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CHARACTERIZATION OF THE ANDROGEN-SENSITIVE MDA-KB2 CELL LINE FOR ASSESSING COMPLEX ENVIRONMENTAL MIXTURES

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Abstract—Synthetic and natural steroidal androgens and estrogens and many other non-steroidal endocrine-active compounds commonly occur as complex mixtures in aquatic environments. It is important to understand the potential interactive effects of these mixtures to properly assess their risk. Estrogen receptor agonists exhibit additivity in mixtures when tested in vivo and in vitro. Little is known, however, concerning possible mixture interactions of androgen receptor agonists. In these studies we used the MDA-kb2 cell line, a human breast cancer cell line with endogenous androgen receptors and a stably transfected luciferase reporter gene construct to quantify the androgenic activity of seven natural and synthetic androgens: 17β-trenbolone, dihydrotestosterone, methyltestosterone, testosterone, trendione, 17α-trenbolone, and androstenedione. We tested combinations of these androgens and compared the observed activity to expected androgenic activity based on a concentration addition model. Our analyses support the hypothesis that androgen receptor agonists cause additive responses in a mixture. Binary mixtures of 17β-trenbolone with 17β-estradiol or triclocarban (an antimicrobial found in the environment) were also tested. 17β-Estradiol induced androgenic activity, but only at concentrations 600-fold greater than those found in the environment. Triclocarban enhanced the activity of 17β-trenbolone. Additionally, three anti-androgens were each paired with three androgens of varying potencies. The relative potencies of the antagonists were a vinclozolin metabolite (M2) > procymidone > prochloraz regardless of the androgen used. The results of our studies demonstrate the potential utility of the androgen-responsive MDA-kb2 cell line for quantifying the activity of mixtures of endocrine-active chemicals in complex wastes such as municipal effluents and feedlot discharges. Environ. Toxicol. Chem. 2010;29:1367–1376. © 2010 SETAC

Keywords—Endocrine-active chemicals Concentration addition Androgen receptor (ant)agonist Triclocarban Estrogen

INTRODUCTION

Environmental contaminants that affect endocrine function through interactions with the hypothalamic-pituitary-gonadal axis have the potential to impact key aspects of reproduction and development in fish and wildlife [1,2]. Endocrine-disrupting chemicals (EDCs) have been associated with a variety of products (e.g., pharmaceuticals, pesticide formulations, plastics, cleaning supplies) and sources, and can act through many different mechanisms of action. Of particular concern are chemicals that bind to estrogen or androgen receptors, in part because these receptors are highly conserved across species [3–5]. For example, effluent from wastewater treatment plants (WWTPs) can contain low concentrations of the potent synthetic steroidal estrogen ethynylestradiol (EE2), which is used as a human contraceptive [2]. When released to the aquatic environment, even at low concentrations, EE2 binds to and activates fish estrogen receptors, feminizing male fish [2,6], and can affect fish populations [7]. Not only can male fish be feminized by environmental estrogens, but female fish can be masculinized by androgenic compounds found, for example, in discharges from pulp and paper mill plants or concentrated animal feeding operations [8], leading to possible population-level impacts [9,10].

Typical point sources of EDCs to aquatic environments such as WWTPs, pulp and paper mills, and farms invariably release complex mixtures of chemicals, several of which could affect the hypothalamic-pituitary-gonadal axis. For example, WWTPs release not only estrogens such as EE2, 17β-estradiol (E2), alkylphenols, phytoestrogens [11], but other endocrine-active compounds such as antimicrobials [12], steroidal androgens [13], and certain pesticides [14]. Pulp and paper mill discharges have been reported to have androgenic and/or estrogenic properties, likely associated with complex mixtures of plant sterols, sterol metabolites, and various additives used in processing the wood pulp [15–17]. Depending on the type of feedlot, endocrine-active wastes from concentrated animal feeding operations could include natural and synthetic steroidal androgens such as 17β- and 17α-trenbolone, trendione, testosterone and its metabolites, estrogens such as E2 and its metabolites, as well as certain types of endocrine-active pesticides such as antifungals registered for use as drugs in livestock [8,18,19]. Hence, most real-world exposures to EDCs are complex mixtures comprised of materials with similar as well as dissimilar endocrine mechanisms of action.

