Modulation of steroidogenic gene expression and hormone production of H295R cells by pharmaceuticals and other environmentally active compounds

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Abstract

The H295R cell bioassay was used to evaluate the potential endocrine disrupting effects of 18 of the most commonly used pharmaceuticals in the United States. Exposures for 48 h with single pharmaceuticals and binary mixtures were conducted; the expression of five steroidogenic genes, 3βHSD2, CYP11β1, CYP11β2, CYP17 and CYP19, was quantified by Q-RT-PCR. Production of the steroid hormones estradiol (E2), testosterone (T) and progesterone (P) was also evaluated. Antibiotics were shown to modulate gene expression and hormone production. Amoxicillin up-regulated the expression of CYP11β2 and CYP19 by more than 2-fold and induced estradiol production up to almost 3-fold. Erythromycin significantly increased CYP11β2 expression and the production of P and E2 by 3.5- and 2.4-fold, respectively, while production of T was significantly decreased. The β-blocker salbutamol caused the greatest induction of CYP17, more than 13-fold, and significantly decreased E2 production. The binary mixture of cyproterone and salbutamol significantly down-regulated expression of CYP19, while a mixture of ethynylestradiol and trenbolone, increased E2 production 3.7-fold. Estradiol production was significantly affected by changes in concentrations of trenbolone, cyproterone, and ethynylestradiol. Exposures with individual pharmaceuticals showed the possible secondary effects that drugs may exert on steroid production. Results from binary mixture exposures suggested the possible type of interactions that may occur between drugs and the joint effects product of such interactions. Dose–response results indicated that although two chemicals may share a common mechanism of action the concentration effects observed may be significantly different.

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Keywords: Bioassay; Steroidogenesis; Pharmaceuticals; Endocrine disruptors; Drug mixtures; Dose–response

Introduction

According to the U.S. Food and Drug Administration (USFDA), approximately 82,000 drugs are registered in the U.S. for human use, accounting for more than 3000 active ingredients. Adjuvants and, in some instances, pigments and dyes are also components of the formulated drug product. After administration to humans and animals, pharmaceuticals are excreted in waste products and many unused medications are disposed in drains or sewage systems. Sewage treatment facilities, depending on their technology and a chemical’s physicochemical properties, are not always effective in removing active chemicals from wastewater. As a result, pharmaceuticals find their way into the environment, where they can directly affect terrestrial and aquatic organisms and can be incorporated into food chains (Díaz-Cruz et al., 2003; Cecchini and LoPresti, 2007).

Despite extensive and detailed reports about residues of pharmaceuticals in the environment have been published (Jorgensen...
and Halling-Sorensen, 2000; Hereber, 2002; Sanderson et al., 2004; Jones et al., 2004), the potential ecological effects associated with the presence of these compounds have been largely ignored. In the European Union, discharge of pharmaceutical products is regulated through mandatory submission of Environmental Risk Assessments (ERAs) that accompany Marketing Authorization Approval. Most of the methods used today for the identification and quantification of pharmaceuticals in the environment and the first attempts at eco-toxicity evaluations of these active compounds have been developed in European countries (Commission of the European Communities, 1992). Notably, the European Union has taken the lead in banning the use of the majority of growth-promoting antibiotics in livestock on the basis of the “Precautionary Principle” (Casewell et al., 2003).

Among the frequently detected substances in rivers are β-blockers such as metoprolol (at concentrations up to 1.5 μg/l) and β-sympathomimetics (Hirsch et al., 1996; Sedlak and Pinkston, 2001). Analgesic and anti-inflammatory drugs like diclofenac have been observed in several studies at concentrations up to 1.2 μg/l (Ternes, 1998; Stumpf et al., 1998; Buser et al., 1998); estrogens such as 17β-estradiol have been found at concentrations up to 13 ng/l (Kuch and Ballenschmitter, 2000). In addition, antibiotics such as erythromycin have been reported to occur at concentrations as high as 1.7 μg/l (Hirsch et al., 1999; Lindsey et al., 2001). Estrogenic compounds have also been identified in rivers of southern and central Germany (Adler et al., 2001), as well as lipid-lowering agents such as clofibrac acid at concentrations as great as 0.2 μg/l (Ollers et al., 2001), and antiepileptic drugs such as carbamazepine at concentrations up to 2.1 μg/l (Mohle et al., 1999).

During 1999–2000, the U.S. Geological Survey conducted the first nationwide investigation of the occurrence of pharmaceuticals, hormones and other organic contaminants in 139 streams in 30 states (Kolpin et al., 2002). A total of 95 residues were targeted including antibiotics, prescription and non-prescription drugs, steroids and hormones, 82 of which were found in at least one sample. Although the authors cautioned that sites were chosen based on their increased susceptibility to contamination from urban or agricultural activities, a surprising 80% of streams sampled were positive for one or more of the targeted pharmaceuticals. Furthermore, 75% of the streams contained two or more of the targeted pharmaceuticals, 54% had more than five, while 34% had more than 10 and 13% tested positive for more than 20 targeted contaminants. Similar reconnaissance studies are ongoing all over the world to evaluate the presence of pharmaceuticals in groundwater and surface water sources of drinking water. Identification of the environmental exposure routes of these drugs is crucial for a realistic environmental assessment of pharmaceuticals because it is the prescribed drug dose and the duration of treatment that provides an estimate of environmental loading. The fact that the same drug may be used for several applications and that exposure routes may vary in different environmental matrices means that the fate of the drug may also vary, resulting in quite different environmental concentrations.

Pharmaceuticals are sometimes thought to be easily (bio) degraded in the environment, but it has been established that large proportions of many pharmaceuticals can be excreted from the body un-metabolized and enter wastewater as biologically active substances (Fent et al., 2006; Kummerer, 2001). Some drugs which have been metabolized can be converted back to the parent compound in the environment (Pickrell, 2002). This has been demonstrated for the glucuronide metabolite of chloramphenicol and the acetylated metabolite of sulphadimidine in samples of liquid manure (Berger et al., 1986). Thus, it is often not only the parent compound which should be the subject for a risk assessment but also the major metabolites. Additionally, drug residues found in the environment, especially in aquatic systems, usually occur as mixtures rather than as single contaminants, and their possible interactions should therefore be considered in risk assessments.

Since pharmaceuticals are specifically designed to be biologically active, they may have unintended effects on non-target organisms in the environment, even at low concentrations. However, there is a lack of information about effects other than the original innate function for which the chemical or pharmaceutical was designed and/or produced. Furthermore, the paucity of information concerning ecotoxicity of pharmaceuticals (van Wezel and Jager, 2002) also makes it difficult to characterize and assess the environmental risk of these compounds.

