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Quantitative RT-PCR Methods for Evaluating Toxicant-Induced Effects on Steroidogenesis Using the H295R Cell Line

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Gene expression profiles show considerable promise for the evaluation of the toxic potential of environmental contaminants. For example, any alterations in the pathways of steroid synthesis or breakdown have the potential to cause endocrine disruption. Therefore monitoring these pathways can provide information relative to a chemical's ability to impact endocrine function. One approach to monitoring these pathways has been to use a human adrenocortical carcinoma cell line (H295R) that expresses all the key enzymes necessary for steroidogenesis. In this study we have further developed these methods using accurate and specific quantification methods utilizing molecular beacon-based quantitative RT-PCR (Q-RT-PCR). The assay system was used to analyze the expression patterns of 11 steroidogenic genes in H295R cells. The expression of gene transcripts was measured using a real-time PCR system and quantified based on both a standard curve method using a dilution series of RNA standards and a comparative C_t method. To validate the optimized method, cells were exposed to specific and nonspecific model compounds (inducers and inhibitors of various steroidogenic enzymes) for gene expression profiling. Similar gene expression profiles were exhibited by cells treated with chemicals acting through common mechanisms of action. Overall, our findings demonstrated that the present assay can facilitate the development of compound-specific response profiles, and will provide a sensitive and integrative screen for the effects of chemicals on steroidogenesis.

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Introduction

There has been increasing public and scientific concern about environmental chemicals that may alter development, reproduction, and the endocrine systems of wildlife and humans (*1, 2*). These so-called "endocrine disrupting chemicals" (EDCs) can operate through several different mechanisms of action. First, EDCs can act through receptor-mediated processes that control sexual development and homeostasis. Of particular concern have been those chemicals that interact directly with steroid hormone receptors such as the estrogen receptor (ER), androgen receptor (AR), and thyroid hormone receptor (ThR). Currently, effects of EDCs and methods to screen for them have focused on these three receptors; however, there are a number of other receptor-mediated processes that control sexual development and homeostasis. Second, there are a number of nonreceptor-mediated processes that may alter endocrine function. There are compounds that can modulate steroid hormone production or breakdown and cause endocrine disruption without acting as direct hormone mimics. These nonreceptor-mediated effects are often exerted indirectly via effects on common signal transduction pathways. They can also act as direct or indirect stimulators or inhibitors of the enzymes involved in the production, transformation, or elimination of steroid hormones.

Steroid hormones are derivatives of cholesterol that are synthesized in a variety of tissues, most prominently the adrenal gland and gonads. The adrenal cortex is unique in its steroidogenic biosynthetic capabilities as it can synthesize all the major classes of steroid hormones (mineralocorticoids, glucocorticoids, and sex steroids, including androgens and estrogen). Consequently, the adrenal gland is vital to health and has a role in reproduction and development (*3, 4*). In the adult adrenal cortex, a battery of oxidative and other enzymes located in both the mitochondria and endoplasmic reticulum of the three phenotypically distinct zones are involved in the biosynthesis of steroid hormones (Table 1). Steroid hormone biosynthesis in the adrenal cortex involves the coordinated transcription of the numerous genes encoding steroidogenic enzymes. Chemical agents that alter expression of these steroidogenic enzymes have the potential to alter hormone biosynthesis. Because of its unique steroidogenic capability and its role in responses to environmental insults, the adrenal cortex has been suggested to be the most common and perhaps the most susceptible endocrine target organ for EDCs (*5*). Nevertheless, current testing strategies to assess the endocrine disrupting properties of chemicals have not adequately examined adrenocortical function.

The H295R human adrenocortical carcinoma cell line (a subpopulation of the H295 cell line that forms a monolayer in culture) has been characterized in detail and shown to express all the key enzymes necessary for steroidogenesis (*6*−*8*). As a result it has been investigated as a potential system for in vitro screening of chemical effects on steroidogenesis (*9*). The system has been demonstrated to assist in mechanistic interpretation and to provide relevant data for risk assessment based on measuring effects on specific enzymes such as aromatase (*10*−*12*).

Due to the rapid development of reagents and hardware, real-time quantitative RT-PCR (Q-RT-PCR) has emerged as a sensitive and powerful technique for the quantification of gene expression. Q-RT-PCR can quantify alterations in mRNA concentrations undetectable by earlier techniques such as northern blotting, RNase protection assays, or gel-based end-point detection RT-PCR. In addition, compared to the use

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TABLE 1. Endpoint Enzymes Involved in Steroidogenesis or Cholesterol Production

gene	Genbank ID	enzyme	function
CYP11A	NM_000781	desmolase (20,22 desmolase)/CYP11A (P450scc)	cholesterol side-chain cleavage
CYP11B1	NM_000497	steroid 11β hydroxylase/CYP11B1 (P450c11B1, P450c11β)	making cortisol from 11-deoxycortisol
CYP11B2	NM_000498	aldosterone synthetase/ CYP11B2 (P450c11B2, P450 18, P450c11aldo, P450c11AS)	11- and 18-hydroxylation, 18-oxidation
CYP17	M14564	steroid 17α-hydroxylase and/or 17,20 lyase (17−20 Desmolase); (P450c17)	17-hydroxylation, scission of the C-17,20 carbon bond
CYP19	NM_000103	CYP19 (p450c19, P450arom)/aromatase	conversion of androgens to estrogens
CYP21	NM_000500	CYP21 (P450c21)/ steroid 21-hydroxylase	21-hydroxylation
3βHSD1	NM_000862	hydroxy-delta-5-steroid dehydrogenase/ 3 beta-and steroid delta-isomerase 1	3β-hydroxysteroid dehydrogenation and isomerization
3βHSD2	NM_000198	hydroxy-delta-5-steroid dehydrogenase/ 3 beta-and steroid delta-isomerase 2	3β-hydroxysteroid dehydrogenation and isomerization
17βHSD	NM_000413	17β hydroxysteroid dehydrogenase (17 ketoreductase)	NAD(H)- and/or NADP(H)-dependent enzymes that catalyze the oxidation and reduction of 17,-hydroxy- and 17,-ketosteroids, respectively.
HMGR	NM_000859	hydroxymethylglutaryl CoA reductase	4-electron reduction of HMG CoA into CoA and mevalonate-step leading to the cholesterol synthesis
StAR	NM_000349	steroidogenic acute regulatory protein	mediates cholesterol transport to the inner mitochondrial membrane (to CYP11A)

of microarray analysis and other techniques that evaluate gene expression changes on a genome-wide scale, Q-RT-PCR offers a more specific, sensitive, comprehensive, and reliable method for the investigation of multiple functionally related genes. The dye method based on SYBR Green and molecular beacons are two different detection chemistries for real time PCR. SYBR Green is a nonspecific dye that binds double-stranded DNA and thus can be used to detect and quantify PCR products in solution in real-time. Molecular beacons form a stem-loop structure when free in solution with a fluor and a quencher attached to opposite ends of the DNA oligonucleotide. When unbound in solution the proximity of the fluor and quencher results in fluorescence resonance energy transfer (FRET) which quenches the fluoresence. When a molecular beacon hybridizes to its target DNA, the fluor and quencher are separated, and the fluorescent dye emits light upon irradiation (*13*).

In a previous study we demonstrated the use of SYBR Green-based Q-RT-PCR to measure expression of 10 steroidogenic enzymes altered by exposure to chemicals in H295R cells (*12*). In this study we report the development of molecular beacon-based Q-RT-PCR, a more specific and reproducible method, to quantify the chemical-induced gene expression of steroidogenic enzymes, in both semiquantitative and quantitative manners. We also report the measurement of expression for two additional enzymes, 3βHSD1 (3β-hydroxysteroid dehydrogenase 1) and CYP11B1 (steroid 11β hydroxylase), in H295R cells. Utilizing the developed Q-RT-PCR procedure and selected model chemicals, we further evaluated the use of the H295R human adrenocortical tumor cell line for examining the effects of environmental chemicals on steroidogenesis.

Materials and Methods

Chemicals. The chemicals used in this study were chosen on the basis of their variety of known effects on steroid metabolism as previously described (*12*). Briefly, aminoglutethimide is an aromatase inhibitor; lovastatin is metabolized to produce a specific hydroxymethylglutaryl-CoA reductase (HMGR) inhibitor; 8BrcAMP and forskolin increase cellular cAMP concentrations; phorbol-12-myristate-13-acetate (PMA) is a diacylglycerol analogue that activates protein kinase C; ketoconazole works principally by the inhibition of cytochrome P450 14a-demethylase (*P45014DM*); and daidzein is a weak estrogen receptor agonist. Forskolin, 8Br-cAMP, PMA, lovastatin, ketoconazole, DL-aminoglutethimide, and spironolactone were obtained from Sigma (St. Louis, MO); daidzein was from MP Biomedicals Inc. (Aurora, OH).

Cell Culture and Chemical Exposures. H295R cells (ATCC, Beltsville, MD) were cultured in Dulbecco's modified Eagle's medium supplemented with Ham's nutrient mixture F-12 (Sigma) with 1 mL/100 mL ITS+ Premix (BD Bioscience, San Jose, CA) and 2.5% BD Nu-Serum (BD Bioscience) at 37 °C in a 5% CO_2 atmosphere. Cells were exposed to chemicals dissolved in dimethyl sulfoxide (DMSO; Sigma) for 48−72 h after plating. To minimize the potential effect of hormones in the serum during compound exposures, Nu-serum was replaced with 2.5% charcoal dextran-treated FBS (HyClone Laboratories, Inc. Logan, UT) immediately before dosing.