Combinations of in vivo and in vitro assays and instrumental analytical chemistry techniques can be used successfully to help assess risks associated with complex mixtures of EDCs where

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any single approach may not suffice. For example, although instrumental analyses are needed to measure concentrations of discrete chemicals, complex mixtures can be difficult to assess using analytical chemistry due to difficulty in detecting low concentrations of potentially potent chemicals and/or the presence of unsuspected EDCs. Similarly, although in vivo assays are critical for integrating mixtures and discerning adverse effects, it is often difficult to use in vivo tests to determine specific mechanisms of action of chemicals that might be present. To help address shortcomings of instrumental analyses and/or in vivo tests in dealing with mixtures, targeted in vitro assays have been developed to sensitively detect chemicals with specific endocrine mechanisms of action. An in vitro system of particular utility in this regard is the MDA-kb2 cell line. These cells were developed from the parent cell line MDA-MB-453 of human breast cancer cells, which naturally express high levels of the androgen receptor [20]. The cells were stably transfected with an androgen-responsive luciferase reporter gene, which enables quantification of concentration/potency of androgen receptor agonists based on intensity of luminescence. Due to its ability to integrate androgenic activity of samples, this cell line is potentially well suited for analysis of complex mixtures.

To support use of the MDA-kb2 cell line for assessing androgenicity of complex environmental mixtures, responses of the cells should be characterized using defined mixtures reflective of what might occur in the environment. For example, natural and synthetic androgens can simultaneously be present in concentrated animal feeding operations (CAFO) discharges, and we hypothesize they should act additively based on previous mixture studies of EDCs with other mechanisms of action such as estrogens [21]. These studies have shown mixtures of chemicals with the same mechanism of action to be additive when evaluated using the Loewe additivity model of concentration addition [21]. This model is based on the assumption that the components of the mixture act in a similar manner (for the MDA-kb2 cells binding to and activating the androgen receptor) and that replacing one or more of the chemicals totally, or in part, with equivalent concentrations the other mixture components will produce the same overall effect [22,23]. To examine whether androgens would cause additive effects in the MDA-kb2 cells, the first objective of our present study was to evaluate responses of the cell line to a mixture of seven different steroidal androgens that either have been detected or could occur in environmental samples.

In characterizing the utility of the MDA-kb2 cells for assessing environmental mixtures, it is also important to understand how mixtures of EDCs with different mechanisms of action might affect assay results. For example, estrogens such as EE2 or E2 can occur in mixtures with steroidal androgens [13,24]. Steroidal estrogens could bind to the androgen receptor in MDA-kb2 cells, thereby potentially interfering with response of the cells to androgens [3,20]. Another compound commonly present in U.S. waterways is the antimicrobial triclocarban [12,25]. Recent studies have shown that triclocarban can enhance the activity of androgens (although not through binding to the androgen receptor) both in vitro and in vivo [26,27]; hence, we were interested in ascertaining whether the MDA-kb2 cells could detect this enhancement. To address how EDCs with dissimilar mechanisms of action might affect interpretation of MDA-kb2 data, our study objectives included characterization of responsiveness of the cell line to a model environmental androgen, (17β-trenbolone) in binary mixtures with E2 or triclocarban.

A diverse group of environmental contaminants can act as androgen receptor antagonists, inhibiting the activity of androgens through competitive or noncompetitive binding to different sites on the androgen receptor [8]. The final objective of this study was to characterize the effects of several known antiandrogens on the responsiveness of MDA-kb2 cells to androgens. Specifically, we evaluated three androgen receptor antagonists, selected based on their potency and known/possible occurrence in the environment: M2 (a metabolite of vinclozolin), procymidone, and prochloraz. All three are current-use pesticides [8,28] that range in affinity for the androgen receptor, M2 > procymidone > prochloraz [29,30]. Inhibition response curves were developed for each antagonist with three androgens also of varying potency: 17β-trenbolone > testosterone > androstenedione [31,32]. These data provide insights on how sensitive the MDA-kb2 cell line might be to different combinations of androgen receptor agonists and antagonists that could be present in environmental samples.

MATERIALS AND METHODS

Test chemicals

The seven androgens used for our experiments were 17β-trenbolone (Sigma; 98% purity), 17α-trenbolone (Sigma; 99.9% purity), androstenedione (Steraloids; 97% purity), testosterone (Sigma; >98% purity), trendione (Aventis Pharma;), methyl testosterone (Sigma; 99.7% purity), and dihydrotestosterone (Sigma; 99% purity) (Table 1). Hydroxy-flutamide (gift of Schering-Plough Research Institute) was used as a model androgen receptor antagonist to confirm the mechanism of action of the androgen receptor agonists. Chemicals used in binary mixture experiments with a model androgen (17β-trenbolone) were E2 (Sigma; 99.8% purity) and triclocarban (Sigma; 99% purity). Three androgens (17β-trenbolone, testosterone, and androstenedione) also were tested in binary mixtures with three different androgen receptor antagonists: vinclozlin metabolite M2 (Applied Pharma; >99% purity), procymidone (Sigma; 99% purity), and prochloraz (Sigma; 99.4% purity).

MDA-kb2 assay protocol

Initiating, maintaining, passing, and chemical dosing of the MDA-kb2 cells were generally conducted as described previously [20]. To start a culture for testing, the cells were thawed and a 1-ml aliquot of the cell suspension was added to a 25-cm^2 cell culture flask containing 10 ml of L-15 media (Gibco) supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Gibco). Cultures were incubated at 37°C under an ambient atmosphere.