The objective of the present study was to evaluate the potential effects of 18 of the most used human and veterinary pharmaceuticals in the United States on steroidogenesis. Using the H295R cells as a study model, the effects of five antibiotics, four growth promoters (two of which are also used as antibiotics), one corticosteroid, one anti-cancer and one birth control drug, two analgesic and anti-inflammatory drugs, one anti-lipid, one antidepressive, one β-blocker and one insect repellent, on the expression of five steroidogenic genes encoding for the four rate-determining enzymes controlling the production of the three main hormones in the steroidogenic pathway was evaluated by use of Q-RT-PCR. The genes studied included 3βHSD2, CYP11β1, CYP11b2, CYP17 and CYP19. In addition, the production of the hormones estradiol (17β-estradiol, E2), testosterone (T), and progesterone (P) was quantified using ELISA methods and related to gene expression. Dose–response curves were also developed to evaluate the effects of chemical concentration on both gene expression and hormone production.

Methods

Test chemicals. The 18 pharmaceuticals used for this study are depicted in Table 1. All chemicals were obtained from Sigma (St. Louis, MO, USA), except for amoxicillin, cephalaxin hydrate and erythromycin, which were obtained from BioChemika (St. Louis, MO, USA). Doxycycline hyclate was used in this study. Purity of all test chemicals from Sigma exceeded 98% while that of chemicals from BioChemika exceeded 97%. The chemicals used in this study were selected based on the list of the top 300 prescription drugs dispensed in the USA during 2005 (Rx List, www.rxlist.com) and also by their prevalence in surface waters. A set of high and low concentration exposures was run with the purpose of testing the range of response of the H295R cell system. High concentrations were established as higher than 3 × 10⁻³ μg/l and low concentrations were established to be less than 1 μg/l.

Experimental design. The H295R human adrenocortical carcinoma cell line was obtained from the American Type Culture Collection (ATCC # CRL-2128, ATCC, Manassas, VA, USA) and cells were grown in 75 cm² flasks with 12.5 ml of supplemented medium at 37 °C with a 5% CO₂ atmosphere. Supplemented
Table 1  
Pharmaceuticals and environmentally active compounds used in H295R cell exposures\textsuperscript{a,b}  

<table>
<thead>
<tr>
<th>Compound</th>
<th>Therapeutic use</th>
<th>Conc.\textsuperscript{a} (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>Analgesic</td>
<td>$3 \times 10^5$</td>
</tr>
<tr>
<td>Clobfibrate</td>
<td>Lipid agent</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Corticosteroid</td>
<td>$2 \times 10^5$</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Antibiotic</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>DEET</td>
<td>Pesticide</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Antibiotic</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>NSAID</td>
<td>$25 \times 10^4$</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>Antibiotic</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>Tylolosin</td>
<td>Antibiotic\textsuperscript{c}</td>
<td>$3 \times 10^5$</td>
</tr>
<tr>
<td>DEET</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fludrocortisone</td>
<td>Corticosteroid</td>
<td></td>
</tr>
<tr>
<td>HEPES; 6.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jose, CA, USA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Final component concentrations in the medium were: 15 mM HEPES, pH 6.25; 6.25 mM Na\textsubscript{2}CO\textsubscript{3}, ITS+Premix (BD Bioscience, 1 ml Premix/100 ml medium), and 12.5 ml/500 ml NuSerum (BD Bioscience, San Jose, CA, USA). Final component concentrations in the medium were: 15 mM HEPES, 6.25 µg/ml insulin; 6.25 µg/ml transferrin; 6.25 ng/ml selenium; 1.25 mg/ml bovine serum albumin; 5.35 µg/ml linoleic acid; and 2.5% NuSerum. The medium was changed 2–3 times per week and cells were detached from flasks for sub-culturing using sterile 1× trypsin–EDTA (Life Technologies Inc.). For exposure, cells were harvested into a final volume of 10 ml of medium. Cell density was determined using a hemacytometer. For dosing, 3 ml of cell suspension containing approximately 10⁶ cells/ml were placed in each well of 6-well tissue culture plates (Nalgene Nunc Inc., Rochester, NY, USA). Cells were exposed for 48 h to different groups of pharmaceuticals and several other compounds of relevant environmental importance dissolved in DMSO or methanol. The studied genes include 3α-oxidoreductase, CYP11β-1 a n d C Y P 1 1 β - 2 , which work directly on cortisol production and aldosterone synthesis respectively; CYP17 required for androgen production and regulation of substrate supplies for aromatization, as well as for cortisol biosynthesis; and CYP19 gene encoding for the aromatase enzyme, which mediates the aromatization of C18 estrogenic steroids from C19 androgens. CYP11β-1 was measured only for the analysis of dose responses of cyproterone and trenbolone exposures. The analysis of gene expression Real-time PCR (quantitative PCR) was performed by the Smart Cycler System (Cepheid, Sunnyvale, CA, USA) in 25 µl sterile tubes using a master mix containing 25 mM MgCl\textsubscript{2}, 1U/µl AmpliErase (Applied Biosystems, Foster City, CA, USA), 5 nM [α\textsuperscript{32}P]dCTP, 1 µM primers, and 1 µl of cDNA. The thermal cycling program included an initial denaturing step at 94 °C for 10 min, followed by 25–35 cycles of denaturing (95 °C for 15 s), primer annealing (at 60–64 °C for 40–60 s), and cDNA extension (72 °C for 30 s); a final extension step at 72 °C for 5–10 min was also included. Melting curve analyses were performed immediately following the final PCR cycle to differentiate between the desired amplicons and any primer-dimers or DNA contaminants. For quantification of PCR results the threshold cycle (C\textsubscript{t}) was calculated using the stepwise method.

**Cell viability/cytotoxicity.** Before nucleic acid isolation and hormone analysis, cell viability was determined. Cells were visually inspected under a microscope to evaluate viability and cell number. In addition, to establish the range of chemical concentrations that could be used without producing cytotoxic damage to the cells, live/dead cell viability assay kit (Molecular Probes, Eugene, OR, USA) was used. In instances where exposure resulted in cell death or decreased viability (less than 85%) the data were not used to evaluate gene expression or hormone production. **RNA isolation.** For nucleic acid extraction, cells were lysed in the culture plate after removal of the medium by the addition of 580 µl/well of lysis buffer–β-mercaptoethanol (β-ME) mixture (Stratagene, La Jolla, CA, USA) and RNA was isolated as previously described (Hilscherová et al., 2004). Briefly, lysed cells were mixed and then centrifuged in a pre-filter spin cuvette. The filtrate was diluted with 70% ethanol and vortexed. The mixture was transferred to an RNA spin cupe and centrifuged for 1 min. The filtrate was discarded and the spin cup was washed with a low-salt buffer and then centrifuged for 1 min. RNase-Free DNase I solution (Stratagene, La Jolla, CA, USA) was added to the fiber matrix inside the spin cup and the sample was incubated at 37 °C for 15 min. The sample was then washed with a high-salt followed by a low-salt buffer. After each wash cycle, the filtrate was discarded. After the final wash, the sample was centrifuged and nuclease-free water added directly to the fiber matrix inside the spin cup. The tube was incubated for 2 min at room temperature and centrifuged. This elution step was repeated to maximize the yield of RNA. The purified RNA was used immediately or stored at −80 °C until needed. An appropriate dilution of the RNA sample (1:50) was prepared for RNA quantification. The absorbance of the RNA solution was measured at 260 nm and 280 nm and the 260/280 ratio was calculated. The concentration of total RNA was estimated using the A\textsubscript{260} value and a standard with A\textsubscript{260} of 1 that was equivalent to 40 µg RNA/mL.