Cell Viability Assay. To assess cytotoxicity, H295R cells were seeded to 96-well ViewPlates (Packard Instruments) at a concentration of 1×10^5 cells/mL in 250 μL of medium per well. After attachment, compounds were added and cell viability was examined by the LIVE/DEAD Viability/Cytotoxicity Assay Kit (Molecular Probes, Eugene, OR) after 48 h. Fluorescence was subsequently measured using a plate-reading fluorescence measurement system (Cytofluor 2300/2350, Millipore, Bedford, MA).

RNA Isolation and First-Strand cDNA Synthesis. The schematic diagram of the experimental procedures is shown in Figure 1A. H295R cells were grown in 6-well plates at 37 °C until 70% confluence; based on cell viability assays, 4 or 5 different concentrations of test chemicals were dissolved in fresh hormone-free medium and cells were cultured for a further 48 h. At the end of chemical exposure, RNA was isolated from cells harvested from each single well using the SV Total RNA Isolation System (Promega, Madison, WI) following manufacturer's specifications. Three RNA replicates for each treatment were isolated from three different wells. In this study, three RT-PCR measurements for each treatment were made for one of the replicate RNA samples. Purified RNA was stored at −80 °C until analysis. First-strand cDNA synthesis was performed using N-MLV RT (H-) reverse transcriptase (Promega). A 1-μg aliquot of cellular total RNA (purified by the SV total RNA kit) or RNA standard was combined with 1.25 μL of 10 μM Oligo$(dT)_{17}$ and RNase-free water to a final volume of 14 μL. Mixes were denatured at 70 °C for 5 min and then quickly cooled on ice for 5 min. Reverse transcription was performed in 1× first-stand synthesis buffer, 0.5 mM dNTP mix (Amersham Pharmacia Biotech, Piscataway, NJ), 2 U/μL RNaseOUT (Invitrogen,

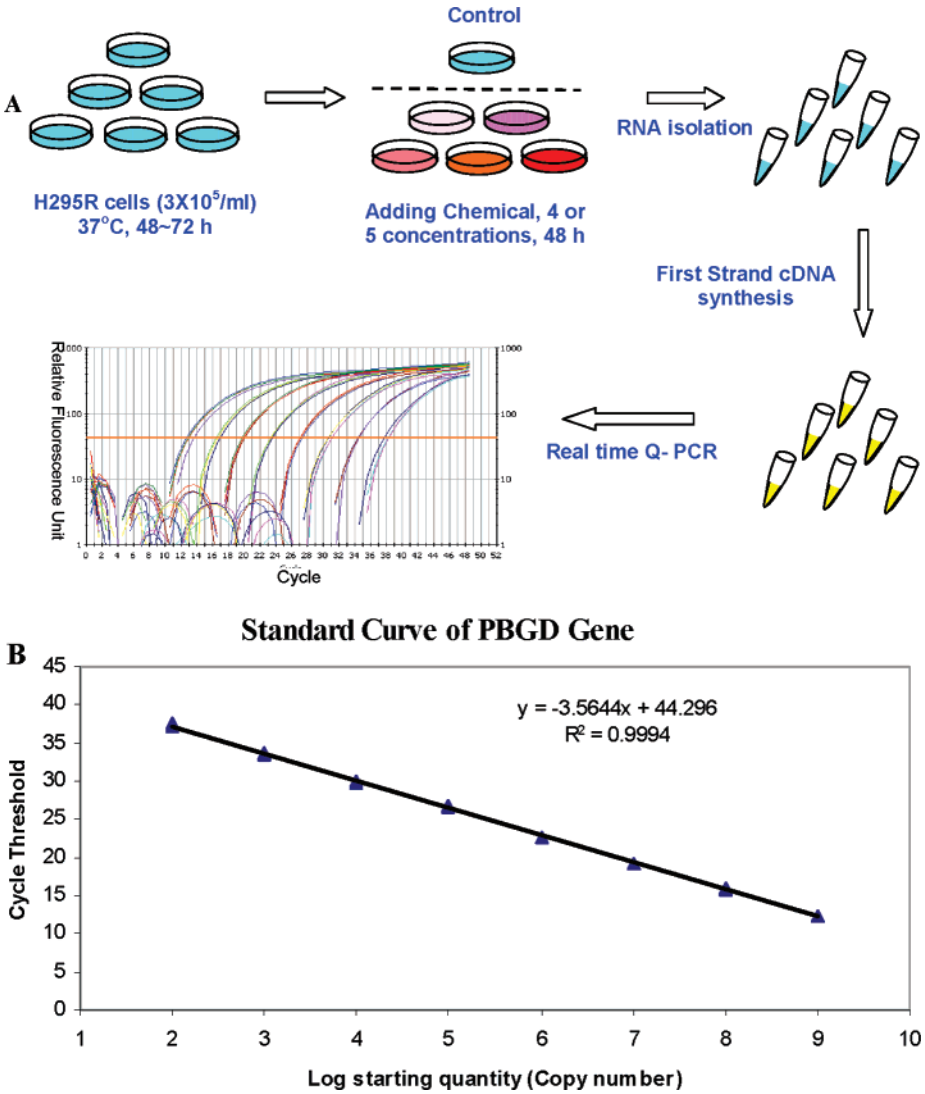

FIGURE 1. (A) Schematic diagram of experimental procedures. H295R cells were grown in 37 °C until 70% confluence; 4 or 5 different concentrations of tested chemicals were dissolved in fresh hormone-free medium and cells were cultured for a further 48 h. RNA were isolated at the end of the exposure. First-strand cDNA were synthesized for each RNA sample. The mRNA expression was measured based on the C_t (cycle threshold) value using a standard curve method and was calculated as fold change compared to control. (B) PBGD standard curve showing a linear increase of C_t for 8 dilutions of RNA standard from 1×10^2 to 1×10^8 copies/reaction, with a correlation coefficient of 0.9994 and a PCR efficiency of 91%.

Carlsbad, CA), 12.5 U/μL N-MLV RT (H-) reverse transcriptase, and RNase-free H_2O to a final volume of 25 μL. Reactions were incubated at 42 °C for 50 min and, on completion, were inactivated at 70 °C for 15 min. To digest RNA, 1.25 μL RNase H (Invitrogen) was added before incubation at 37 °C for 30 min. First-strand cDNAs were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's protocol and finally eluted with 40 μL of sterile water.

Synthesis of RNA Standards. For quantification of gene expression and optimization of PCR conditions, RNA standards representing the gene amplicon sequences were synthesized by in vitro transcription. RT-PCR amplicons were first cloned into the pGEMT vector (Promega) to create RNA polymerase promoter sites on cDNAs. Sequence authenticity of the cloned amplicons was verified by automatic DNA sequencing and followed by a BLAST2 (National Center for Biotechnology Information (NCBI), Bethesda, MD) analysis with their corresponding sequences in Genbank. To produce linear DNA templates for in vitro transcription, PCR amplification was performed using a pair of vector-specific primers (M13-F and M13-R) which flank the T7 and SP6 RNA polymerase promoter sites, respectively. To purify the DNA templates, phenol-chloroform extraction and the Wizard SV Gel and PCR Clean-Up System (Promega) were used, respectively, to remove potential RNase contamination and unincorporated nucleotides and primers present in the PCR products. In vitro transcription was performed using 400 ng of purified DNA template with an appropriate RNA polymerase (Invitrogen) to produce sense RNA. Residual DNA was removed from RNA samples by incubation with RNase-free DNase (Promega) followed by extraction with TRIZOL reagent (Invitrogen). RNA quality was assessed by formaldehyde gel-electrophoresis, and RNA concentration was accurately determined using the RiboGreen RNA Quantification Kit (Molecular Probes, Eugene, OR) and converted to copy number/μL according to the following formula:

$$\text{No. of molecules} = (\text{RNA concentrated in ng}/\mu\text{L} \times 2.941 \div \text{RNA size in base}) \times 6.023 \times 10^{11}$$

For PCR optimization when required a dilution series of the RNA standards (1×10^2 to 1×10^8 copies/reaction) was freshly prepared in nuclease-free water.

Design of PCR Primers and Molecular Beacons. Primers and molecular beacons (see Table 1 in Supporting Information) were designed based on Genbank gene sequences (Table 1) using the Beacon Designer 2 program (PREMIER Biosoft Intl., Palo Alto, CA) and the mFold 3.0 (2002) program (available at http://www.idtdna.com/biotools /mfold/mfold.asp). All primers were tested for specificity with BLASTn (NCBI) against the human genome. They were excluded if there was any potential for false-priming which could cause unwanted PCR amplification. Primer specificity was further verified by the production of a single distinct peak in melting-curve analysis (performed after a SYBR Green-based real-time PCR) and also by DNA sequencing of the PCR amplicons produced.

The molecular beacons were labeled with 6-FAM (or HEX for the reference gene prophobilinogen deaminase, *PBGD*), the reporter, at the 5′-end and with DABCYL, the quencher, at the 3′-end. Each molecular beacon possessed a 7 nucleotide based paired arm at each end of the probe and a 21−25 nucleotide probe sequence. The arm sequences were designed to form a stable hairpin stem at the annealing temperature of the PCR, ensuring that nonhybridized beacons do not fluoresce when free in solution. The probe sequences were designed to hybridize to a target strand and form probe-target hybrids that are longer and more stable than the hairpin stem, causing the separation of the reporter and quencher and emission of fluorescence. Before using the molecular beacons in subsequent Q-RT-PCR assays, melting curve analysis was carried out to determine the optimal hybridization temperature for the probe−target duplexes.