To initiate an assay, cells were removed from flasks, counted, and plated in a 96-well, clear-bottomed, opaque-sided plate (NUNC) at a density of 50,000 cells per well and incubated for 6 h at 37°C. Following this, cells were dosed with chemical (described below) with ethanol as a carrier solvent. The final ethanol concentration in the media was never greater than 0.2% and varied when testing concentration response/inhibition curves, but remained at 0.2% when testing binary mixtures of 17β-trenbolone with E2 or triclocarban. Stock solutions were prepared in 100% ethanol at 2,000-fold the desired final concentration in the well and 2 μl of the solution was added to 998 μl of L-15 supplemented media. Each experiment included ethanol (vehicle) controls and a positive control that consisted of cells treated with 17β-trenbolone. The positive

Table 1. Response of the MDA-kb2 cell assay to seven androgens, listed in order of potency

Androgen	PC50 [a] nM (LL[b], UL[c])	PC50 ng/mL	17β-TB Eq.[d]	Hill Slope	EC50[e] nM	Detection limit[f] nM	Env. concn.[g] nM	Reference for env. concn.
17β-Trenbolone	0.107 (0.105, 0.109)	0.0289	1.00	1.41	0.110	0.0117	0.0740	[18]
Dihydrotestosterone	0.190 (0.177, 0.205)	0.0515	1.78	1.25	0.178	0.0181	0.114	[40]
Methyltestosterone	0.412 (0.364, 0.466)	0.111	3.85	1.24	0.229	0.0277	0.0231	[42]
Testosterone	0.552 (0.502, 0.606)	0.149	5.16	1.23	0.505	0.0503	0.0971	[41]
Trendione	2.12 (1.96, 2.30)	0.574	19.9	1.29	2.19	0.200	0.0596	[44]
17α-Trenbolone	5.18 (4.25, 6.33)	1.40	48.4	0.891	3.34	0.149	0.444	[18]
Androstenedione	59.3 (18.7, 65.7)	16.0	554	1.03	41.3	3.05	0.367	[13]

Parameters of the Hill models are listed including slope and 50% effective concentrations relative to the maximum and minimum of the positive control (PC50) and the individual androgen (EC50). 17β-Trenbolone (TB) equivalents were calculated using the effective concentration at the PC50. Also shown are detection limits for the androgens along with concentrations of the compounds detected in environmental samples as reported in the literature.

[a] Effective concentration at 50% of the positive control.
[b] Lower limit of 95% confidence interval.
[c] Upper limit of 95% confidence interval.
[d] 17β-Trenbolone equivalencies calculated based on PC50.
[e] Effective concentration at 50% of the maximum of the individual chemical.
[f] Solvent control plus three standard deviations.
[g] Environmental concentrations.

control curve ranged from 0.005 nM to 10 nM using a 50% dilution series. The positive control enabled evaluation of among-experiment variability and the calculation of androgen (17β-trenbolone) equivalents to support data analyses.

Cells were incubated for 16 h at 37°C with chemical. Before luciferase was quantified, cytotoxicity was analyzed using a Live/Dead® assay (Molecular Probes), which does not interfere with luciferase activity [33,34]. The cytotoxicity assay was performed as described previously by the manufacturer [35] and is based on two reagents, calcein AM and ethidium homodimer-1. To conduct the cytotoxicity assay the L-15 supplemented media was removed from the cells and rinsed twice with 25 μl Dulbecco's phosphate-buffered saline (DPBS) solution to remove any extracellular esterase activity. Fifty μl of DPBS and 50 μl of the Live/Dead reagent were added to each well, giving a final concentration of 4 μM ethidium homodimer-1 and 2 μM calcein AM in the wells. The dead cell positive control consisted of a 50-μl treatment with ethanol instead of the DPBS buffer. The treated plates were incubated for 15 min at room temperature and fluorescence then was quantified. This assay was used for all mixture experiments; we saw no evidence of cytotoxicity in our experiments.

Following the cytotoxicity test the Live/Dead reagent was removed and the cells were lysed with 25 μl of Luciferase Cell Culture Lysis Reagent (Promega) and incubated at room temperature for 30–60 min prior to quantification of luciferase with a luminometer (Synergy 2/4; BioTek). Luminescence (in relative light units) of each well was measured after addition of 25 μl of a reaction buffer (25 mM glycyglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg/ml bovine serum albumin, pH 7.8) and 25 μl of 1 mM luciferin (Promega) prepared in distilled water. Luminescence in each well was read using an integration time of 5 s.