### cDNA preparation

Total RNA (1–5 µg) was combined with 50 µl oligo(dT)\textsubscript{20}, 10 mM dNTPs, and diethylpyrocarbamate (DEPC)-treated water to a final volume of 12 µl. RNA and primers were denatured at 65 °C for 5 min and then incubated on ice for 5 min. Reverse transcription was performed using 8 µl of a master mix containing 5× cDNA synthesis buffer (Carlshad CA, USA) and RNase/DNase free water. Reactions were incubated at 50 °C for 45 min and were terminated by incubation at 85 °C for 5 min. Samples were either used directly for PCR or were stored at −20 °C until analyzed.

**Gene expression using real-time PCR.** The studied genes include 3β/5βSD encoding for the enzyme catalyzing the production of the first biologically important steroid in the pathway, progesterone; CYP11β1 and CYP11β2, which work directly on cortisol production and aldosterone synthesis respectively; CYP17 required for androgen production and regulation of substrate supplies for aromatization, as well as for cortisol biosynthesis; and CYP19 gene encoding for the aromatase enzyme, which mediates the aromatization of C18 estrogenic steroids from C19 androgens. CYP11β1 was measured only for the analysis of dose responses of cyproterone and trenbolone exposures. The analysis of gene expression Real-time PCR (quantitative PCR) was performed by the Smart Cycler System (Cepheid, Sunnyvale, CA, USA) in 25 µl sterile tubes using a master mix containing 25 mM MgCl\textsubscript{2}, 1U/µl AmpliErase (Applied Biosystems, Foster City, CA, USA), 5 nM [α\textsuperscript{32}P]dCTP, 1 µM primers, and 1 µl of cDNA. The thermal cycling program included an initial denaturing step at 94 °C for 10 min, followed by 25–35 cycles of denaturing (95 °C for 15 s), primer annealing (at 60–64 °C for 40–60 s), and cDNA extension (72 °C for 30 s); a final extension step at 72 °C for 5–10 min was also included. Melting curve analyses were performed immediately following the final PCR cycle to differentiate between the desired amplicons and any primer-dimers or DNA contaminants. Specifics of the assay parameters such as primers used and annealing temperatures have been published previously (Hilscherová et al., 2004).

**For quantification of PCR results the threshold cycle (C\textsubscript{t}) was calculated using the stepwise method.**
expression was measured in triplicate for each control or exposed cell culture and each exposure was repeated at least three times.

Hormone quantification. Hormone extraction and quantification were conducted by ELISA were conducted as previously described (Hecker et al., 2006). Briefly, after exposure cell medium was collected from each well prior to cell lysis for RNA extraction and stored in 1 ml aliquots at −80 °C until needed. For analysis, frozen medium samples were thawed on ice, and the hormones were extracted twice with diethyl ether (5 ml) in glass tubes. To determine extraction recoveries a trace amount of 1H-T was added to each sample prior to extraction. The solvent extract was separated from the water phase by centrifugation at 2000×g for 10 min and transferred into small glass vials. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in EIA buffer from Cayman Chemical Company and either immediately measured or frozen at −80 °C for later hormone determination. Concentrations of hormones in media were measured by competitive ELISA using Cayman Chemical® hormone EIA kits (Cayman Chemical Company, Ann Arbor, MI, USA; P [P; Cat # 582201]; T [T; Cat # 582701]; 17β-estradiol [E2; Cat # 582251]). Because the antibody to P exhibits 61% cross-reactivity with pregnenolone and the method does not allow for the separation of these two hormones, P concentrations are expressed as P/pregnenolone. Hormones in all media samples were measured in triplicate. The working ranges for the determination of steroid hormones in H295R media were, P: 7.8–1000 pg/ml; T: 3.9–500 pg/ml; E2 estradiol: 7.8–1000 pg/ml. Media extracts were diluted 1:25 and 1:100 for T, while dilutions for P and E2 were 1:50 to 1:100 and 1:2 to 1:10, respectively.

Statistical analysis. Statistical analyses of gene expression profiles were conducted using SYSTAT (SYSTAT Software Inc., Point Richmond, CA, USA). Differences in gene expression and hormone production were evaluated by ANOVA followed by Tukey’s Test. Differences with p<0.05 were considered significant. Statistical correlations between gene expression and hormone production were established by Pearson correlation analysis followed by Bonferroni probability test. Correlations with p<0.05 were considered significant.

Results

Antibiotic exposure

Gene expression

Gene expression responses to the exposures conducted with seven of the most commonly used antibiotics in human medicine and for veterinary purposes are given in Table 2. The responses of gene expression for the blank and solvent control exposures were consistent. Treatment of the H295R cells with environmentally relevant or greater concentrations of the selected antibiotics resulted in significant changes in the expression of several genes. Exposure of H295R cells to environmentally relevant concentrations of amoxicillin, cephalaxin, oxytetracycline and tylosin significantly altered the expression pattern of the four target genes relative to that of solvent-exposed cells. Because oxytetracycline was not soluble in DMSO methanol was used to dissolve this compound. A methanol control was also included among the exposures. Amoxicillin significantly increased the expression of CYP17 and CYP19 more than 4-fold compared to solvent controls, while cephalaxin and oxytetracycline significantly increased expression of CYP19 more than 2-fold. Oxytetracycline was also the only antibiotic shown to affect the expression of the progesterogenic gene 3βHSD2. Tylosin increased expression of the aldosteronogenic gene CYP11b2 approximately 10-fold. Erythromycin, doxycycline and trimethoprim were used at non-relevant environmental concentrations of 3 to 10 μg/ml. Erythromycin increased the expression of CYP11b1 approximately 7-fold while doxycycline induced the expression of CYP19 almost 3-fold.

Hormone production

While the concentrations of all the hormones measured were very consistent in the blank, DMSO and methanol exposures (Table 2), some of the pharmaceuticals produced changes in production of hormones. Erythromycin increased the production of P and E2 more than 2- and 3-fold respectively, and reduced the production of T by more than 50%. In contrast, tetracyclines did not significantly affect hormone production. Tylosin decreased the production of T and E2, while cephalaxin only decreased T production and amoxicillin increased production of E2 more than 2-fold.