Real-Time PCR Assays. Real-time Q-RT-PCR was performed by using an iCycler system (Bio-Rad Laboratories, Hercules, CA) in sterile 96-well PCR plates (Bio-Rad). PCR reaction mixtures contained 1× PCR buffer II (Applied Biosystems, Foster City, CA), 200 μM dNTP mix (Promega), 5.5 mM $MgCl_2$ (Applied Biosystems), 200 nM molecular beacon, 0.05 U/μL DNA polymerase AmpliTaq Gold (Applied Biosystems), and 200 nM or 400 nM sense/anti-sense gene-specific primers. Genes used with a primer concentration of 200 nM were *PBGD*, *StAR*, *CYP19*, *3βHSD1*, *CYP11B1*, and *17βHSD*; genes used with a primer concentration of 400 nM were *CYP17*, *CYP11A*, *HMGR*, *CYP21*, *CYP11B2*, and *3βHSD2*. A final reaction volume of 20 μL was made up with 10 μL of diluted cDNA and nuclease-free distilled water (Invitrogen). The PCR reaction mix was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile was (1) denaturation for 15 s at 95 °C; (2) annealing for 30 s at 60 °C (all genes, except *17βHSD* at 58 °C); and (3) extension for 30 s at 72 °C. A total of 50 PCR cycles were used. PCR efficiency, uniformity, and linear dynamic range of each Q-RT-PCR assay were assessed by the construction of standard curves using RNA standards.

Statistical Analysis. The quantification of target gene expression was based on both an absolute standard curve method and a comparative cycle threshold (C_t) method (see Calculation of Gene Expression Levels, Supporting Information). Statistical analyses of gene expression profiles were conducted using SYSTAT 10 (SPSS Inc, Chicago, IL). Differences in gene expression were evaluated by ANOVA followed by Tukey's test. Differences with $p < 0.05$ were considered to be significant.

Results

Linearity of PCR Assays. To measure gene expression in absolute quantities (i.e., target mRNA copy number/μg total RNA), a 10-fold dilution series of cDNA prepared from a known copy number of RNA standards, was used for standard curve construction. The cDNA dilution series represented the range of "input" mRNA copy numbers from 1×10^2 to 1×10^8. To maximize the sensitivity, uniformity and linearity of the PCR assays, optimization of the procedures of cDNA preparation (e.g., post-RT RNase digestion and column purification), and PCR components/conditions (e.g., primer, $MgCl_2$ and *Taq* pol. concentrations and PCR annealing temperature) was carried out. The resulting standard curves (generated by plotting the C_t values against the logarithm of the initial copy numbers) exhibited high correlation coefficients (0.999−1.000) over the 7-order dilution range (Figure 1B). In addition, PCR efficiencies were calculated to be ≥90% in all assays (see Table 2 in Supporting Information).

Gene Expression Profile of Resting Cells. Using the standard curves above, copy numbers of the steroidogenic gene transcripts in the control and solvent- and chemical (inducer)-treated cells were quantified (Table 2). In the control (unexposed) cells, expression of the different genes was highly heterogeneous, with copy numbers per μg of RNA as low as 2.02×10^4 for *CYP11B1* and as high as 2.76×10^8 for *CYP11A*, a difference in expression levels of 4 orders of magnitude. The 11 steroidogenic genes could be grouped into three categories according to their transcript abundance: (1) highly expressed genes (1×10^7 to 1×10^9 copies/μg RNA) *CYP11A*, *StAR*, *HMGR*, *CYP17*, and *CYP21*; (2) moderately expressed genes (1×10^5 to 1×10^6 copies/μg RNA) *17βHSD1*, *3βHSD2*, *CYP19*, and *3βHSD1*; and (3) rarely expressed genes (1×10^4 to 1×10^5 copies/μg RNA) *CYP11B1* and *CYP11B2*.

Standard Curve Method vs Comparative C_t Method. Sample-to-sample variations in the amount of RNA input, the efficiencies of cDNA synthesis and purification, and the presence of PCR inhibitors can lead to either over- or under-estimation of gene transcript copy number. To normalize these intrinsic variations, expression of the reference gene, *PBGD*, was used as an internal reference. For each steriodogenic gene, the expression ratio (ER) for the blank, solvent control, 300 μM 8Br-cAMP- and 30 μM forskolin-treated samples were computed by dividing the steriodogenic gene copy number by the reference gene copy number. Fold-changes, representing differential expression, of a target gene upon solvent or chemical exposure (with reference to the control group) were calculated using formula 3 (see Supporting Information) (Table 2). These fold-change values were used for pairwise comparison with those generated by the comparative C_t method.

For the comparative C_t method, E (a factor of PCR amplification efficiencies) and C_t of the target and reference gene transcripts were substituted into formula 4 and formula 6 (see Supporting Information) to compute the fold changes representing differential gene expression (Table 3). The comparative C_t data set and the data set obtained using the standard curve method (Table 3) were highly comparable, indicating the high compatibility of these two calculation methods (Figure 2). Since the standard curve method is more tedious than the comparative C_t method in terms of experimental manipulation, we selected the latter method as the quantification algorithm for the remainder of these studies.

Gene Expression Profile of Inducer-Treated Cells. To examine how the expression pattern of the 11 steroidogenic genes was altered by chemical "inducers" of steroidogenesis, H295R cells were exposed to 4 or 5 different concentrations of PMA, forskolin, 8Br-cAMP, lovastatin, and spironolactone, over time periods up to 48 h. PMA, at 1 nM concentration, did not cause significant differential gene expression except suppression of *CYP17* (0.61-fold) (see Table 3 in Supporting Information). At 10 nM PMA, *CYP17* was further suppressed to 0.11-fold, while *CYP19* and *CYP21* were significantly induced by 1.90- and 4.32-fold, respectively. When H295R cells were exposed to 100 nM PMA, *CYP17* was suppressed greatly to 0.02-fold compared to control. In contrast, this concentration caused 5.13-, 8.60-, 2.90-, 25.9-, and 12.8-fold

TABLE 2. Gene Expression Data Computed by the Standard Curve Method[a,b,c]

	Treatment							
	Copy number/μg RNA				Fold change			
gene	control	solvent	8Br-cAMP (300 μM)	forskolin (30 μM)	control	solvent	8Br-cAMP (300 μM)	forskolin (30 μM)
PBGD	3.06E+05 (3.06E+04)	1.83E+05 (1.63E+04) *	3.76E+05 (1.85E+04) *	4.50E+05 (2.41E+04) *				
CYP11A	2.76E+08 (3.65E+07)	2.37E+08 (1.46E+07)	9.20E+08 (5.43E+07) *	1.03E+09 (7.44E+07) *	1.00 (0.13)	1.43 (0.09)*	2.72 (0.16)*	2.54 (0.18)*
CYP17	1.51E+07 (6.43E+05)	1.25E+07 (1.07E+06)	5.38E+07 (6.22E+06)*	2.71E+07 (3.28E+06) *	1.00 (0.04)	1.39 (0.12)	2.91 (0.34)*	1.23 (0.15)
CYP19	1.29E+05 (2.16E+04)	8.35E+04 (9.97E+03)	4.14E+06 (2.54E+05) *	5.13E+06 (8.83E+05) *	1.00 (0.17)	1.08 (0.13)	26.1 (1.60)*	27.0 (4.65)*
CYP21	1.00E+07 (5.19E+05)	7.08E+06 (7.06E+05)	1.63E+08 (1.81E+07) *	1.97E+08 (9.89E+06) *	1.00 (0.05)	1.18 (0.12)	13.2 (1.47)*	13.4 (0.67)*
StAR	2.11E+08 (2.16E+07)	1.55E+08 (8.25E+06)	7.77E+08 (7.40E+07) *	9.52E+08 (7.20E+07) *	1.00 (0.10)	1.23 (0.07)	3.00 (0.29)*	3.08 (0.23)*
HMGR	3.33E+07 (4.64E+06)	3.47E+07 (4.48E+06)	6.23E+07 (4.23E+06) *	5.01E+07 (4.42E+06) *	1.00 (0.14)	1.74 (0.22)*	1.52 (0.10)*	1.02 (0.09)
17βHSD1	1.47E+06 (9.30E+04)	1.20E+06 (3.18E+04)	1.34E+06 (2.22E+05)	1.27E+06 (1.27E+05)	1.00 (0.06)	1.36 (0.04)*	0.75 (0.12)*	0.59 (0.06)*
3βHSD1	1.06E+05 (2.23E+04)	8.57E+04 (2.16E+04)	2.00E+05 (5.95E+04) *	2.03E+05 (1.82E+04) *	1.00 (0.21)	1.36 (0.34)	1.55 (0.46)	1.31 (0.12)
3βHSD2	1.88E+05 (3.11E+04)	1.70E+05 (4.30E+04)	3.83E+07 (2.82E+06) *	3.34E+07 (3.45E+06) *	1.00 (0.17)	1.51 (0.38)	166 (12.18)*	121 (12.5)*
CYP11B1	2.01E+04 (1.76E+03)	2.91E+04 (4.23E+03)	2.34E+05 (4.00E+04) *	3.66E+05 (7.81E+04) *	1.00 (0.09)	2.42 (0.35)	9.50 (1.62)*	12.4 (2.65)*
CYP11B2	2.02E+04 (3.48E+03)	1.38E+04 (2.52E+03)	6.53E+05 (7.87E+04) *	9.51E+05 (1.82E+05) *	1.00 (0.17)	1.15 (0.21)	26.4 (3.18)*	32.1 (6.15)*

[a] Cell exposures were for 48 h. [b] Gene activity is expressed as means and standard deviations. [c] Asterisk (*) indicates statistically different from control ($p < 0.05$), $n = 3$.