Individual androgens

Initial experiments focused on defining concentration response relationships for the seven individual androgens. Prior to designing and conducting the mixture study it was necessary to fully define chemical-specific, 50% effective concentration (EC50) values, and minimum and maximum activity plateaus. The initial concentrations tested ranged from 0.01 to 10 nM for 17β-trenbolone, 0.01 to 100 nM for testosterone, 0.000001 to 100,000 nM for androstenedione, and 0.0000001 to 10,000 nM for dihydrotestosterone, methyltestosterone, trendione, and 17α-trenbolone. In these experiments there were four replicate wells per treatment concentration, with 12 concentrations for each androgen, in addition to a 17β-trenbolone standard curve and ethanol control. Each of the seven individual androgen response curves was tested four times, where each replicate used a different passage of cells. Concentration ranges varied slightly from experiment to experiment to achieve a maximum number of concentrations along the Hill slope of the luciferase response curve.

For analysis of data from the individual chemicals, concentration scales were $\log_{10}$-transformed. The relative light units (luciferase response) were normalized to the average maximum and minimum of the standard curve for a 0 to 100% value. The curve of each individual chemical was fit to the logistic equation [36,37] with SAS NLMIXED (SAS Institute) using combined data from the repeated exposures to determine the maximum, minimum, slope, x% effective concentration relative to the individual chemical maximum and minimum (ECx), x% effective concentration relative to the maximum and minimum response of the positive control (PCx) [38], and error in the ECx and PCx values. Nonhomogeneous variances were included in the fitted models. Detection limits were calculated using the average of the vehicle control plus three times the standard deviation.

Androgen mixture

The concentration addition model [22,23] is used to describe a mixture of similarly acting compounds:

$$\mathrm{PC}x_{\mathrm{mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{\mathrm{PC}x_i} \right)^{-1} \quad (1)$$

where $\mathrm{PC}x_{\mathrm{mix}}$ and the $\mathrm{PC}x_i$ are the concentrations at which the mixture of chemicals or the individual chemical, respectively, induces x% of the highest observed effect. Here p_i is the proportion of the ith fraction of the mixture concentration of chemical i, relative to the sum of the concentrations of all of the chemicals in the mixture being studied.

To evaluate mixture additivity using this model, a predicted response was compared to observed data from an actual mixture study. For the mixture experiment the cells were dosed with a fixed-ratio mixture comprised of the seven androgens that had

been tested singly. The concentrations selected for the 100% mixture were 0.848 (17β-trenbolone), 1.59 (dihydrotestosterone), 5.69 (methyltestosterone), 4.67 (testosterone), 17.0 (trendione), 71.0 (17α-trenbolone), and 610 nM (androstenedione). The androgens were combined in a 100% ethanol solution to produce a final concentration in the stock at 2,000-fold the aforementioned concentrations. Then, 2 μl of the stock was added to 998 μl media and the chemical/media was diluted twofold to achieve a full concentration–response curve before addition to the wells. The ratios of the chemicals in the mixture remained the same in each dilution and these ratios were used to calculate the predictive model [21]. The mixture experiment was repeated four times. The observed data were fit to the Hill model following the procedure described for the individual androgens. We also used hydroxyl flutamide in cotreatment with the mixture to confirm androgenicity of the seven androgens. This was achieved by including plates containing 10 μM hydroxy flutamide in duplicate in the media with the mixture and repeated twice. Concentration addition (Eqn. 1) was then used to predict the induction of luciferase by the mixture. The chemicals would be considered additive if the estimated response was consistent with that observed in the empirical mixture experiment, synergistic if the 50% effective concentration relative to the maximum and minimum response of the positive control (PC50) of the observed mixture was higher than predicted, and antagonistic if the observed PC50 was lower than the prediction [23]. Propagation of error was used to derive 95% confidence intervals (CIs) for the concentration addition prediction (see Supplemental Data for derivation) for comparison with the observed mixture of seven androgens [39]. To test for differences between the concentration addition model and the observed data, PC*x* values were compared using a normal table ($z = 1.96$).

Estradiol or triclocarban and 17β-trenbolone mixtures

A similar experimental design was used to test interactions of combinations of E2 or triclocarban and 17β-trenbolone. Five concentrations of E2 (ranging from 1.25–250 nM) or triclocarban (ranging from 125–2,000 nM) were each combined with the EC50, 0.11 nM, or the EC100, 2.5 nM, of 17β-trenbolone. All test concentrations of the estrogen, antimicrobial, and androgen also were assayed individually. Treatments were run in duplicate wells on each plate and the experiments were repeated three times. The binary mixtures were analyzed with linear regression (Prism; GraphPad Software). The data were grouped by 17β-trenbolone treatment with luciferase activity as the dependent variable and E2 or TCC concentration the independent variable. Slopes were tested for equivalence to zero and compared to one another. To obtain equal variance and normality, E2 or TCC concentrations were log transformed and luciferase (y) data were transformed (y') from a percent of the positive control:

$$y' = \log(y + 10) \quad (2)$$

Detection limits were calculated as described above.