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gene expression</th>
<th>DMSO</th>
<th>MeOH</th>
<th>AMOXI</th>
<th>CEPHA</th>
<th>ERYT</th>
<th>OXYTC</th>
<th>DOXYC</th>
<th>TRIME</th>
<th>TYLO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1%</td>
<td>0.1%</td>
<td>71 μg/l</td>
<td>73 μg/l</td>
<td>3×10^1 μg/l</td>
<td>81 μg/l</td>
<td>1×10^6 μg/l</td>
<td>3×10^3 μg/l</td>
<td>3×10^5 μg/l</td>
</tr>
<tr>
<td>CYP11b2</td>
<td></td>
<td>1.00±0.33</td>
<td>1.00±0.19</td>
<td>0.95±0.24</td>
<td>0.77±0.33</td>
<td>6.91±0.97*</td>
<td>1.72±0.31</td>
<td>0.69±0.73</td>
<td>0.64±0.05</td>
<td>9.99±0.19*</td>
</tr>
<tr>
<td>CYP19</td>
<td></td>
<td>1.00±0.61</td>
<td>1.00±0.16</td>
<td>4.45±0.55*</td>
<td>2.87±1.13*</td>
<td>0.54±0.14</td>
<td>2.58±0.25*</td>
<td>2.87±0.40*</td>
<td>0.58±0.08</td>
<td>0.49±0.33</td>
</tr>
<tr>
<td>CYP17</td>
<td></td>
<td>1.00±0.10</td>
<td>1.00±0.16</td>
<td>4.48±0.35*</td>
<td>1.74±0.56</td>
<td>0.75±0.06</td>
<td>1.89±0.071</td>
<td>0.85±0.04</td>
<td>1.90±0.08</td>
<td>1.03±0.04</td>
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<td>3βHSD2</td>
<td></td>
<td>1.00±0.13</td>
<td>1.00±0.16</td>
<td>1.42±0.75</td>
<td>0.72±0.52</td>
<td>0.92±0.25</td>
<td>2.51±0.17*</td>
<td>1.00±0.08</td>
<td>0.60±0.36</td>
<td>1.32±0.27</td>
</tr>
<tr>
<td>Hormone</td>
<td></td>
<td>0.1%</td>
<td>0.1%</td>
<td>71 μg/l</td>
<td>73 μg/l</td>
<td>3×10^1 μg/l</td>
<td>81 μg/l</td>
<td>1×10^6 μg/l</td>
<td>3×10^3 μg/l</td>
<td>3×10^5 μg/l</td>
</tr>
<tr>
<td>Testosterone</td>
<td></td>
<td>1.00±0.27</td>
<td>1.00±0.34</td>
<td>0.71±0.02</td>
<td>0.11±0.03*</td>
<td>0.44±0.08*</td>
<td>1.23±0.007</td>
<td>1.55±0.085</td>
<td>0.42±0.09*</td>
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<tr>
<td>Progesterone</td>
<td></td>
<td>1.00±0.01</td>
<td>1.00±0.13</td>
<td>0.63±0.13</td>
<td>0.89±0.04</td>
<td>3.55±0.58*</td>
<td>1.32±0.55</td>
<td>1.26±0.20</td>
<td>1.30±0.28</td>
<td></td>
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<tr>
<td>Estradiol</td>
<td></td>
<td>1.00±0.32</td>
<td>1.00±0.60</td>
<td>2.5±0.61*</td>
<td>0.51±0.30</td>
<td>2.46±0.33*</td>
<td>0.15±0.05</td>
<td>2.04±0.60</td>
<td>0.12±0.08*</td>
<td></td>
</tr>
</tbody>
</table>

All exposures were conducted for 48 h under standard conditions. All gene expression and hormone production values for fold-change relative to DMSO the solvent control (=1.0), given as means and standard deviations. DMSO: Dimethylsulfoxide; MeOH: Methanol; AMOXI: Amoxicillin; ERYT: Erythromycin; OXYTC: Oxytetracycline; TRIME: Trimethoprim; TYLO: Tylosin.

* Compared to MeOH as solvent control.

* Indicates statistically significant differences at p<0.05.
Hormone therapy drugs

None of the four hormone therapy drugs that are commonly used for cancer treatment, birth control, inflammatory processes and as growth promoters in animal production significantly affected the expression of 3β-HSD2 (Table 3). Environmentally relevant concentrations of the cancer therapy drug cyproterone induced the expression of CYP19 more than 4-fold and the expression of the androgenic gene CYP17 3-fold. Dexamethasone and EE2 exposures induced expression of CY11β2 approximately 5-fold. The growth promoter trenbolone only increased the expression of CYP19 by about 3-fold.

Hormone production

Hormone therapy drugs also affected the hormone production (Table 3). EE2 significantly increased P and E2 production by more than 2-fold and at the same time significantly decreased T production by about 66%. Trenbolone and cyproterone decreased T production by approximately 50% and 66% respectively. However, no chemical except of EE2 affected E2 or P production.

Other pharmaceuticals and environmentally active compounds

Gene expression

A significant up-regulation of CYP17 was observed after the exposure to the β2-agonist salbutamol, which increased the expression of this gene more than 10-fold (Table 4). None of the other genes studied were affected by this compound. Of the analgesics studied, only acetaminophen significantly affected the expression of CYP11β2 by increasing it approximately 4-fold. CYP11β2 was induced approximately 5-fold by the anti-lipidic clofibrate and the antidepressant fluoxetine, and ap-

Table 3
Gene expression and hormone production in H295R cells exposed to single hormone therapy drugs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gene</th>
<th>DMSO</th>
<th>CYPROT</th>
<th>DEXAM</th>
<th>EE2</th>
<th>TRENB</th>
<th>ZEARA</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CYP11β2</td>
<td>1.00±0.33</td>
<td>1.34±0.52</td>
<td>5.39±0.50*</td>
<td>4.88±0.97*</td>
<td>0.81±0.43</td>
<td>0.22±0.03</td>
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<tr>
<td></td>
<td>CYP19</td>
<td>1.00±0.61</td>
<td>4.62±1.51*</td>
<td>0.98±0.19</td>
<td>0.54±0.14</td>
<td>2.56±0.55*</td>
<td>0.32±0.10</td>
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<tr>
<td></td>
<td>CYP17</td>
<td>1.00±0.10</td>
<td>2.81±0.5*</td>
<td>0.71±0.04</td>
<td>0.75±0.06</td>
<td>1.79±0.41</td>
<td>0.20±0.04</td>
</tr>
<tr>
<td></td>
<td>3βHSD2</td>
<td>1.00±0.13</td>
<td>0.90±0.32</td>
<td>0.64±0.09</td>
<td>0.92±0.25</td>
<td>0.77±0.15</td>
<td>0.14±0.03*</td>
</tr>
<tr>
<td>Hormone</td>
<td>DMSO</td>
<td>0.1%</td>
<td>62 μg/l</td>
<td>2×10^3 μg/l</td>
<td>1 μg/l</td>
<td>25 μg/l</td>
<td>2.8×10^3 μg/l</td>
</tr>
<tr>
<td></td>
<td>CYPROT</td>
<td>1.00±0.27</td>
<td>0.29±0.03*</td>
<td>0.25±0.05*</td>
<td>0.36±0.12*</td>
<td>0.48±0.09*</td>
<td>1.06±0.15</td>
</tr>
<tr>
<td></td>
<td>DEXAM</td>
<td>1.00±0.01</td>
<td>0.12±0.30</td>
<td>0.74±0.05</td>
<td>2.71±0.39*</td>
<td>0.72±0.20</td>
<td>1.02±0.21</td>
</tr>
<tr>
<td></td>
<td>EE2</td>
<td>1.00±0.32</td>
<td>1.25±0.47</td>
<td>1.4±0.26</td>
<td>2.33±0.27*</td>
<td>1.6±0.25</td>
<td>0.17±0.03</td>
</tr>
</tbody>
</table>
| All exposures were conducted for 48 h under standard conditions. All gene expression and hormone production values are expressed as fold-change relative to the solvent control DMSO (=1.0), given as means and standard deviations. DMSO: Dimethylsulfoxide; CYPROT: Cyproterone; DEXAM: Dexamethasone; EE2: Ethynylestradiol; TRENB: Trenbolone Acetate; ZEARA: α-Zearalanol. * Indicates statistically significant differences at p<0.05.