TABLE 3. Gene Expression Data Computed by the Comparative C_t Method[a,b,c]

	Treatment			
gene	control	solvent	8Br-cAMP (300 μM)	forskolin (30 μM)
CYP11A	1.00 (0.21)	1.42 (0.06) *	2.68 (0.04) *	2.51 (0.20) *
CYP17	1.00 (0.13)	1.39 (0.23)	2.89 (0.28)*	1.21 (0.08)
CYP19	1.00 (0.24)	1.06 (0.04)	25.8 (1.67) *	26.5 (3.13) *
CYP21	1.00 (0.14)	1.17 (0.02)	13.1 (1.00) *	13.3 (0.07) *
StAR	1.00 (0.18)	1.23 (0.15)	2.97 (0.16) *	3.05 (0.24) *
HMGR	1.00 (0.18)	1.73 (0.19) *	1.51 (0.03) *	1.02 (0.13)
17βHSD1	1.00 (0.16)	1.35 (0.09) *	0.74 (0.09) *	0.58 (0.07) *
3βHSD1	1.00 (0.24)	1.33 (0.21)	1.53 (0.46)	1.30 (0.17)
3βHSD2	1.00 (0.24)	1.48 (0.27)	164 (3.99) *	119 (5.79) *
CYP11B1	1.00 (0.12)	2.43 (0.52)	9.41 (1.14) *	12.4 (3.08) *
CYP11B2	1.00 (0.15)	1.14 (0.10)	26.3 (1.87) *	32.3 (7.52) *

[a] Cell exposures were for 48 h. [b] Gene activity is expressed as means and standard deviations. [c] Asterisk (*) indicates statistically different from control ($p < 0.05$), $n = 3$.

induction of *CYP19*, *CYP21*, *3βHSD1*, *3βHSD2*, and *CYP11B2*, respectively. The expression profile of the cells treated with 1 μM PMA was similar to that of cells treated with 100 nM. At the highest concentration (10 μM), expression of *StAR*, *CYP19*, *CYP21*, *3βHSD1*, *CYP11B2*, and *CYP11B1* was further increased by 2.91-, 4.59-, 13.9-, 4.26-, 61.02-, and 7.11-fold compared to control.

Forskolin resulted in concentration-dependent induction of most steroidogenic genes except *17βHSD1* (see Table 4 in Supporting Information), after 48 h of exposure. Three concentrations (1, 3, and 10 μM) induced *CYP17*, *StAR*, *CYP11A*, *CYP21*, *HMGR*, and *3βHSD1* by 2−13.4-fold, *CYP19*, *CYP11B2*, and *CYP11B1* by 15−78-fold, and *3βHSD2* by 124−168-fold. Expression levels of *CYP11B1* and *CYP11B2* were remarkably elevated when forskolin concentrations increased from 1 to 10 μM. Expression of *CYP17*, *CYP19*, *CYP21*, *3βHSD1*, and *3βHSD2* was also moderately increased in this concentration-dependent manner.

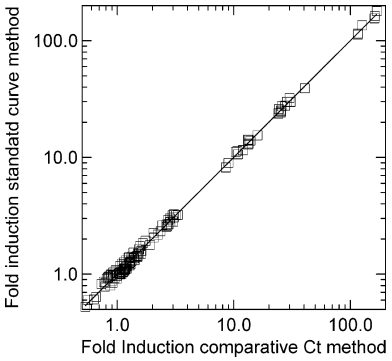

FIGURE 2. Correlation between the gene expression derived from the standard curve method and the comparative C_t method.

8Br-cAMP led to statistically significant increases in expression mostly when greater concentrations (100 μM) were used (see Table 5 in Supporting Information). *CYP17*, *StAR*, *CYP11A*, *CYP21*, and *3βHSD1* were moderately induced (1.5−3.6-fold), while *CYP19*, *CYP11B2*, and *CYP11B1* were induced by 6.8−8.6-fold. Of all the steroidogenic genes, *3βHSD2* was up-regulated at the greatest extent (33.6-fold). However, expression of *17βHSD1* and *HMGR* were not significantly altered by 8Br-cAMP at any of the concentrations tested.

Lovastatin caused statistically significant increases in gene expression mostly at 10 and 30 μM (see Table 6 in Supporting Information). Greater than 2-fold induction was only exhibited by *CYP11B2* and *CYP11B1* at 10 μM and *CYP17*, *StAR*, *CYP11B2*, and *CYP11B1* at 30 μM. Concentration-dependent induction (>2-fold) was only exhibited by *CYP11B2* and *CYP11B1*.

Gene Expression Profile of Inhibitor-Treated Cells. The effects of some chemical inhibitors (spironolactone, DL-

TABLE 4. Fold Differences in Gene Expression for H295R Cells Exposed to Model Chemicals[a]

Chemical	CYP17	StAR	CYP11A	CYP19	CYP21	HMGR	17βHSD1	3βHSD1	3βHSD2	CYP11B2	CYP11B1
Inducers											
8-Br-cAMP		↑		↑↑	↑↑↑			↑	↑↑↑	↑↑↑	↑↑↑
PMA	↓↓↓	↑		↑↑	↑↑↑		↓	↑	↑↑↑	↑↑↑	↑↑
forskolin	↑	↑	↑	↑↑	↑↑↑	↑		↑↑	↑↑↑	↑↑↑	↑↑↑
lovastatin	↑	↑						↓		↑↑	↑
Inhibitors											
spironolactone				↑							↑
DL-aminoglutethimide					↓		↑	↑	↓		↑↑↑ Λ
daidzein		↓			↑		↑ Λ	↑ Λ	↑ Λ	↑ Λ	↑↑↑ Λ
ketoconazole			↑ Λ		↑ Λ		↑ Λ	↑ Λ		↑	↑
spironolactone				↑							↑
DL-aminoglutethimide					↓		↑	↑	↓		↑↑↑ Λ
daidzein		↓			↑		↑ Λ	↑ Λ	↑ Λ	↑ Λ	↑↑↑ Λ
ketoconazole			↑ Λ		↑ Λ		↑ Λ	↑ Λ		↑	↑

[a] Symbols indicate fold difference relative to control; ↑=2-fold or more; ↑↑=5-fold or more; ↑↑↑=10-fold or more. Λ= response recovered at highest concentration. All other differences are less than 2-fold.

aminoglutethimide, daidzein, and ketoconazole) on gene expression patterns were investigated after 48 h of exposure. Spironolactone resulted in significant inhibition of *CYP17*, *HMGR*, *17βHSD1*, and *3βHSD1* gene expression but no gene was inhibited more than 2-fold (see Table 7 in Supporting Information). Concentration-dependent gene suppression was not clearly demonstrated. Spironolactone induced *StAR*, *CYP19*, *CYP11B2*, and *CYP11B1* at some concentrations, but only *CYP19* and *CYP11B1* expression had more than 2-fold increase at 30 μM.

Treatment with DL-aminoglutethimide resulted in a complicated gene expression profile. At 1 μM, *CYP21*, *HMGR*, *3βHSD1*, and *3βHSD2* were significantly suppressed to a mild extent (>2-fold) (see Table 8 in Supporting Information). When the concentration was elevated to 100 μM, all these genes (except *3βHSD1*) were further inhibited by about 2-fold. In addition, significant inhibition was also exhibited by *CYP19* and *CYP11B2* (>2-fold). Increased expression (>2-fold) was only significantly shown by *17βHSD1*, *3βHSD1*, and *CYP11B1* at 10 μM.

Daidzein significantly suppressed *StAR* expression at all tested concentrations (see Table 9 in Supporting Information) and about a 5-fold decrease was observed at the highest concentration (100 μM). Interestingly, mRNA levels of *CYP21*, *17βHSD1*, *3βHSD1*, *3βHSD2*, *CYP11B2*, and *CYP11B1* were significantly increased (over 2-fold) at 10 μM daidzein.

Ketoconazole significantly reduced expression of *CYP17* and *17βHSD1* only at 0.3 and 10 μM, respectively (see Table 10 in Supporting Information). More than 2-fold gene induction was demonstrated by *CYP11A*, *17βHSD1*, *3βHSD1*, and *CYP11B1* at 3 μM and by *CYP11B2* at 10 μM ketoconazole.

The overall effects of chemical inducers and inhibitors on the expression profile of the 11 steroidogenic genes are summarized in Table 4.

Comparison Between the SYBR Green- and the Molecular Beacon-Based Q-RT-PCR. The SE_{MNE}% derived from the SYBR Green- and the molecular beacon-based Q-RT-PCR were computed using formula 5 (see Supporting Information) (Table 5). This evaluates the standard error of the quantitative measurements of expression of both the target and reference genes. For the 9 common target genes (*CYP17*, *StAR*, *CYP11A*, *CYP19*, *CYP21*, *HMGR*, *17βHSD1*, *3βHSD2*, and *CYP11B2*) studied in both assays, the molecular beacon-based method resulted in a smaller mean SE_{MNE}% than the SYBR Green method. This indicates that the molecular beacon-based method provides a more precise procedure than the SYBR Green method, for measuring expression of the steroidogenic genes in H295R cells. However, *CYP11B1* and *3βHSD1* were only studied using the molecular beacon-based method, and they had the two largest average SE_{MNE}% (18.40 and 24.13%, respectively); this might be due to the relatively low expression levels of these two genes in H295R cells.