Antiandrogen and androgen mixtures

Procymidone, prochloraz, and M2 were tested to determine their effects on activity of different androgen receptor agonists of varying potency in the MDA-kb2 cells. In these studies the androgen concentration remained constant and the antiandrogen was applied at 10 serially diluted concentrations to obtain a range of responses. The androgens 17β-trenbolone, testosterone, and androstenedione were tested near their EC50 (0.11, 0.58, and 76 nM, respectively). Procymidone concentrations ranged from 20 to 10,000 nM, prochloraz ranged from 39 to 20,000 nM, and M2 ranged from 4.9 to 2,500 nM. The controls included a media control, a carrier control (0.2% ethanol), androgen alone, and antiandrogen alone. Treatments were assayed in duplicate in each experiment and experiments were repeated three times. For each combination of androgen (at its EC50) and antiandrogen (at multiple concentrations), a concentration inhibition curve (Hill model with negative Hill slope) and 95% CI were calculated with SAS NLMIXED.

RESULTS

Individual androgens

The luciferase reporter gene was activated by all the putative androgens tested in the MDA-kb2 cell assay (Table 1). Potency of the seven androgens varied widely; the synthetic steroid 17β-trenbolone was the most potent with a PC50 of 0.107 nM. The least potent was androstenedione, with a PC50 of 59.3 nM (Table 1). Four of the seven androgens tested induced a near maximal response (94–100%). Methyltestosterone, 17α-trenbolone, and androstenedione produced 74, 83, and 84%, respectively, of the maximum response of the positive control. As evidenced by the narrow 95% CI obtained for each of the individual androgens (Table 1, Fig. 1), the nonlinear Hill model used to fit the data performed quite well. In general, the variability along individual curves was not equal, but rather increased as chemical test concentration increased.

Androgen mixture

There was excellent correspondence between the measured versus predicted activity of the seven androgen mixture (Fig. 2). The observed and predicted confidence intervals overlapped from the PC5 to the PC70, indicating the curves were similar. For example, the PC50 of the observed mixture was 9.72 (8.85, 10.7) nM and the predicted PC50 based on concentration addition was 10.1 (9.03, 11.2) nM. Using the normal table, the observed and predicted PC*x* values did not differ significantly from one another at the PC5 to the PC65, whereas the PC70 values of the two lines were significantly different ($z = 2.2$). Overall, there was no evidence of antagonism or synergism. Both the prediction and empirical data did not reach the 100% maximum of the positive control. Hydroxy-flutamide (10 μM) blocked the induction of luciferase by the androgen mixture; this was not due to cytotoxicity (data not shown).

Estradiol or triclocarban and 17β-trenbolone mixtures

Estradiol showed both agonism and antagonism in the MDA-kb2 cell line, but only at high concentrations (Fig. 3). An induction of luciferase activity was found in the cell line at 12.5, 25, and 250 nM E2 alone, increasing the response from baseline to 12, 20, and 28% of maximal, respectively (slope was significantly greater than zero). When E2 was combined with 17β-trenbolone at the EC50 of the androgen, antagonism was observed in the binary mixtures; with 250 nM E2, luciferase response decreased from 51 to 29% (slope significantly less than zero; Fig. 3). At the highest 17β-trenbolone treatments (2.5 nM; EC100) E2 also showed antagonism as demonstrated by a slope which was significantly less than zero (Fig. 3). Again, the decrease in luciferase response was not due to cytotoxicity (data not shown).

Both alone and in combination with 17β-trenbolone there was a trend toward increased induction of luciferase activity