Table 4
Gene expression and hormone production in H295R cells exposed to single pharmaceuticals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gene</th>
<th>DMSO</th>
<th>ACETA</th>
<th>IBUPR</th>
<th>SALBU</th>
<th>CLOFI</th>
<th>DEET</th>
<th>FLUOX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP11β2</td>
<td>1.00±0.33</td>
<td>3.66±0.89*</td>
<td>2.6±0.77</td>
<td>2.00±0.38</td>
<td>4.67±1.24*</td>
<td>8.20±1.30*</td>
<td>5.69±0.77*</td>
</tr>
<tr>
<td></td>
<td>CYP19</td>
<td>1.00±0.61</td>
<td>0.88±0.10</td>
<td>0.72±0.01</td>
<td>1.88±0.62</td>
<td>1.30±0.15</td>
<td>0.5±0.04</td>
<td>1.41±0.09</td>
</tr>
<tr>
<td></td>
<td>CYP17</td>
<td>1.00±0.10</td>
<td>0.64±0.08</td>
<td>0.55±0.08</td>
<td>13.64±0.98*</td>
<td>1.04±0.09</td>
<td>1.26±0.13</td>
<td>0.90±0.05</td>
</tr>
<tr>
<td></td>
<td>3βHSD2</td>
<td>1.00±0.13</td>
<td>0.45±0.20</td>
<td>0.37±0.23</td>
<td>1.05±0.19</td>
<td>0.66±0.03</td>
<td>1.04±0.44</td>
<td>0.69±0.07</td>
</tr>
<tr>
<td>Hormone</td>
<td>DMSO</td>
<td>0.1%</td>
<td>3×10^5 μg/l</td>
<td>25×10^4 μg/l</td>
<td>5×10^−2 μg/l</td>
<td>3×10^3 μg/l</td>
<td>1 μg/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACETA</td>
<td>1.00±0.27</td>
<td>1.12±0.04</td>
<td>0.88</td>
<td>0.51±0.17</td>
<td>0.68±0.13*</td>
<td>0.39±0.01*</td>
<td>0.75±0.01</td>
</tr>
<tr>
<td></td>
<td>IBUPR</td>
<td>1.00±0.01</td>
<td>2.3±0.15*</td>
<td>1.84</td>
<td>0.30±0.30</td>
<td>0.75±0.09</td>
<td>1.68±0.48</td>
<td>1.37±0.08</td>
</tr>
<tr>
<td></td>
<td>SALBU</td>
<td>1.00±0.32</td>
<td>0.50±0.2</td>
<td>0.42</td>
<td>0.32±0.32*</td>
<td>1.55±0.05</td>
<td>0.17±0.10*</td>
<td>1.30±0.30</td>
</tr>
</tbody>
</table>
| All exposures were conducted for 48 h under standard conditions. All gene expression and hormone production values are expressed as fold-change relative to the solvent control DMSO (=1.0), given as means and standard deviations. DMSO: Dimethylsulfoxide; ACETA: Acetaminophen; IBU: Ibuprofen; SALBU: Salbutamol; CLOFI: Clofibrate; DEET: N,N-diethyl-3-methylbenzamide; FLUOX: Fluoxetine. * Indicates statistically significant differences at p<0.05.
proximately 4-fold by the analgesic acetaminophen. The insect repellent, DEET also increased the expression of this gene more than 8-fold. Although the natural phytoestrogen α-zearalanol decreased the expression of the four evaluated genes, only the decrease in 3βHSD2 was statistically significant. The non-steroidal analgesic anti-inflammatory drug (NSAAID) ibuprofen did not produce any significant changes in the expression of any of the steroidogenic genes studied.

**Hormone production**

Ibuprofen and fluoxetine did not cause significant changes in the production of any of the analyzed hormones compared to blank and solvent controls (Table 4). However, T production was decreased by clofibrate and DEET, while E2 production was significantly inhibited by salbutamol and DEET. Moreover, the analgesic acetaminophen produced a 2-fold increase in P concentrations.

**Pattern of responses to chemical mixtures**

**Gene expression**

When H295R cells were exposed to binary mixtures where one of the two components was EE2 the gene expression responses were very diverse (Table 5). In the response to trenbolone exposure CYP19 was up-regulated approximately 2.5-fold. Exposure to a mixture of trenbolone and EE2 caused a decrease in CYP19 expression of as much as 50% compared to solvent control. Individually cyproterone up-regulated expression of CYP19 more than 4-fold, but when cells were exposed to a mixture of cyproterone and EE2, expression of this gene was not significantly different from that of the control. Although tylosin reduced CYP19 expression this reduction was not statistically significant when compared to that of cells exposed to the solvent only. The tylosin–EE2 mixture did not produce changes in the expression of this gene compared to controls. Cypreterone significantly up-regulated the expression of the aromatase gene CYP19 up to 4.6-fold and salbutamol did not produce any effects on this gene, but when these two compounds were mixed together the expression of CYP19 was almost completely inhibited.

**Hormone production**

Salbutamol caused induction of CYP17 of more than 13-fold; while cyproterone also significantly induced this gene almost 3-fold. Neither tylosin nor EE2 affected the expression of CYP17, and moreover none of the binary mixture studied significantly affected the expression of this gene.

3βHSD2 was the least affected by any of the treatments. Individual exposures with the chosen chemicals did not produce any significant changes in expression of this gene. However, the cyproterone/salbutamol mixture significantly decreased the expression of 3βHSD2.