TABLE 5. SE_{MNE} of the SYBR Green and Molecular Beacon Q-RT-PCR

	SYBR Green Q-RT-PCR [a]		Molecular beacon Q-RT-PCR [b]	
	SE_{MNE} % (n = 41)[e]		SE_{MNE} % (n = 48)[e]	
gene	mean	SD	mean	SD
CYP17	16.87%	8.85%	10.45%	5.50%
StAR	17.83%	9.25%	12.75%	6.34%
CYP11A	19.97%	12.85%	12.80%	6.83%
CYP19	19.64%	10.11%	13.75%	8.24%
CYP21	18.54%	9.53%	11.97%	5.94%
HMGR	20.18%	11.00%	10.24%	5.13%
17βHSD1	22.17%	16.47%	12.24%	6.18%
3βHSD2	22.01%	9.56%	13.63%	6.33%
CYP11B2	18.49%	8.86%	14.62%	9.21%
17βHSD4[c]	19.08%	14.35%		
3βHSD1[d]			18.40%	8.98%
CYP11B1[d]			24.13%	17.82%

[a] β-Actin was chosen as the reference gene in the previous reported SYBR Green Q-RT-PCR. [b] *PBGD* was chosen as the reference gene in the molecular beacon Q-RT-PCR. [c] *17βHSD4* was studied only in SYBR Green Q-RT-PCR. [d] *3βHSD1* and *CYP11B1* were studied only in Molecular beacon Q-RT-PCR. [e] SE_{MNE} was calculated using Formula 5 (see Supporting Information).

To assess the "portability" (i.e., the ability for the method to be established and results repeated in different laboratories) of the cell culture bioassay procedure, results obtained in the current study were compared with those previously reported (*12*). Due to the differences in exposure concentration and duration, only the data from a 48-h exposure of 10 μM forskolin could be directly compared (Figure 3). In general, agreement between these two data sets is good, particularly for genes induced by ≤10-fold (*17βHSD1, CYP11A, CYP17, HMGR*, and *StAR*). A significant discrepancy between the measurements is revealed only in the 3βHSD2 expression (about a 100-fold difference). However, this gene has also been demonstrated to show a high degree of time dependence in gene expression (Gracia et al., unpublished data) and so any small deviation in exposures times could result in relatively great differences in gene expression.

Discussion

The H295R cell line has been shown to have potential as an in vitro model for screening for the adverse effects of

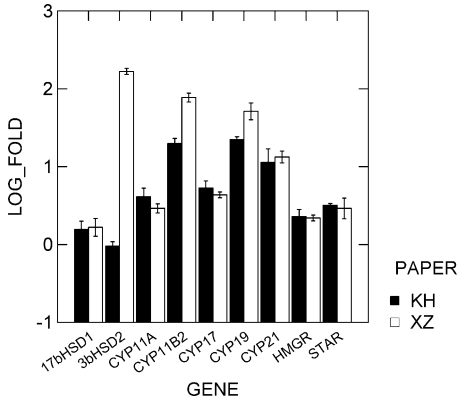

FIGURE 3. Comparison of gene expression profiles obtained by the SYBR Green method (*12*) and molecular beacon method used in the current study. H295R cells were treated with 10 uM forskolin for 48 h ($n = 3$). Solid bar = SYBR Green method; open bar = molecular beacon method.

chemicals on steroidogenesis (*11, 12, 14*). The present study provides reliable methods for accurate and precise quantification of expression of 11 key steroidogenic enzymes in this cell line. Compared to the SYBR Green-based assays reported previously (*12*), the present molecular beacon-based method resulted in expression data with smaller SE values. This indicates that the molecular beacon-based method offers better precision than the SYBR Green-based method for measuring steroidogenic gene expression. The superiority of molecular beacons over SYBR Green can be attributed to the difference in the mechanisms for detecting the PCR amplicons. Since SYBR Green is a nonspecific dye that binds to all double-stranded DNA, an amplification growth curve can represent signals from a specific product as well as nonspecific products such as primer−dimers or amplicons resulting from false-priming. In contrast, molecular beacons fluoresce brightly only when they are bound to amplicons containing their specific target sequences. This distinguishing feature of the molecular beacons offers a higher degree of specificity with better signal-to-noise ratios in amplicon detection compared to SYBR Green. Despite the difference in the precision of the two methods and differences in experimental conditions between previous work (*12*) and the present study, the comparability of expression patterns obtained demonstrates the reproducibility and flexibility of the Q-RT-PCR approach in measuring steroidogenic gene expression. This indicates that the cell culture assays developed so far are sufficiently "portable" to produce highly equivalent results even when different amplicon detection systems (SYBR Green vs molecular beacon), reference genes used in data normalization (β-actin vs PBGD), and instrumentation (Cepheid Smart Cycler vs Bio-Rad iCycler) are used.

The previous study examined the time-dependent expression profiles for model chemicals which are able to modulate steroid metabolism (*12*). Using 48 h as the exposure period, the current work focused on examining the concentration-dependent effects of the chemicals on steroidogenic gene expression. Concentration-dependent changes in gene expression were observed in cells treated with PMA, forskolin, 8Br-cAMP, and lovastatin. Interestingly, in some circumstances, the greatest increases in gene expression occurred not at the highest concentration levels, but at the middle of the concentration range as seen in the cells treated with daidzein, ketoconazole, and DL-aminoglutethimide. However, in the cases of gene suppression, the greatest effect often occurred at the highest concentrations tested. Since all chemical exposures were conducted at noncytotoxic concentrations, the inhibition in gene expression was unlikely a consequence of cytotoxic or other indirect effects. Thus, it would be worthwhile in further studies to use a wider concentration range to better understand the effects of the tested chemicals on the transcriptional regulation of steroidogenic genes.

3β-hydroxysteroid dehydrogenase/$\Delta^{4,5}$-isomerase (3βHSD) is crucial to the biosynthesis of all steroid hormones, including aldosterone, cortisol, and testosterone. *3βHSD1* and *3βHSD2* are two genes located on chromosome 1p13.1, which encode two different 3βHSD isoenzymes (*15*). The structures of *3βHSD1* and *3βHSD2* are highly homologous in DNA sequence and in the length of introns between the coding sequences (*16*). In humans, *3βHSD2* is expressed primarily in the adrenals and gonads, while *3βHSD1* is expressed mainly in the extra-adrenal/extra-gonadal tissues (*15, 17, 18*). In vitro kinetic studies have suggested that the enzyme efficiency of 3βHSD1 toward the steroid substrates is several times greater than 3βHSD2 in converting Δ5 steroids (*17, 19*). There is currently no evidence with regard to the activity of 3βHSD1 in human adrenals (*20*). Data from the present study demonstrate that both genes are expressed in H295R cells, but at different levels. The results also demonstrate that *3βHSD1* expression in H295R cells can be induced by forskolin, 8Br-cAMP, and PMA in the same manner as *3βHSD2*, although to a lesser extent than *3βHSD2*. However, treatment with DL-aminoglutethimide increased expression of *3βHSD1* but down-regulated *3βHSD2*, indicating that despite the similarities in gene sequence and enzymatic function between 3βHSD1 and 3βHSD2, their expression is regulated differently.

CYP11B1 is a mitochondrial cytochrome enzyme which catalyzes the final step in the biosynthesis of cortisol. It shares 90% nucleotide sequence identity in the introns and 95% in the exons with *CYP11B2* and lies in close proximity to *CYP11B2* on chromosome 8q (*21, 22*). However, expression of these two genes is localized in different zonal regions of the adrenal cortex. *CYP11B1* is expressed in the zona fasciculata/reticularis, while *CYP11B2* is found solely in the zona glomerulosa and catalyzes the final step in biosynthesis of aldosterone. It is known that the transcription of both genes is regulated by a cAMP-dependent mechanism. In H295R cells, *CYP11B1* and *CYP11B2* are modulated similarly by forskolin and 8Br-cAMP. However, DL-aminoglutethimide elicited over a 30-fold increase on expression of *CYP11B1*, but not *CYP11B2*. Additionally, daidzein enhances the transcriptional activation of *CYP11B1* up to 10-fold, while *CYP11B2* is only slightly up-regulated, about 2-fold, at the same concentration. In part, this could be explained by the finding that *CYP11B1* and *CYP11B2* are regulated by different pathways. *CYP11B2* is regulated by ACTH/cAMP, angiotensin II, and potassium via protein kinase C and two elements: a steroidogenic factor 1 (SF-1) binding site and a cAMP response element (CRE) (*23, 24*). Alternatively, *CYP11B1* expression is activated by ACTH via cAMP and protein kinase A and dependent on a cAMP response binding protein (CREBP) family member, ATF-2, in addition to SF-1 (*25*).

Ketoconazole is known to inhibit the activities of several CYP enzymes by binding to the heme moiety of the enzyme, thus preventing activation of molecular oxygen (*26*). This chemical has also been reported to inhibit *CYP21*, *CYP17*, *CYP11B1*, and *CYP11A* enzyme activities in in vitro tests using H295R cells (*27*). However, expression of none of these CYP enzymes was inhibited by ketoconazole at the transcription level in our studies. In fact, the mRNA levels of *CYP21*, *CYP11B1*, and *CYP11A* in our study were actually increased by exposure of H295R cells to ketoconazole. These results would clearly indicate a compensatory mechanism where by the cell generates additional enzyme to compensate for the chemical inhibition. Therefore this classic enzyme inhibitor actually appears to be an "inducer" in this assay

system. This might at first appear to be a disadvantage of the assay system. We would suggest that it is in fact an advantage since cells which have compensated for inhibition by induction of gene expression may show no actual alteration in enzyme activity or hormone production. However, the gene expression profile clearly indicates an effect attributable to these chemicals. DL-Aminoglutethimide has also been shown to inhibit the in vitro activities of *CYP11A*, *CYP21*, and *CYP11B1* to various extents in both H295R and other cell lines (*27, 28*). Again, the results of our study demonstrate that the expression of these enzymes was not down-regulated at the mRNA level by DL-aminoglutethimide except for *CYP21* where its expression was suppressed over 2-fold at the highest concentration tested (100 μM). Furthermore, H295R cells treated with 10 μM DL-aminoglutethimide exhibited a 10-fold increase in *CYP11B1* expression. Daidzein, an estrogenic isoflavone, has been shown to suppress dibutyryl cAMP-stimulated cortisol production by H295R cells and to strongly and significantly inhibit *3βHSD2* and *CYP21* activities at concentrations of 1−25 μM (*14*). It has also been reported that the reduction in cortisol production caused by daidzein is not mediated through the estrogen receptor. In addition, while daidzein has been shown to decrease cortisol synthesis by suppressing *CYP21* enzymatic activity in cultured human adrenocortical cells, transcription of the CYP21 gene was unaffected (*29*). Our results demonstrate that the mRNA levels of both *CYP21* and *3βHSD2* were enhanced more than 2-fold by 10 μM daidzein. Taken together these observations support the hypothesis that those compounds called "steroidogenic enzyme inhibitors" based on an inhibitory effect on selected enzyme activities, do not inhibit expression of the enzyme at the mRNA level. In addition, these results also indicate that inhibition of enzyme activities may elicit a compensatory up-regulation of gene expression. Finally, because many of the in vitro assay systems used to evaluate steroidogenesis use ATCH/cAMP stimulated cells the importance and relevance of such inhibitory effects need further evaluation in an in vivo system.