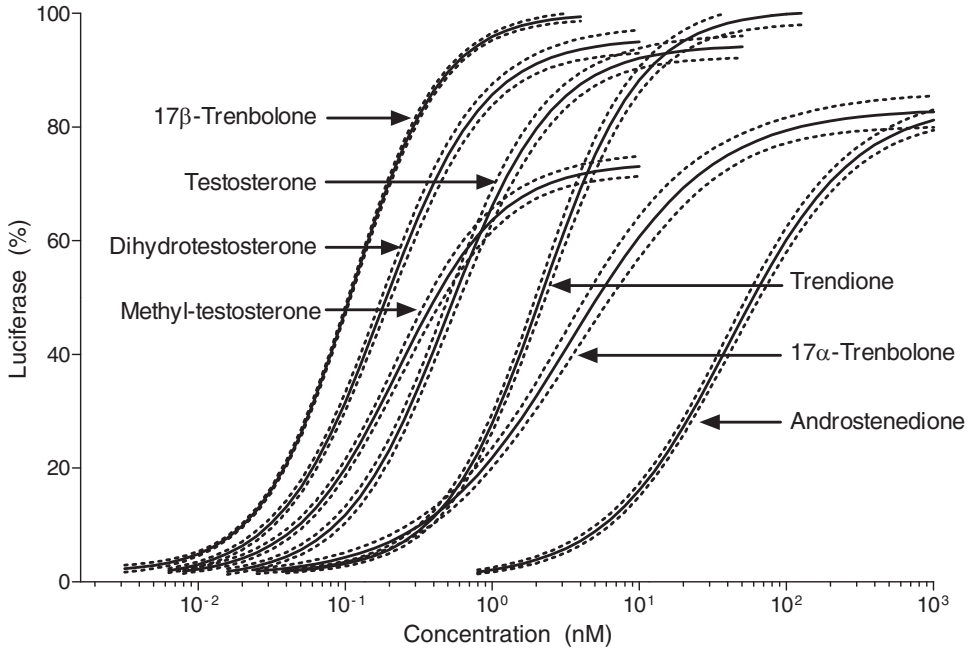

Fig. 1. Concentration–response curves of seven androgens assessed using the MDA-kb2 cell assay. The seven androgens tested in the mixture study included (from most to least potent): 17β-trenbolone, dihydrotestosterone, methyltestosterone, testosterone, trendione, 17α-trenbolone, and androstenedione. The data were fit with the Hill model (solid lines) and 95% confidence intervals along the curves (dashed lines).

with increasing concentrations of triclocarban (Fig. 4). Alone, triclocarban increased luciferase activity from 0 to 4.4%, just below the formal detection limit of the assay (PC5.7). When triclocarban was mixed with 0.11 nM 17β-trenbolone the luciferase response increased from 52 to 71% (Fig. 4). When triclocarban was in combination with 2.5 nM of the potent androgen, the response increased from 100 to 121%. The slopes of the regressions were significantly greater than zero and not different from one another.

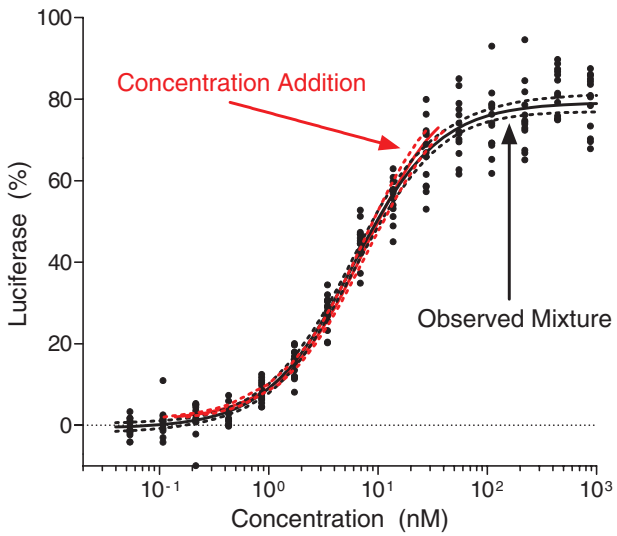

Fig. 2. Response curve for androgenic activity (luciferase induction) in MDA-kb2 cells treated with an equipotent mixture of seven androgens compared to the concentration addition model prediction based on data from the single chemical responses (Fig. 1). The concentration addition model is represented with a red line (predicted) and 95% confidence interval (CI) along its curve (red dashed lines). Empirical data are plotted as individual observations (black dots) which were fit with the nonlinear Hill model, and represented by the black line (observed). Dashed black lines along the observed curve represent 95% CI. The *x* axis represents the sum of individual androgen concentrations that made up the mixture.

Antiandrogen and androgen mixtures

Binary mixtures of androgen receptor antagonists and androgens of varying potency were used to help determine the degree to which environmental antiandrogens might interfere with the detection of androgenic activity by the MDA-kb2 assay (Fig. 5). When tested alone, none of the three antagonists (M2, procymidone, prochloraz) activated the androgen receptor. The three androgens tested alone produced responses close to their targeted EC50 values: 41, 53, and 68% (of maximal) for 17β-trenbolone, testosterone, and androstenedione, respectively. The vinclozolin metabolite (M2) was the most potent antagonist, followed by procymidone, with the least potent prochloraz (Fig. 5). The 50% inhibitory concentrations (IC50) of the antiandrogens varied by almost 100-fold (Table 2). For example, the IC50 of M2, procymidone, and prochloraz in combination with 17β-trenbolone were 59, 310, and 4,100 nM, respectively. Interestingly, there was an increase in the amount of antagonist needed to repress activation of the luciferase reporter as the androgen decreased in potency (i.e., androstenedione required higher concentrations of antiandrogens than 17β-trenbolone to result in a 50% decrease in luciferase activation). Cytotoxicity was not responsible for the reduction in luciferase response caused by the antagonists (data not shown).

