dose-dependent reduction (up to 4.7-fold) in E2 production by H295R cells was observed for FAD concentrations greater than 0.1 μM (Fig. 4). However, no comparable response pattern was observed for T and P with the exception that at the greatest dose, 100 μM, both mean T (p<0.001) and P (p<0.05) concentrations were significantly reduced relative to the solvent controls. Mean T production was suggestive of a dose-dependent decrease (maximum reduction ≈12-fold) at FAD concentrations greater than 1 μM, but due to the relatively great variability in the hormone concentrations, only the greatest dose was statistically significant when compared to the solvent control. Although statistically significant, the reduction of P production at 100 μM FAD was not great and was approximately 0.5-fold that of the control levels.

**Aminogluthetimide (AMG)**

Treatment with AMG resulted in a dose-dependent change in hormone concentrations (Fig. 5). T concentrations were significantly greater than the SCs at AMG concentrations greater than 1 μM with a maximum response being observed (3-fold greater) at 10 μM. Concentrations of E2 were significantly less relative to the SCs at AMG concentrations of 10 μM or greater with a more than 4-fold reduction at the greatest dose tested (30 μM). Concentrations of P in the medium decreased in a manner similar to that observed for E2 except that the only statistically significant reduction from SCs was observed at the greatest dose, 30 μM.

**Forskolin (FOR)**

FOR exposure increased all hormone concentrations (Fig. 6). The greatest increase relative to the SCs (7-fold) was observed for E2 at 30 μM. E2 concentrations were greater than 1 μM with a maximum response being observed (3-fold greater) at 10 μM. Concentrations of E2 were significantly less relative to the SCs at AMG concentrations of 10 μM or greater with a more than 4-fold reduction at the greatest dose tested (30 μM). Concentrations of P in the medium decreased in a manner similar to that observed for E2 except that the only statistically significant reduction from SCs was observed at the greatest dose, 30 μM.
significantly greater than the SCs at all doses greater than 0.1 μM. P and T concentrations increased in a dose-dependent manner but the relative increases were less than those observed for E2. At FOR concentrations greater than 0.1 μM, the concentrations of P and T were statistically greater than the SCs. The maximum concentration of both P and T (3-fold above SCs) was observed in cells treated with 30 μM FOR.

Vinclozolin (VIN)

Cell viability appeared to be slightly reduced upon exposure to the three greatest VIN concentrations tested (100, 300, 1000 μM), resulting in approximately 70 to 73% viable cells in these treatments. However, since the reduction in cell viability was not dose-dependent, all data except the 1000 μM exposure were used for hormone analyses. Exposure of H295R cells to VIN resulted in a maximum increase in E2 concentration that was less than 1.5-fold, while the maximum decreases for T and P were 2- and 3-fold, respectively (Fig. 7). Decreases in P and T concentrations were characterized by a relatively high variation among the different experiments conducted. Changes in the relative P, T, and E2 concentrations were statistically significant in cells exposed to 30 and 100 μM vinclozolin.

Discussion

General test performance

The ELISA determination of P, T, and E2 concentrations was accurate and precise. However, it was necessary to extract hormones from the medium prior to determination of P and E2 because the antibodies used in these ELISAs appear to cross-react with unknown compounds in the non-extracted medium. In fact, some of the antibodies used with the hormone ELISA systems utilized in this study cross-react with steroid metabolites and conjugates such as sulfates and glucuronates. Such compounds are likely to be produced by the cells, and are removed during the extraction process. However, the hormone metabolizing capabilities of H295R cells have not yet been described, and therefore, the exact cause for the observed differences remains unclear. Additional experiments are needed that describe steroid metabolism by these cells. It is also important to note that since the antibody used to measure P has a cross-reactivity to pregnenolone (approximately 61%), it is likely that the P concentrations measured in this study were primarily pregnenolone since it has been reported that H295 cells produce approximately 75-fold more pregnenolone than progesterone (Gazdar et al., 1990). Absolute P concentrations measured in our study (mean = 7.0 ng/2 × 10^5 cells/48 h) were comparable to those of pregnenolone previously described (Gazdar et al., 1990) (15.6 ng/2 × 10^5 cells/48 h) after applying a correction factor (0.61) for cross-reactivity. Since the goal of this study was to develop a test system to assess the effects of chemicals on E2 and T production, it can be assumed that the P assay as used in our study was sufficient to evaluate the status of the precursor pool involved in the synthesis of androgens and estrogens. As a result, changes in the concentrations of P would be diagnostic of up-stream events even if the assay was not measuring only progesterone.