In this study, mRNA levels of the 11 steroidogenic enzymes varied greatly in response to the selected model chemicals. The enzymes whose mRNA levels were altered to the greatest degree by the various chemical treatments were CYP11B1, CYP11B2, and 3βHSD2. In general, concentrations of HMGR, CYP11A1, and StAR mRNAs were not markedly altered. We hypothesize that these differences in the magnitude of gene expression alterations are related to the basal mRNA abundance of these enzymes. For those enzymes with higher basal transcription levels, such as *HMGR*, *CYP11A*, and *StAR*, mild adjustment in transcriptional regulation could result in a substantial change in mRNA level, and this alteration might in turn affect the level of translated protein and consequently the biological activity. Alternatively, it might be suggested that the genes are in effect steroidogenesis "housekeeping genes": genes whose activity is generally always in excess. As a result, these genes may not represent critical control points once the steroidogenic pathway has been supplied with substrate. This issue needs further study, and, in particular, the identification of critical control points in the steroidogenic pathways within the H295R cell system. These data can then be used to interpret the results from the H295R system as well as serve as the basis for comparison to in vivo models.

Although the transcriptional regulation of steroidogenic genes is multi-factorial, chemicals that had the same mechanism of action elicited similar gene expression patterns. For the eight chemicals tested in this study, similar expression patterns were observed for forskolin and 8Br-cAMP which are known to stimulate cAMP regulated pathways (*11, 12*). It follows that changes in patterns of relative expression may be useful in classifying chemicals with unknown mechanisms of action.

The Q-RT-PCR method was developed to examine alterations in gene expression of 11 key steroidogenic genes induced by different chemicals in H295R cells. Gene expression was quantitatively measured utilizing molecular beacon methods. The chemical-induced gene expression patterns observed were similar to those previously determined using a SYBR Green method (*12*). Together, these studies demonstrate the robust nature of the bioassessment technique since a range of techniques can be used to observe the endpoints of interest.

In summary, this study and previous work (*12*) have demonstrated that the H295R bioassay procedure provides a flexible and robust technique for the assessment of effects of chemical contaminants on the expression of steroidogenic enzymes. The RT-PCR techniques used provide the most cost-effective means of determining effects on a wide range of different enzymes, and a variety of different RT-PCR techniques provide equivalent results in the assay procedure.

Acknowledgments

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Supporting Information Available

Method description of calculation of gene expression levels, table of linear regression parameters for Q-RT-PCR standard curves, and tables of expression of steroidogenic genes in H295R cells exposed to PMA, forskolin, 8Br-cAMP, lovastatin, spironolactone, DL-aminoglutethimide, daidzein, and ketoconazole (pdf). This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information

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Calculation of Gene Expression Levels

The quantification of target gene expression was based on both an absolute standard curve method and a comparative cycle threshold (C_t) method. In the absolute standard curve method, a standard curve for each transcript was generated from a dilution series of synthesized RNA standards and linear regression was used to analyze the data (Equation 1):

$$Y = aX + b \qquad (1)$$

where: Y=mean C_t value; a=slope of the standard curve; X=log (copy number); b=y-intercept.

The mean C_t value for each gene was based on triplicate Q-RT-PCR measurements. The copy numbers of the target genes and the reference gene in the isolated total RNA were calculated from the mean value of C_t (Y), the y-intercept (b), and the slope of the standard curve (X) of the corresponding PCR run, according to the above Equation. To compensate for variations of RNA input and reverse transcription efficiency, the copy numbers of target genes were normalized to that of the internal reference gene (*PBGD*). *PBGD*, compared to other commonly used reference genes (e.g. β-actin), is not subject to interferences caused by processed pseudogenes/DNA (1, 2, 3, 4, 5, 6). Studies have also demonstrated that *PBGD* mRNA is consistently expressed in some complex tissues as well as in several specific cell types making it well suited as an internal standard for Q-RT-PCR (5, 6).

Expression ratio (ER) of mRNA copy numbers between target genes and the reference gene in the same sample was further calculated using Formula 2,

ER = mRNA copy no. of target gene/mRNA copy no. of reference gene (2)

Fold change of the target gene expression in a chemical-exposed culture was then derived using Formula 3:

$$\text{Fold change} = ER_{exp}/ER_{con} \quad (3)$$

where ER_{exp} and ER_{con} are the expression ratio of the gene of interest in the chemical-exposed and control cultures, respectively.

In the comparative C_t method, the normalization algorithm used the differences in the PCR amplification efficiencies for the target and reference genes. The mean normalized expression (MNE) was calculated based on the mean C_t of the reference and target genes using Formula 4 (7):

$$MNE = \frac{(E_{reference})^{C_{t\ reference,\ Mean}}}{(E_{target})^{C_{t\ target,\ Mean}}} \quad (4)$$

where $E = 10^{-1/X} - 1$ and %PCR efficiency = $(E-1)\times 100$.

Standard errors (SE) were calculated as a measure for the precision of the assay by applying the differential equation of Gauss for error propagation. SE_{MNE} was calculated based on the SE for the C_t of the reference and target genes using Formula 5 (7).

$$SE_{MNE} = MNE \cdot ((\ln (E_{target}) \cdot SE_{Ct\ target\ mean})^2 + (\ln (E_{reference}) \cdot SE_{Ct\ reference\ mean})^2)^{0.5} \quad (5)$$

The expression of the target gene in a chemical-exposed culture was expressed as the "fold change" from the control culture using Formula 6:

$$\text{Fold change} = MNE_{exp}/MNE_{con} \quad (6)$$

where MNE_{exp} and MNE_{con} are the mean normalized expression of the gene of interest in the chemical-exposed and control cultures, respectively.

Table 1. Sequences of molecular beacons and primers*

Gene	Sense primer	Anti-sense primer
3βHSD1	5′-AGCATCTTCTGTTTCCTGGTG-3′ (85)	5′-TCTCCTTCAGCTCCTTCTCCTT-3′ (225)
3βHSD2	5′-AGCATCTTCTGTTTCCTGGCA-3′ (96)	5′-TCTCCTTCAGTTCCTTCTCTTC-3′ (237)
17βHSD1	5′-TTCATGGAGAAGGTGTTGG-3′ (1545)	5′-AAGACTTGCTTGCTGTGG-3′ (1648)
CYP11A	5′-GAGATGGCACGCAACCTGAAG-3′ (1065)	5′-CTTAGTGTCTCCTTGATGCTGGC-3′ (1201)
CYP11B1	5′-GGTTTGCCAGGCTAAGC-3′ (1679)	5′-CAAACTGCCCAGAGGACAG-3′ (1791)
CYP11B2	5′-TCCAGGTGTGTTCAGTAGTTCC-3′ (2705)	5′-GAAGCCATCTCTGAGGTCTGTG-3′ (2850)
CYP17	5′-GGCACCAAGACTACAGTGATTG-3′ (246)	5′-AGAGTCAGCGAAGGCGATAC-3′ (392)
CYP19	5′-TTGGAAATGCTGAACCCGATAC-3′ (148)	5′-GCCAGTGAGGAGCAGGAC-3′ (240)
CYP21	5′-ACCTCAGTTTCTCCTTTATTGC-3′ (1663)	5′-AGAGCCAGGGTCCTTCAC-3′ (1756)
HMGR	5′-TTCAGGTTCCAATGGCAACAAC-3′ (1702)	5′-GCCACGAGTCATCCCATCTG-3′ (1823)
StAR	5′-ATGAGTAAAGTGGTCCCAGATG-3′ (451)	5′-ACCTTGATCTCCTTGACATTGG-3′ (593)
PBGD	5′-CTGGAGGAGTCTGGAGTCTAG-3′ (866)	5′-TGGAATGTTACGAGCAGTGATG-3′ (1002)
Gene	Molecular beacons	
3βHSD1 & *3βHSD2*	5′ 6-FAM d(CGCGATCCCTCCTGCTCCTGTCACAAGGCGATCGCG) DABCYL 3′ (169 for *3BHSD1*; 181 for *3BHSD1*)	
17βHSD1	5′ 6-FAM d (CGCGATCGACATCCACACCTTCCACCGCTGATCGCG) DABCYL 3′ (1593)	
CYP11A	5′ 6-FAM d (CGCGATCTGCCGCGCAGCCAAGACCTCTGATCGCG) DABCYL 3′ (1126)	
CYP11B1	5′ 6-FAM d (CGCGATCCTGACCTTGTCCCCAGCCCCAGATCGCG) DABCYL 3′ (1729)	
CYP11B2	5′ 6-FAM d (CGCGATCCCGCAGGTTGCTTTCCCACCCTGATCGCG) DABCYL 3′ (2760)	
CYP17	5′ 6-FAM d (CGCGATCAAGGGCAAGGACTTCTCTGGGCGGATCGCG) DABCYL 3′ (306)	
CYP19	5′ 6-FAM d (CGCGATCTCGTGCCTGAAGCCATGCCTGGATCGCG) DABCYL 3′ (188)	
CYP21	5′ 6-FAM d (CGCGATCAGGAAGCCTTCTCTGCCAGCGAGATCGCG) DABCYL 3′ (1724)	
HMGR	5′ 6-FAM d (CGCGATCGCTGGCACCTCCACCAAGACCTAGATCGCG) DABCYL 3′ (1790)	
StAR	5′ 6-FAM d (CGCGATCCGGCTGGAGGTCGTGGTGGACGATCGCG) DABCYL 3′ (487)	
PBGD	5′ HEX d (CGCGATCACAAGAGACCATGCAGGCTACCATCGATCGCG) DABCYL 3′ (903)	

* Numbers in bracket are the nucleotide positions of the primers/beacons in the Genbank gene sequences with the accession numbers shown in Table 1. The Genbank accession number of *PBGD* is X04217. Primers and beacons were synthesized through custom service from Invitrogen Inc. (Carlsbad, CL) and Biosearch Technologies Inc.(Novato,CA), respectively.