DISCUSSION

Risks associated with complex mixtures of chemicals in the environment can be difficult to assess using only analytical techniques and in vivo assays. Mixture assessments can sometimes be aided, however, by focusing on a single mechanism of action/biological activity with transcriptional activation assays, such as the androgen-responsive MDA-kb2 cell line. The current study addressed several potential questions that may arise while

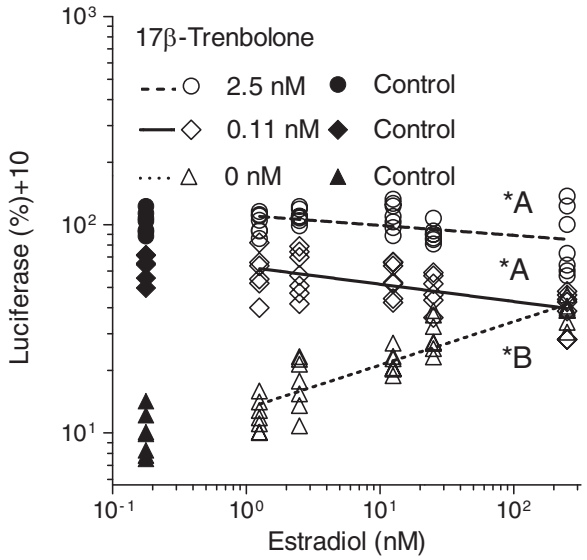

Fig. 3. Effects of estradiol (E2) and binary mixtures of E2 and 17β-trenbolone on luciferase expression in androgen-responsive MDA-kb2 cells. The triangle, diamond, and circle are individual observations and correspond to different 17β-trenbolone treatments of 0, 0.11, and 2.5 nM, respectively. The fitted lines are indicated by dotted, solid, or dashed lines indicating the androgen treatment (0, 0.11, and 2.5 nM, respectively). Asterisks indicate a significant difference in the slope from zero, $p < 0.05$ (*), after data were transformed (Eqn. 2). Letters indicate significant differences between slopes of differing 17β-trenbolone treatments. Controls (vehicle and positive) are indicated by solid shapes corresponding to the appropriate 17β-trenbolone treatments.

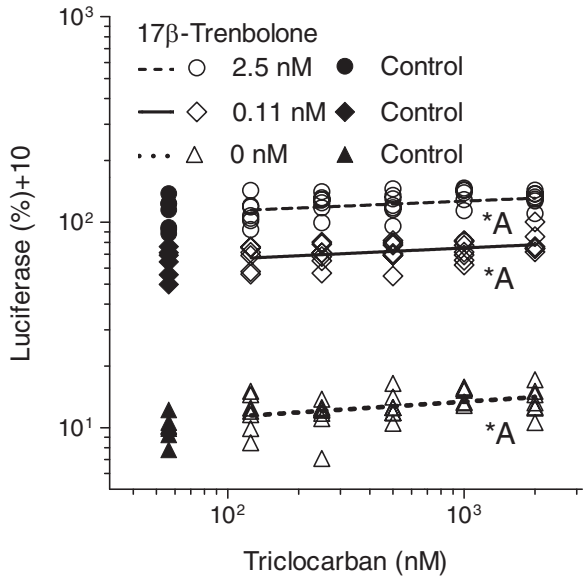

Fig. 4. Effects of triclocarban and binary mixtures of triclocarban and 17β-trenbolone on luciferase expression in androgen-responsive MDA-kb2 cells. The triangle, diamond, and circle are individual observations and correspond to different 17β-trenbolone treatments of 0, 0.11, and 2.5 nM, respectively. The fitted lines are indicated by dotted, solid, or dashed lines indicating the androgen treatment (0, 0.11, and 2.5 nM, respectively). Asterisks indicate a significant difference in the slope from zero, $p < 0.05$ (*), after data were transformed (Eqn. 2). Letters indicate significant differences between slopes of differing 17β-trenbolone treatments. Controls (vehicle and positive) are indicated by solid shapes corresponding to the appropriate 17β-trenbolone treatments.

testing complex environmental samples using MDA-kb2 cells. As discussed in greater detail below, these studies demonstrate that androgens of different potencies activate the assay at environmentally relevant concentrations and act additively; at levels found in the environment, binary mixtures of an androgen and an estrogen most likely will reliably reflect activity of the androgen; some environmental chemicals, such as triclocarban, could