A variety of responses was also observed for CYP11β2. Expression of this gene was increased significantly by around 5-fold after exposure to EE2. Mixtures of cyproterone and trenbolone each with EE2 did not affect the expression of CYP11β2, and although tylosin treatment significantly induced (10-fold) the expression of this gene, the tylosin–EE2 mixture did not produce any significant change.

**Dose–response analysis**

**Gene expression**

Dose–response curves were constructed after 48 h of exposure to trenbolone, EE2, and cyproterone in the ranges of 0–39 μg/l,

### Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CYP17</th>
<th>CYP19</th>
<th>3βHSD2</th>
<th>CYP11β2</th>
<th>PROG</th>
<th>TEST</th>
<th>ESTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>1.04 (0.05)</td>
<td>0.99 (0.02)</td>
<td>0.94 (0.09)</td>
<td>1.00 (0.01)</td>
<td>1.00 (0.01)</td>
<td>1.00 (0.27)</td>
<td>1.00 (0.32)</td>
</tr>
<tr>
<td>Ethynylestradiol</td>
<td>1.05 (0.18)</td>
<td>1.05 (0.18)</td>
<td>0.76 (0.1)</td>
<td>4.88 (0.88)*</td>
<td>2.71 (0.39)*</td>
<td>0.36 (0.12)*</td>
<td>2.33 (0.27)*</td>
</tr>
<tr>
<td>Ethynylestradiol+Trenbolone</td>
<td>0.55 (0.50)</td>
<td>0.57 (0.50)</td>
<td>0.45 (0.25)</td>
<td>1.46 (1.16)</td>
<td>2.72 (1.67)</td>
<td>0.77 (0.02)</td>
<td>3.77 (1.13)*</td>
</tr>
<tr>
<td>Ethynylestradiol+Cyproterone</td>
<td>0.88 (0.01)</td>
<td>0.99 (0.03)</td>
<td>0.43 (0.33)</td>
<td>1.28 (0.39)</td>
<td>2.49 (0.42)</td>
<td>0.70 (0.14)</td>
<td>1.69 (0.19)</td>
</tr>
<tr>
<td>Ethynylestradiol+Tylosin</td>
<td>0.69 (0.43)</td>
<td>1.04 (0.44)</td>
<td>0.54 (0.47)</td>
<td>2.33 (0.27)</td>
<td>1.39 (0.95)</td>
<td>0.85 (0.17)</td>
<td>0.74 (0.09)</td>
</tr>
<tr>
<td>Cyproterone+Salbutamol</td>
<td>0.76 (0.27)</td>
<td>0.01 (0.00)*</td>
<td>0.33 (0.06)*</td>
<td>2.53 (1.34)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Trenbolone</td>
<td>1.79 (0.41)</td>
<td>2.56 (0.55)*</td>
<td>0.77 (0.15)</td>
<td>0.81 (0.43)</td>
<td>0.72 (0.20)</td>
<td>0.48 (0.09)*</td>
<td>1.60 (0.25)</td>
</tr>
<tr>
<td>Tylosin</td>
<td>2.81 (0.50)*</td>
<td>4.62 (1.51)*</td>
<td>0.90 (0.32)</td>
<td>1.34 (0.52)</td>
<td>1.02 (0.30)</td>
<td>0.29 (0.03)*</td>
<td>1.25 (0.47)</td>
</tr>
<tr>
<td>Cyproterone</td>
<td>1.03 (0.04)</td>
<td>0.49 (0.03)</td>
<td>1.32 (0.27)</td>
<td>9.99 (1.09)*</td>
<td>1.03 (0.04)</td>
<td>0.42 (0.09)*</td>
<td>0.12 (0.08)*</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>13.64 (0.5)*</td>
<td>1.00 (0.19)</td>
<td>1.88 (0.62)</td>
<td>2.00 (0.38)</td>
<td>0.30 (0.30)</td>
<td>0.51 (0.17)*</td>
<td>0.32 (0.32)*</td>
</tr>
</tbody>
</table>

Concentrations of single chemicals and mixtures exposures were: DMSO (0.1%), Ethynylestradiol (1 μg/l), Trenbolone (25 μg/l), Cyproterone (62 μg/l), Tylosin (3 × 10^7 μg/l), Salbutamol (50 × 10^7 μg/l). ND: No Data.

* All exposures were conducted for 48 h under standard conditions. All gene expression and hormone production values are expressed as fold-change relative to the solvent control DMSO (=1.0), given as means and standard deviations in parenthesis. TEST: Testosterone, PROG: Progesterone, ESTR: Estradiol.

* Indicates statistically significant differences at p < 0.05.
0–150 μg/l and 0–45 μg/l respectively. Relative expression of 3βHSD2, CYP11β2, CYP17 and CYP19 values normalized to β-actin were compared to solvent controls. 17βHSD1 responses were also analyzed for the cyproterone and trenbolone only.

In the EE2 exposure (Fig. 1) 3βHSD2, CYP17 and CYP19 were not affected by the different concentrations of this chemical, however, CYP11β2 expression increased between 1.5 and 15 μg/l EE2 were constant between 15 and 75 μg/l EE2, and rose again between 75 and 150 μg/l EE2. For the cyproterone exposure (Fig. 2), 3βHSD2, CYP19 and 17BHSD1 expression remained at basal levels, while the androgenic gene CYP17 increased in expression by approximately 3-fold at a concentration of 0.9 μg/l. Exposure to trenbolone at concentrations ranging from 0 to 780 μg/l did not affect expression of any of the five genes studied in the dose–response exposures (Fig. 3). Most of the genes fluctuated positively around basal values of expression except for CY11β2, which showed a decrease in expression at 78 μg/l but returned to basal values at greater concentrations such as 780 μg/l.

**Hormone production**

For the EE2 exposure, the dose–response curve for the production of T was constant and did not change (Fig. 1).
Moreover, P production started to increase at 0.15 μg/l EE2, reaching an 8-fold maximum induction at 1.5 μg/l EE2. P production then returned to basal levels at 15 μg/l EE2.

The dose–response curve for hormone production in response to cyproterone exposure was bimodal through the concentration range of 0 to 45 μg/l (Fig. 2). P and T production were slightly greater than control upon exposure to 0.09 μg/l cyproterone, then decreased at 0.9 μg/l and increased again at 9 μg/l, remaining constant up to 45 μg/l cyproterone. E2 production followed the same pattern as P and T but the fold-change was greater. Maximum E2 production was observed to be 2.5-fold when exposed to 9 μg/l of cyproterone, while for P and T the maximum was less than 1.5-fold. Production of P and T remained at control values for mental relevant concentrations of pharmaceuticals have the capacity when examining effects of environmentally relevant doses of pollutants on steroid production. To ascertain the validity of these correlations Bonferroni probabilities were also calculated. The results of these analyses indicated that by pooling all treatments together only one correlation was statistically significant and it was the negative correlation between the responses of the aromatase gene CYP19 and the aldosterone gene CYP11β2. No statistically significant correlations between hormone production and gene expression were observed for the combined treatments.