To date, most studies with H295R cells have measured hormone products of the mineralocorticoid (aldosterone) and glucocorticoid (cortisol) pathways as well as certain androgens such as androstenedione (Ohno et al., 2002; Rainey et al., 1993; Rainey et al., 1994; Thomson et al., 2003; Li et al., 2004; Li and Wang, 2005). The major androgen production pathways are thought to include formation of 17α-hydroxyprogesterone and subsequent synthesis of androstenedione.

Fig. 6. Effect of forskolin treatment on hormone release by H295R cells. Cells were treated for 48 h with the indicated concentrations of forskolin. Hormone data are expressed as relative changes compared to solvent controls (SC=0). Values represent the mean±SD. Significant differences for progesterone (*), testosterone (+), and estradiol (@) relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol= p<0.05; 2 symbols= p<0.01; 3 symbols= p<0.001.

Fig. 7. Effect of vinclozolin treatment on release of hormones by H295R cells. Cells were treated for 48 h with the indicated concentrations of vinclozolin. Hormone data are expressed as relative changes compared to solvent controls (SC=0). Values represent the mean±SD. Significant differences for progesterone (*), testosterone (+), and estradiol (@) relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol= p<0.05; 2 symbols= p<0.01; 3 symbols= p<0.001.
(Gazdar et al., 1990). Although Gazdar et al. (1990) hypothesized that cells secrete small amounts of T and E2, they only quantified the production of androstenedione. However, other studies have reported T production by H295R cells (Danesi et al., 1996). In fact, concentrations measured for androstenedione (0.11 ng/2 × 10^5 cells/48 h) (Gazdar et al., 1990) are less than the T concentrations measured in our study (1.6 ng/2 × 10^5 cells/48 h). To our knowledge, there have been no reports quantifying estrogen production in H295R cells even though aromatase activity has previously been measured (Sanderson et al., 2002). While the production of E2 by H295R cells was demonstrated in our study, E2 concentrations were approximately 20- and 6-fold less than those of P and T, respectively.

Hormone production of H295R cells was variable among different batches/passages of cells. This variability may be due to several factors; first, it has been shown in various stably transfected cell lines that physiological and biochemical fluctuations can be linked to the cell cycle (McFerran et al., 2001; Barker et al., 2004). It has also been reported that with increasing age (cell passage), cells may gradually lose their capacity to maintain specific functions such as albumin secretion, drug metabolism capabilities, and the functionality of the urea cycle (Guillouzo, 1998; Priesner et al., 2004). Finally, differences in cell density may have also contributed to the variability in hormone production. To account for some of this variability, hormone concentrations in the treated wells were normalized to those measured in relevant SCs. Repeated exposure experiments with model chemicals showed that relative changes in hormone production were more reproducible than absolute hormone concentrations among experiments and were a reliable way to express effects of compounds on steroid hormone production. Overall, based on the data for the changes of hormone production as a function of cell passage, it is recommended to use cells up to passage 10. Past this passage, basal production of E2 declines to the extent that detecting decreases of this steroid can be limited. Furthermore, due to the fluctuations and changes in cell-specific functions discussed above changes or shifts in hormone homeostasis cannot be excluded with increasing age of the cells. Further studies are needed to better elucidate the changes in hormone production patterns and steroid homeostasis as a function of cell age.

Cell culture medium had some effect on hormone production by H295R cells with the greatest effect observed for E2. The use of dextran-coated charcoal-stripped (DCC) medium reduced E2 concentrations relative to those of cells grown in non-stripped medium. Previous studies have demonstrated that changes in medium components can affect production of hormones by H295R cells; culture of cells in serum-free medium resulted in a reduction of C21 steroids when compared to cells grown in medium containing 2% serum (Gazdar et al., 1990). The basis for these differences has not yet been investigated but may involve the presence of cholesterol and trace levels of other steroid hormones in the supplemented medium that serve as precursors in the production of androgens and estrogens. The fact that the differences observed for T and P at the lower cell confluence tested (30%) were no longer apparent when these hormones were measured in cells exposed at 70% confluence is likely due to a more rapid depletion of these precursors under these conditions. In addition, components within the serum may also alter the aldosterone and/or cortisol biosynthesis pathways such that the androgen/estrogen pathways are enhanced. However, the significance of these factors in affecting the production of the steroids monitored under the culture conditions developed in this study needs to be evaluated prior to the final validation of this bioassay. It is important to note that since prochloraz, an inhibitor of estrogen synthesis, reduced E2 concentrations in both DCC and regular supplemented medium to near the sample specific limit of quantification (S-LOQ), it is unlikely that the differences in basal hormone production were due to contamination of regular medium with hormones.