Table 2. Linear regression parameters for Q-RT-PCR standard curves

Gene	Slope	Intercept	Correlation Coefficient	PCR efficiency	
				E[a]	(%)[b]
CYP11A	-3.363	48.48	1.000	1.983	98.3%
CYP17	-3.504	44.37	1.000	1.929	92.9%
CYP19	-3.397	42.15	1.000	1.970	97%
CYP21	-3.425	47.29	1.000	1.959	95.9%
StAR	-3.524	48.14	0.999	1.922	92.2%
HMGR	-3.472	45.13	1.000	1.941	94.1%
17βHSD1	-3.581	45.16	1.000	1.902	90.2%
3βHSD1	-3.500	44.49	0.999	1.931	93.1%
3βHSD2	-3.387	41.38	1.000	1.973	97.3%
CYP11B1	-3.575	47.44	0.999	1.904	90.4%
CYP11B2	-3.370	43.41	1.000	1.980	98%
PBGD	-3.552	44.28	1.000	1.912	91.2%

[a] $E = 10^{(-1/\text{slope})}$

[b] % efficiency = $(E-1)\times 100\%$

Table 3. Expression of steroidogenic genes in H295R cells exposed to PMA [ab]

Gene	Treatment					
	Control[c]	1 nM	10 nM	100 nM	1 μM	10 μM
CYP17	1.00 ± 0.10	0.61 ± 0.05*	0.11 ± 0.01*	0.02 ± 0.00*	0.05 ± 0.01*	0.07 ± 0.00*
StAR	1.00 ± 0.00	1.05 ± 0.21	1.43 ± 0.32	1.94 ± 0.25	2.21 ± 0.40	2.91 ± 0.92*
CYP11A	1.00 ± 0.13	1.32 ± 0.21	1.37 ± 0.05*	0.60 ± 0.02*	0.83 ± 0.19	1.03 ± 0.08
CYP19	1.00 ± 0.04	1.54 ± 0.10	1.90 ± 0.00*	5.13 ± 0.33*	3.74 ± 0.24*	4.59 ± 0.60*
CYP21	1.00 ± 0.07	1.85 ± 0.33	4.32 ± 0.57*	8.60 ± 0.85*	7.31 ± 0.28*	13.9 ± 0.52*
HMGR	1.00 ± 0.14	0.97 ± 0.07	0.92 ± 0.14	0.75 ± 0.03*	0.97 ± 0.07	0.74 ± 0.27
17βHSD1	1.00 ± 0.26	1.09 ± 0.22	0.66 ± 0.07	0.78 ± 0.16	0.43 ± 0.06*	0.81 ± 0.09
3βHSD1	1.00 ± 0.04	0.78 ± 0.03	0.87 ± 0.09	2.90 ± 0.11*	2.34 ± 0.43*	4.26 ± 0.29*
3βHSD2	1.00 ± 0.00	1.03 ± 0.04	3.72 ± 0.52	25.9 ± 1.94*	25.5 ± 10.3*	23.5 ± 0.89*
CYP11B2	1.00 ± 0.04	0.77 ± 0.00	4.28 ± 0.47	12.8 ± 0.99*	16.6 ± 2.76*	61.0 ± 6.17*
CYP11B1	1.00 ± 0.86	2.27 ± 1.74	0.73 ± 0.59	3.51 ± 1.54	4.16 ± 2.23	7.11 ± 2.85*

[a] Cell exposures were for 48 h.

[b] Relative gene activity is expressed as means and standard deviations.

[c] Blank control.

* Statistically different from control (p<0.05), n=3.

Table 4. Expression of steroidogenic genes in H295R cells exposed to forskolin [ab]				
Gene	Treatment			
	Control[c]	1 μM	3 μM	10 μM
CYP17	1.00 ± 0.13	3.35 ± 0.26*	3.65 ± 0.24*	4.35 ± 0.29*
StAR	1.00 ± 0.17	2.98 ± 0.11*	3.26 ± 0.43*	2.96 ± 0.67*
CYP11A	1.00 ± 0.10	2.45 ± 0.25*	2.38 ± 0.09*	2.92 ± 0.29*
CYP19	1.00 ± 0.10	38.1 ± 2.80*	45.1 ± 2.94*	52.0 ± 9.22*
CYP21	1.00 ± 0.10	8.63 ± 0.57*	10.3 ± 0.39*	13.4 ± 1.76*
HMGR	1.00 ± 0.14	2.24 ± 0.15*	1.96 ± 0.00*	2.19 ± 0.14*
17βHSD1	1.00 ± 0.18	1.40 ± 0.24	1.64 ± 0.30	1.68 ± 0.34
3βHSD1	1.00 ± 0.00	4.68 ± 0.68*	4.62 ± 0.66*	6.53 ± 0.62*
3βHSD2	1.00 ± 0.08	124 ± 14.7*	144. ± 23.1*	168 ± 11.2*
CYP11B2	1.00 ± 0.20	19.7 ± 1.45*	46.9 ± 3.61*	77.6 ± 7.61*
CYP11B1	1.00 ± 0.37	15.4 ± 3.58*	27.9 ± 2.76*	48.2 ± 1.85*

[a] Cell exposures were for 48 h.
[b] Relative gene activity is expressed as means and standard deviations.
[c] Blank control.
* Statistically different from control (p<0.05), n=3.

Table 5. Expression of steroidogenic genes in H295R cells exposed to 8Br-cAMP [ab]

Gene	Treatment			
	Control[c]	10 μM	30 μM	100 μM
CYP17	1.00 ± 0.13	1.16 ± 0.00	1.35 ± 0.05*	1.59 ± 0.10*
StAR	1.00 ± 0.17	2.24 ± 0.64	1.95 ± 0.15	2.69 ± 0.38*
CYP11A	1.00 ± 0.10	1.71 ± 0.17*	1.59 ± 0.16*	1.51 ± 0.00*
CYP19	1.00 ± 0.10	1.22 ± 0.13	1.79 ± 0.12	8.55 ± 2.75*
CYP21	1.00 ± 0.10	0.79 ± 0.09	1.14 ± 0.16	3.61 ± 0.37*
HMGR	1.00 ± 0.14	1.34 ± 0.31	1.26 ± 0.12	1.45 ± 0.09
17βHSD1	1.00 ± 0.18	1.17 ± 0.24	1.16 ± 0.17	0.79 ± 0.09
3βHSD1	1.00 ± 0.00	1.25 ± 0.05	1.56 ± 0.51	2.13 ± 0.42*
3βHSD2	1.00 ± 0.08	0.75 ± 0.03	1.49 ± 0.10	33.6 ± 2.23*
CYP11B2	1.00 ± 0.20	1.14 ± 0.37	1.24 ± 0.22	6.81 ± 0.95*
CYP11B1	1.00 ± 0.37	2.30 ± 0.45	1.34 ± 0.81	7.02 ± 1.93*

[a] Cell exposures were for 48 h.
[b] Relative gene activity is expressed as means and standard deviations.
[c] Blank control.
* Statistically different from control (p<0.05), n=3.

Table 6. Expression of steroidogenic genes in H295R cells exposed to lovastatin [ab]

Gene	Treatment				
	Control[c]	1μM	3 μM	10 μM	30 μM
CYP17	1.00 ± 0.07	0.89 ± 0.10	1.16 ± 0.21	1.57 ± 0.10*	2.02 ± 0.00*
StAR	1.00 ± 0.04	0.99 ± 0.07	1.40 ± 0.09	1.75 ± 0.07*	2.02 ± 0.08*
CYP11A	1.00 ± 0.08	0.83 ± 0.03	1.20 ± 0.05	0.85 ± 0.35	1.07 ± 0.08
CYP19	1.00 ± 0.04	1.05 ± 0.07	0.83 ± 0.06	0.80 ± 0.09*	0.45 ± 0.06*
CYP21	1.00 ± 0.07	0.95 ± 0.07	1.53 ± 0.10*	1.23 ± 0.10	0.98 ± 0.07
HMGR	1.00 ± 0.04	1.56 ± 0.10*	1.89 ± 0.46*	1.75 ± 0.11*	1.33 ± 0.00
17βHSD1	1.00 ± 0.10	1.01 ± 0.04	0.77 ± 0.06*	0.84 ± 0.10	0.63 ± 0.02*
3βHSD1	1.00 ± 0.12	0.85 ± 0.03	0.82 ± 0.09	1.47 ± 0.10*	1.67 ± 0.00*
3βHSD2	1.00 ± 0.07	1.40 ± 0.29	1.50 ± 0.40	1.01 ± 0.08	0.90 ± 0.09
CYP11B2	1.00 ± 0.07	0.81 ± 0.08	1.28 ± 0.00	4.45 ± 0.29*	7.92 ± 0.52*
CYP11B1	1.00 ± 0.23	1.04 ± 0.04	0.69 ± 0.12	2.44 ± 0.09*	2.90 ± 0.46*

[a] Cell exposures were for 48 h.
[b] Relative gene activity is expressed as means and standard deviations.
[c] Solvent control.
* Statistically different from control ($p<0.05$), n=3.