Correlations for the group of antibiotics not only showed the negative correlation established before between CYP19 and CYP11β2 but also a positive correlation between CYP19 and CYP17; both correlations were statistically significant. Again, no significant correlations between gene expression and hormone production were observed. A single positive significant correlation was observed between expression of CYP19 and CYP17 for the group of chemicals used for hormone therapy, but the negative correlation between CYP11β2 and CYP19 was not observed.

Discussion

Previous studies have demonstrated the effectiveness of the H295R assay in identifying the potential effects that compounds may exert at different points in the steroidogenic pathway (Hilscherova et al., 2004; Hecker et al., 2006; Zhang et al., 2005; Gracia et al., 2006; Blaha et al., 2006). With the H295R cell culture system not only is it possible to analyze gene expression and hormone production, but also to evaluate enzyme activity. Moreover, this cell system has proven useful in identifying chemical mechanisms of action, in establishing patterns of gene and hormone responses, and also in the analysis of different interactions between chemicals when present in complex mixtures. Results from the experiments conducted in the present study confirm the effectiveness of the H295R screening system and its capacities when examining effects of environmentally relevant doses of pollutants on steroid production.

Pattern of responses by group of chemicals

Antibiotics

The present in vitro study demonstrates that environmentally relevant concentrations of pharmaceuticals have the
potential to interfere with the normal pathway of steroid production. In particular, antibiotics were shown to have a broad range of effects on steroidogenesis. Although the semisynthetic β-lactam antibiotics amoxicillin and cephalexin have a similar therapeutic mechanism of action, they affected steroidogenic gene expression and hormone production quite differently. In the case of the semi-synthetic macrolide antibiotics, erythromycin and tylosin, both caused the same gene expression profile, and yet they differed in their hormone production profile. Amoxicillin and cephalexin both have a β-lactam ring in their chemical structures (Saderm et al., 2007), while erythromycin and tylosin both have a macrolide ring, and these small differences in chemical structure may be responsible for the discrepancies observed in gene expression and hormone production profiles.

Since the effects of antibiotics on steroid production have not been previously studied, the mechanisms by which these compounds exert their effects on steroidogenesis are unknown. Given the extensive use of antibiotics and their loadings to the environment, endocrine disruption resulting from these pharmaceutical chemicals should be considered along with the promotion of antibiotic resistance and the potential of these compounds to influence growth in humans (Ternak, 2004) and other non-target organisms.

**Hormone therapy group**

Drugs employed as hormone therapy agents have a broad range of medical uses. Pharmaceuticals of this group are used in cancer treatment, birth control, in diagnostic procedures, and as growth promoters, among other uses. Cyproterone is a steroidal anti-androgen with weak progestagenic activity used in the treatment of prostate cancer (Wirth et al., 2007). This drug exerts its functions by suppressing androgen action both by binding directly to the androgen receptor and by inhibiting the positive feedback of androgens on the pituitary ultimately resulting in reduced production of sex steroids (Sharpe et al., 2004). The anti-androgenic properties of cyproterone were observed in the results for hormone analysis where concentrations of T were reduced by up to one-third. It is noteworthy that the expression of CYP19 and CYP17 were increased, probably in response to depletion of T in the medium. Induction of CYP17 would drive steroidogenesis towards the production of androgens while increase in CYP19 activity would ensure that E2 was produced despite small concentrations of substrate. The significant and strong negative correlation observed for these two genes for this group of pharmaceuticals supports the idea of a coordinated expression system.

EE2 is the most common and most potent estrogenic compound found in sewage effluents (Sarmah et al., 2006). This synthetic E2 analog is used in combination with other estrogenic substances in the manufacturing of contraceptive pills. Studies have demonstrated the effects of EE2 on the survival, sex ratio, gonadal growth, spawning and sexual differentiation of aquatic organisms, especially in fish (Scholz and Gutzeit, 2000). In H295R cells exposed to 1 μg/l EE2 the production of P and E2 in H295R cells doubled, while T production was greatly reduced. The observed decrease in T production may be a reaction to the increased production of E2 since T production may be substrate-limited.

Trenbolone acetate (TBA) is a synthetic steroid hormone commonly used to enhance growth in beef cattle. TBA is quickly metabolized to the potent androgen 17β-trenbolone (Durhan et al., 2006). Despite high affinity of 17β-trenbolone for the human androgen receptor, an affinity which is known to be similar to that of dihydrotestosterone (Bauer et al., 2001), decreases in T production in the H295R cells were observed. We speculate that these results may be an indication of the capability of the 17β-trenbolone metabolite for blocking other pathways directly or indirectly related to T production or for inducing pathways leading to T metabolism. At the same time the cell response to this lack of T is the induction in the expression of the aromatase gene CYP19 trying to keep E2 concentrations at normal levels.

The estrogenic equivalent of 17β-trenbolone is α-zearalanol; this chemical is the active metabolite of the mycotoxin zearalenone that is obtained from Fusarium spp. (Sheehan et al., 1984). This compound is also used in veterinary medicine as a growth promoter. T and P production were not affected by this chemical nor was the expression of the steroidogenic genes studied, except for 3βHSD2. Although both EE2 and α-zearalanol can strongly bind to estrogen receptor (ER) (Takemura et al., 2007) their observed effects on E2 production were very different. EE2 doubled E2 production whereas α-zearalanol reduced E2 production by almost 6-fold. As it was speculated before for the results of trenbolone exposure, we hypothesize that this reduction in E2 production may be the result of the activation or inhibition of other pathways not related to E2 receptors binding.

Together these results indicate that extensive attention must be directed to the use and fate of pharmaceuticals with hormonal properties since this is a group of chemicals that will surely produce significant effects when reaching non-target organisms. This is especially the case for compounds used for veterinary purposes which may be excreted in their active forms by treated animals and then reach aquatic ecosystems via runoff (Lange and Dietrich, 2002).

**Other pharmaceuticals**

Over-the-counter analgesics and anti-inflammatory drugs such as acetaminophen and ibuprofen did not produce significant changes in gene expression or hormone responses. No steroidogenic effects have been demonstrated for acetaminophen and even its exact mechanism of action as an analgesic is unknown. Antilipidic drugs such as clofibrate are commonly used to treat hyperlipidemia, a condition considered a major risk factor of cardio and cerebro-vascular diseases. Despite being withdrawn from the market in most countries in Western Europe, clofibrate concentrations in the ng/l to μg/l range have been reported in several sewage treatment plant effluents (Koutsouba et al., 2003). In H295R cells clofibrate concentrations of 3 μg/ml significantly reduced T production. Reduction of T levels by this drug has also been observed in rat where it was suggested that clofibrate may exert its action through direct effects on the microsomal
enzyme systems responsible for steroid metabolism (Xu et al., 2002).