**Exposure to model compounds**

Agonists of specific steroidogenic pathways can alter hormone profiles in the culture of H295R cells in a manner that is consistent with their mode of action. For instance, angiotensin II or K⁺ selectively promotes the synthesis of aldosterone in H295 cells while adrenocorticotropic hormone (ACTH) and other cAMP pathway inducers are associated with production of cortisol, androgens and estrogens (Rainey et al., 1993; Bird et al., 1993; Pezzi et al., 1996). In our study, exposure of H295R cells to the six model chemicals resulted in different hormone concentration patterns consistent with their modes of action.

Imidazol fungicides, including PRO and KET, have been found to be potent inhibitors of aromatase among the many vertebrate species including humans and fish (Mason et al., 1987; Ankley et al., 2005) as well as being capable of affecting other P450-dependent enzymes (Laignelet et al., 1989). Significant reductions of plasma androgen (T and 11-ketosteroid) concentrations in male fathead minnows (*Pimephales promelas*) and a significant decrease in plasma E2 titers in female fish exposed to 0.3 μM PRO were observed in a study conducted by Ankley et al. (2005). Exposure to PRO resulted in significant decreases in testicular and plasma T concentrations and a significant increase in testicular P concentrations in a study with fetal rats (Vinggaard et al., 2005). A possible explanation for this could be a suppression of 17α-hydroxylase activities. However, this study was not designed to address this question, and therefore future studies will be necessary to investigate the specific effects of PRO on steroidogenic enzyme activities. Regardless, the fact that the effect on E2 production was more potent than that on P and T indicates that aromatase was likely more sensitive to the inhibitory effects of PRO than the upstream target enzymes. While the findings in the above studies were similar to those observed here with H295R cells, effects on T and E2 occurred at three- to ten-fold lesser PRO concentrations compared to the in vivo studies.

The pattern of responses in hormone concentration observed in H295R cells exposed to KET was similar to that
observed in cells exposed to FAD where T and E2 concentrations were reduced in treated cells when compared to controls. In general these response patterns agree with those observed in other studies that evaluated the effects of these compounds on steroidogenesis in several mammalian and fish species. In a 15-day adult male rat study, concentrations of T as well as E2 were significantly reduced from control levels in rats exposed to FAD (O’Connor et al., 2002). However, in a study of male pubertal rats, T concentrations were not different from control levels in rats exposed to 6 mg/kg/d FAD (Marty et al., 2001). The lack of response in the pubertal assay is not surprising in that pre-pubertal animals have been shown to be less responsive than adult animals when exposed to potential endocrine active compounds. Finally, exposure of fathead minnows to FAD resulted in a decrease of brain aromatase in both male and female fish that was also accompanied by an increase in T in male fish (Ankley et al., 2002). While no effects were noted on E2 concentration in exposed male minnows, E2 concentrations in female minnows were decreased in a concentration-dependent manner.

The patterns of response for T and E2 observed with H295R cells exposed to KET differed somewhat from those observed in studies conducted with adult male rats. In these studies, there was a statistically significant decrease in T while E2 concentrations were unaffected (Weber et al., 1991). In the current study, the decrease in P synthesis regardless of species and test system, progestin concentrations would decrease to a lesser extent or be unaffected (Monteiro et al., 2000). Most likely, the differential results observed for T and E2 in these tests were due to the fact that KET has a greater effect on E2. This result is consistent with the greater effect of KET on T in the H295R assay (Johansson et al., 2002; Berman and Laskey, 1993). The lack of response in the pubertal assay is not surprising in that pre-pubertal animals have been shown to be less responsive than adult animals when exposed to potential endocrine active compounds. Finally, exposure of fathead minnows to FAD resulted in a decrease of brain aromatase in both male and female fish that was also accompanied by an increase in T in male fish (Ankley et al., 2002). While no effects were noted on E2 concentration in exposed male minnows, E2 concentrations in female minnows were decreased in a concentration-dependent manner.

The patterns of response for T and E2 observed with H295R cells exposed to KET differed somewhat from those observed in studies conducted with adult male rats. In these studies, there was a statistically significant decrease in T while E2 concentrations were significantly increased compared to controls (O’Connor et al., 2002). In a rat testis explant assay, E2 concentrations were significantly increased compared to studies, there was a statistically significant decrease in T while H295R cells exposed to KET differed somewhat from those decreased in a concentration-dependent manner.