Table 7. Expression of steroidogenic genes in H295R cells exposed to spironolactone [ab]

Gene	Treatment					
	Control[c]	0.3μM	1μM	3 μM	10 μM	30 μM
CYP17	1.00 ± 0.10	0.97± 0.04	1.13 ± 0.07	1.30 ± 0.21*	0.63 ± 0.02*	0.51 ± 0.06*
StAR	1.00 ± 0.04	1.70 ± 0.17*	0.91 ± 0.03	1.04 ± 0.04	1.17 ± 0.09	0.96 ± 0.28
CYP11A	1.00 ± 0.08	1.20 ± 0.16	0.93 ± 0.06	1.14 ± 0.12	0.96 ± 0.14	0.78 ± 0.14
CYP19	1.00 ± 0.10	1.10 ± 0.07	0.83 ± 0.08	0.88 ± 0.14	0.96 ± 0.13	2.36 ± 0.17*
CYP21	1.00 ± 0.28	1.46 ± 0.06	0.87 ± 0.23	0.53 ± 0.19	0.96 ± 0.07	1.58 ± 0.51
HMGR	1.00 ± 0.16	0.76 ± 0.06	0.84 ± 0.18	0.85 ± 0.06	0.61 ± 0.08*	0.71 ± 0.05
17βHSD1	1.00 ± 0.04	0.57 ± 0.04*	0.88 ± 0.13	0.80 ± 0.03*	0.69 ± 0.03*	1.03 ± 0.08
3βHSD1	1.00 ± 0.14	0.50 ± 0.04*	0.62 ± 0.06*	0.78 ± 0.08*	0.57 ± 0.02*	0.56 ± 0.04*
3βHSD2	1.00 ± 0.10	0.83 ± 0.11	1.18 ± 0.28	0.99 ± 0.28	0.80 ± 0.21	1.47 ± 0.10
CYP11B2	1.00 ± 0.13	1.06 ± 0.04	1.05 ± 0.04	1.18 ± 0.04	1.14 ± 0.07	1.50 ± 0.20*
CYP11B1	1.00 ± 0.17	1.13 ± 0.15	1.17 ± 0.08	1.50 ± 0.00*	1.23 ± 0.00	2.03 ± 0.27*

[a] Cell exposures were for 48 h.
[b] Relative gene activity is expressed as means and standard deviations.
[c] Solvent control.
* Statistically different from control ($p<0.05$), n=3.

Table 8. Expression of steroidogenic genes in H295R cells exposed to DL-Aminoglutethimide [ab]

Gene	Treatment					
	Control[c]	1 μM	3 μM	10 μM	30 μM	100 μM
CYP17	1.00 ± 0.10	1.08 ± 0.04	1.44 ± 0.11*	0.97 ± 0.10	1.31 ± 0.05*	1.01 ± 0.15
StAR	1.00 ± 0.26	1.11 ± 0.18	1.11 ± 0.08	1.13 ± 0.15	0.90 ± 0.20	1.30 ± 0.29
CYP11A	1.00 ± 0.04	0.91 ± 0.11	0.87 ± 0.06	1.30 ± 0.13	1.22 ± 0.20	0.90 ± 0.29
CYP19	1.00 ± 0.16	0.74 ± 0.03	0.99 ± 0.04	0.77 ± 0.24	0.90 ± 0.09	0.55 ± 0.02*
CYP21	1.00 ± 0.17	0.63 ± 0.04*	0.70 ± 0.23	1.30 ± 0.05	0.94 ± 0.06	0.47 ± 0.06*
HMGR	1.00 ± 0.00	0.78 ± 0.09*	0.95 ± 0.04	0.67 ± 0.00*	0.74 ± 0.05*	0.57 ± 0.04*
17βHSD1	1.00 ± 0.10	0.89 ± 0.15	1.31 ± 0.22	2.53 ± 0.66*	1.53 ± 0.31	1.22 ± 0.20
3βHSD1	1.00 ± 0.08	0.57 ± 0.08*	0.71 ± 0.07	2.06 ± 0.25*	0.91 ± 0.07	0.68 ± 0.05
3βHSD2	1.00 ± 0.04	0.72 ± 0.03*	0.94 ± 0.17	0.67 ± 0.07*	0.78 ± 0.08	0.41 ± 0.03*
CYP11B2	1.00 ± 0.08	0.88 ± 0.03	1.12 ± 0.16	1.70 ± 0.11*	0.94 ± 0.07	0.68 ± 0.05*
CYP11B1	1.00 ± 0.45	1.66 ± 0.30	2.94 ± 0.30	30.0± 7.44*	2.16 ± 0.63	0.76 ± 0.60

[a] Cell exposures were for 48 h.
[b] Relative gene activity is expressed as means and standard deviations.
[c] Blank control.
* Statistically different from control ($p<0.05$), n=3.

Table 9. Expression of steroidogenic genes in H295R cells exposed to daidzein [ab]

Gene	Treatment					
	Control[c]	1μM	3 μM	10 μM	30 μM	100 μM
CYP17	1.00 ± 0.10	0.83 ± 0.05	1.24 ± 0.08*	1.38 ± 0.05*	1.36 ± 0.05*	0.99 ± 0.04
StAR	1.00 ± 0.10	0.56 ± 0.02*	0.31 ± 0.03*	0.52 ± 0.03*	0.33 ± 0.02*	0.21 ± 0.02*
CYP11A	1.00 ± 0.08	0.71 ± 0.00*	0.55 ± 0.06*	1.18 ± 0.12	0.64 ± 0.09*	0.63 ± 0.02*
CYP19	1.00 ± 0.04	1.25 ± 0.10*	1.92 ± 0.00*	1.82 ± 0.00*	1.60 ± 0.00*	1.29 ± 0.10*
CYP21	1.00 ± 0.04	0.97 ± 0.04	0.75 ± 0.03	2.87 ± 0.22*	1.17 ± 0.08	0.91 ± 0.07
HMGR	1.00 ± 0.07	0.93 ± 0.13	1.45 ± 0.06*	1.39 ± 0.05*	1.52 ± 0.00*	1.41 ± 0.18*
17βHSD1	1.00 ± 0.27	1.20 ± 0.09	1.77 ± 0.24	4.40 ± 1.18*	1.04 ± 0.19	0.97 ± 0.19
3βHSD1	1.00 ± 0.36	1.76 ± 0.07*	2.07 ± 0.17*	4.90 ± 0.37*	1.97 ± 0.08*	1.59 ± 0.26
3βHSD2	1.00 ± 0.11	1.09 ± 0.07	2.14 ± 0.00*	2.07 ± 0.21*	1.53 ± 0.06*	0.82 ± 0.03
CYP11B2	1.00 ± 0.10	1.63 ± 0.27*	1.72 ± 0.11*	2.41 ± 0.16*	1.96 ± 0.28*	0.81 ± 0.14
CYP11B1	1.00 ± 0.49	0.92 ± 0.46	1.26 ± 0.20	10.7 ±2.27*	0.98 ± 0.29	0.56 ± 0.44

[a] Cell exposures were for 48 h.
[b] Relative gene activity is expressed as means and standard deviations.
[c] Blank control.
* Statistically different from control (p<0.05), n=3.

Table 10. Expression of steroidogenic genes in H295R cells exposed to ketoconazole [ab]					
	Treatment				
Gene	Control[c]	0.3 μM	1 μM	3 μM	10 μM
CYP17	1.00 ± 0.13	0.76 ± 0.08*	1.06 ± 0.04	1.12 ± 0.07	1.31 ± 0.09*
StAR	1.00 ± 0.17	1.45 ± 0.30	1.39 ± 0.25	1.12 ± 0.16	1.12 ± 0.04
CYP11A	1.00 ± 0.10	1.30 ± 0.32	2.00± 0.22*	2.13 ± 0.08*	1.38 ± 0.18
CYP19	1.00 ± 0.10	1.03 ± 0.18	1.62 ± 0.21*	1.59 ± 0.12*	1.49 ± 0.24
CYP21	1.00 ± 0.10	0.97 ± 0.06	1.81 ± 0.07*	2.52 ± 0.19*	1.59 ± 0.10*
HMGR	1.00 ± 0.14	0.93 ± 0.07	1.41 ± 0.14*	1.33 ± 0.09*	0.97 ± 0.04
17βHSD1	1.00 ± 0.18	1.07 ± 0.20	2.31 ± 0.15*	2.77 ± 0.21*	0.52 ± 0.04*
3βHSD1	1.00 ± 0.00	0.96 ± 0.17	2.18 ± 0.45*	2.27 ± 0.67*	1.23 ± 0.25
3βHSD2	1.00 ± 0.08	0.70 ± 0.09	1.00 ± 0.00	1.03 ± 0.07	1.02 ± 0.29
CYP11B2	1.00 ± 0.20	1.05 ± 0.08	1.62 ± 0.31	1.16 ± 0.08	3.49 ± 0.35*
CYP11B1	1.00 ± 0.37	2.37 ± 0.79	2.63 ± 0.44	4.16 ± 1.24*	3.03 ± 0.67

[a] Cell exposures were for 48 h.
[b] Relative gene activity is expressed as means and standard deviations.
[c] Blank control.
* Statistically different from control ($p<0.05$), n=3.

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