The results from the exposure with the drug salbutamol, a short-acting, β2-adrenergic receptor agonist used to treat broncho-spasm and in some cases used in obstetrics as a tocolytic to relax uterine smooth muscle and delay premature labor (Blanchard et al., 1993), were especially interesting. Salbutamol binds to β2-adrenergic receptors with greater affinity than β1-receptors; the activation of β2-adrenergic receptors results in relaxation of smooth muscles. Salbutamol is also used in combination with other drugs as a growth promoter in livestock. This β2-adrenergic drug enhances lipolysis and the rate at which fatty acids are oxidized producing leaner animals (Hernández-Carrasquilla, 2003). Thus, we hypothesized that the lipolytic effects of salbutamol could be responsible for the significant decreases of almost 50% in E2 production compared to solvent controls. The most obvious effect of this agonist compound was the increase in expression of the androgenic gene CYP17 by more than 10-fold. More specific studies need to be designed in order to reveal if this increase in the expression of CYP17 is in some way linked to the depletion of E2.

Effects of drug mixtures

From their first use, pharmaceuticals have been entering the environment and have been constantly detected at measurable concentrations; they are ordinarily found in mixtures of active ingredients with a variety of biological activities. Thus, non-target organisms are being exposed to compounds with different biological actions at the same time. Few toxicological studies have been conducted to address chronic toxicity upon exposure to mixtures of biologically active contaminants and the associated risks (Crane et al., 2006). Understanding of the effects of complex mixtures of compounds acting together must become a priority when evaluating the potential risks of pharmaceuticals in the environment. One of the major difficulties in analyzing effects of complex mixtures is the understanding of the different ways in which compounds in the mixture will interact to produce effects.

H295R cells were exposed to four binary mixtures of different pharmaceuticals. EE2, the most common component in birth control pills, was a common component for three of the four binary mixtures prepared. Because of the effects of individual compounds on the four genes studied, cyproterone, trenbolone and tylosin were chosen as the second components in the mixtures with EE2. In addition, due to the effects of cyproterone on gene expression and salbutamol on hormone production, H295R cells were also exposed to a mixture of these two compounds. Gene responses suggested that the chemicals present in these mixtures interact mostly by antagonistic mechanisms, although agonism was also observed in some cases. The dominant effects of EE2 were observed in mixtures with trenbolone and cyproterone. When expression values produced by individual exposures of EE2 were greater than those produced by the second component in the mixture the joint effects observed were similar to those caused by the second component, or in other terms, for the compound that produced lower fold-inductions.

In contrast to gene expression, several types of interactions were observed for the hormone production responses to the mixture treatments. For instance, in the case of P the binary mixtures produced the same effects as produced by EE2 alone, which was more than a 2-fold induction in the production of this hormone, an indication that the EE2 effects prevail in the mixture. On the other hand T production was down-regulated by all the individual treatments but the mixtures did not produce significant changes in the concentration of this hormone showing that these chemicals block each other’s antagonistic effects with respect to T production. On the contrary, the results of E2 measurement showed that an additive effect was produced by the mixture of EE2 and trenbolone; such a response is likely due to the affinity of both these compounds for the ER. These results document that the steroidogenic effects exerted by the binary mixture exposures could not be predicted from the results of exposures of the individual chemicals in question. As an example, cyproterone significantly up-regulated the expression of the aromatase gene CYP19 while salbutamol did not produce significant changes in the expression of this gene, but a mixture of the two compounds resulted in complete suppression of CYP19 expression. These findings corroborate the premise that not only do compounds interact, but their effects are usually different from the responses of individual chemicals. The results show that pharmaceuticals and their mixtures act through additional unknown modes of toxic action that have to be understood in order to truly assess their potential effects as environmental contaminants.

Dose–response analysis

Three pharmaceuticals used in hormone therapy were selected to conduct dose–response studies. The results showed that the dose-dependent changes in gene expression behaved differently for each chemical. Exposure to EE2 affected only CYP11β2 expression. Of the hormones, only T production was not affected by exposure to EE2. E2 concentrations were proportional to the concentration of EE2. Changes were observed even at EE2 concentrations as small as 0.15 μg/l. The positive relationship between E2 production and EE2 in the medium is consistent with the great affinity of EE2 to for the ER. Despite the induction of CYP11β2 by EE2, P production was not affected in the same manner. Increased production of this hormone was only observed between 0.15 and 1.5 μg/l before returning to basal levels.

The anti-androgen cyproterone prevents dihydrotestosterone, the active form of T in mammals, from binding to receptors in carcinoma cells. Thus, induction of CYP17 may be a response to the presence of the anti-androgen that results in an increase in the production of active T to compete for the receptors. E2 was the only hormone to increase proportionally with cyproterone concentration. The mechanisms by which this process occurred are unknown.

Changes in trenbolone concentrations did not produce major effects on the expression of any of the steroidogenic genes, or hormone production except for its effects on E2. The production of E2 was greater than in the control at all trenbolone
concentrations tested, although the expression of the aromatase gene CYP19 was not increased and T production stayed within the basal concentration range. These results suggest the possibility that trenbolone induced the activity of the aromatase enzyme by the activation of other pathways.

Chemicals with the same mechanism of action may have different effects on the expression of steroidogenic genes and the production of steroid hormones. For instance, although cyproterone and trenbolone both interact with the androgen receptor, each caused different effects on gene expression and hormone production. Thus, it appears that each of these chemicals, in addition to its interaction with the androgen receptor, may induce or inhibit other points in the pathway resulting in the observed differences in effects.

The results from this study demonstrated that several pharmaceuticals, including compounds with unknown steroidogenic effects, have the potential to produce changes in steroidogenic gene expression and hormone production at different range of concentrations. Moreover, the steroidogenic effects of mixtures of pharmaceuticals may be different to the effects observed from individual compounds, which leads to conclude that interaction between pharmaceuticals occurs and such interaction has its own particular effects. Although compounds with the same therapeutic mechanism of action may show similar gene expression profiles their effects on hormone production may be different, perhaps due to differences in the particular effects that each compound can exert directly or indirectly into the steroidogenic pathway independently of their genomic effects. Since none statistical correlations were established between gene expression and hormone production it is suggested that not only other factors different to gene expression are influencing the production of steroid hormones, but also that alternatively chemicals may activate or desactivate pathways that may influence the production of steroid hormones. Despite no statistical correlations between gene expression and hormone production were observed, statistical correlations among some genes were shown to be significant, which may suggest that the direct expression dependency between genes, dependency that may only be corroborated through functional genomic analysis.

Pharmaceuticals were just recently classified as environmental contaminants after years of being ignored in environmental studies; the presence of these bioactive compounds in the environment should be controlled and monitored due to the inherent potential biological effects that compounds such as these can exert on non-target organisms. The H295R cell bioassay is a very quick, practical, and sensitive pre-screening method by which the endocrine disruptive effects of environmentally relevant chemicals may be evaluated.

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