Like FAD, AMG is an aromatase inhibitor used in breast cancer treatment to downregulate endogenous estrogen production. AMG can also downregulate other steroidogenic pathways including the synthesis of cortisol and aldosterone (Fassnacht et al., 1998). While AMG consistently decreased E2 synthesis regardless of species and test system, progestin and androgen production in in vivo and in vitro systems can be affected differently. In rat and fish (Platichthys flesus) ovarian cultures, AMG increased the concentration of T and androstendione, respectively (Berman and Laskey, 1993; Monteiro et al., 2000). AMG exposure also resulted in decreased production of P in rat ovarian cell cultures and H295R cells (Johansson et al., 2002; Berman and Laskey, 1993). The decrease in P synthesis is most likely due to suppression of CYP11A activity while decreases in estrogen production and subsequent accumulation of androgens may be due to inhibition of aromatase (Johansson et al., 2002). In contrast, in vivo studies of rats and humans have demonstrated that AMG exposure may decrease androgen production (Tsuitsui, 1992; Lundgren et al., 1996). These conflicting results may be due to the reproductive status of the test organism. Significant differences between effects of AMG during diestrus, proestrus, and estrus stages of cycling rats have been reported (Berman and Laskey, 1993).

Forskolin is a diterpene that can mimic the effects of ACTH by stimulating adenylcyclase and increasing cAMP levels in adrenal cells (Seamon et al., 1981). In our study, exposure of H295R cells to FOR resulted in a general increase in the production of P, T, and E2 that was consistent with effects observed in other studies (Johansson et al., 2002; Rainey et al., 1993; Cobb et al., 1996). However, while the production of all three hormones increased, the greatest relative increase was observed for E2 where FOR exposure resulted in approximately a 7-fold increase in E2 concentrations as compared to a 2–3-fold increase in T and P concentrations. This observation is also consistent with another study that demonstrated the upregulation of aromatase in H295R cells by FOR (Watanabe and Nakajin, 2004).

There are only a few reports on the effects of VIN on P, T, or E2 production in animals. In rats, exposure to 400 mg VIN/kg for 9 days (Yu et al., 2004) and 100 mg VIN/kg for 3–24 h (Kubota et al., 2003) resulted in greater serum T concentrations compared to controls. However, in studies with amphibians (Xenopus laevis) (van Wyk et al., 2003), reptiles (Alligator mississippiensis) (Crain et al., 1997), and birds (Coturnix coturnix japonica) (Niemann et al., 2004), VIN had no effect on sex steroid concentrations. Similarly, exposure of H295R cells to VIN resulted in only minor (< 2 to 3-fold) changes in P, T, and E2 concentrations. However, unlike observations in rats, exposure of H295R cells to VIN resulted in decreased T and P concentrations while E2 levels slightly increased at the greatest VIN concentrations. The basis for these differences has not yet been investigated but may be due to inherent differences that exist between in vivo and in vitro systems where multiple tissues can influence the serum steroid concentrations. It has been reported that the VIN metabolites M1 and M2 are potent androgen receptor antagonists while the parent compound VIN binds only weakly to the receptor (Kelce et al., 1994). Thus, the antiandrogenic action of VIN is unlikely to be mediated directly via effects on steroidogenic pathways but rather may be a function of feedback mechanisms mediated by the inhibition of receptor-mediated processes by VIN metabolites. It is unclear whether H295R cells have the capacity to metabolize VIN to M1 and M2. Furthermore, the extent to which androgen receptors are expressed in H295 cells is not known. Therefore, it remains uncertain whether the trends observed for P, T and E2 in this study can be attributed to the above processes, especially in light of the fact that steroidogenesis in H295R cells can be affected by alterations in cAMP-mediated pathways on which the effect of VIN is unknown.
The results from this study support the use of the H295R cell line as a screening tool to evaluate the effects of chemicals on steroidogenesis. The bioassay is sensitive, reproducible, and effects on steroid hormone production appear to be consistent with most results observed in other models including in vivo models with various mammalian species. In addition, the bioassay provides a convenient and cost-effective tool to evaluate the effects of chemicals on several steroidogenic pathways that include the synthesis of mineralocorticoids, glucocorticoids, and androgens and estrogens. Thus, while the focus of this study was on the synthesis of androgens and estrogens, the H295R assay system can be used as a more comprehensive test system to examine the mode of action of chemicals on molecular and biochemical aspects of steroidogenesis.

While this study emphasized the suitability of the H295R steroidogenesis assay as a possible TIER I screening tool for chemical effects on sex steroid production, uncertainties remain with regard to our understanding of the specific mechanism(s) of action and predicting effects at other levels of organization or biological systems (e.g., different tissues or species). In order for this cell line to become a predictive tool for in vivo effects on hormone production, there is a need for additional research characterizing the autoregulatory processes of steroidogenic pathways and the presence and/or functions of hormone receptors and metabolic capacities of the H295R cells. Currently, studies are underway to elucidate some of these aspects, such as metabolism of sex steroids and expression of hormone receptors. Furthermore, comparative studies are being conducted that compare effects of the model chemicals described in this paper with those on different tissues and whole organisms, including fish and amphibians, to evaluate the relevance of the H295R steroidogenesis system at higher levels of organization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2006.07.007.

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