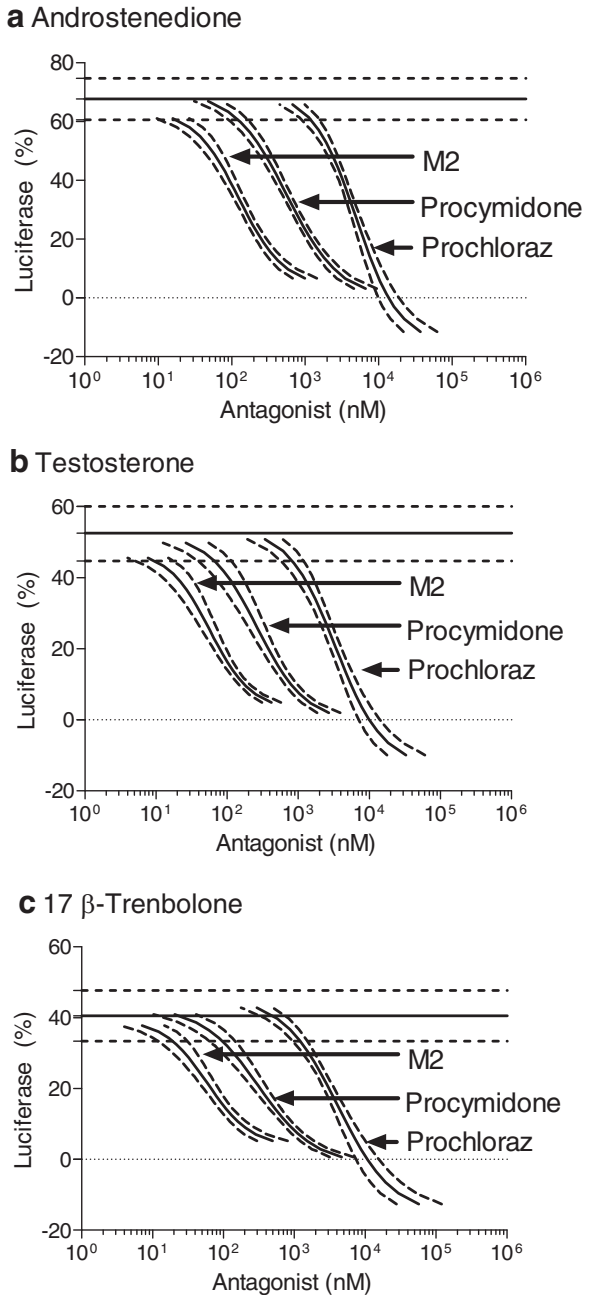

Fig. 5. Inhibition of androgen-induced luciferase activity in MDA-kb2 cells by three androgen receptor antagonists: M2 (vinclozolin metabolite), procymidone, and prochloraz. The three pesticides were tested with three androgen receptor agonists at concentrations that would result in 50% of the maximal induction of luciferase activity in the cells (indicated in parentheses): (**a**) androstenedione (76 nM), (**b**) testosterone (0.58 nM), and (**c**) 17β-trenbolone (0.11 nM). Actual responses of the assay to the androgens alone are indicated by the solid horizontal lines (dashed lines indicate corresponding 95% confidence intervals [CIs]). Response curves are designated by solid curves. The dashed lines indicate 95% CIs associated with each inhibition curve. The 95% CIs and the nonlinear inhibition curves were fit using SAS (SAS Institute). Inhibitory concentrations at 50% as well as 95% CIs are reported in Table 2.

Table 2. The 50% inhibitory concentrations (IC50) of three androgen receptor antagonists (M2 [vinclozolin metabolite], procymidone, and prochloraz) when tested with androstenedione, testosterone, or 17β-trenbolone at androgen concentrations causing a 50% increase in luciferase activity

Androgen	Concentration (nM)	Antagonist IC50[a] values nM (LL[b], UL[c])		
		M2	Procymidone	Prochloraz
Androstenedione	76	130 (110, 150)	560 (490, 650)	5,000 (4,300, 5,700)
Testosterone	0.58	57 (44, 74)	270 (200, 350)	3,400 (2,800, 4,000)
17β-Trenbolone	0.11	59 (46, 76)	310 (240, 400)	4,100 (3,400, 5,000)

[a] 50% Inhibitory effective concentrations.
[b] Lower limit of the 95% confidence interval.
[c] Upper limit of the 95% confidence interval.

enhance androgenicity of a mixture; and at least for the environmental antiandrogens tested herein, comparatively high concentrations would be required to inhibit detection of androgens with varying potencies.

Although data are limited to one or two studies per chemical, several of the individual androgens tested in this study have been quantified in environmental samples via instrumental analysis, often at concentrations sufficient to detect using the MDA-kb2 assay (Table 1). The detection limits of the individual chemicals were calculated to be at the PC5.7 of the individual concentration response curves; these values are shown in Table 1. For example, 17α- and 17β-trenbolone in samples associated with a beef feeding operation were reported at concentrations of 0.44 and 0.074 nM [18], respectively, well within the response range of the cell assay. Similarly, dihydrotestosterone has been detected in waste water effluent with a maximum reported concentration of 0.114 nM, which would be detectable by the assay [40]. The MDA-kb2 cells would also respond to environmental concentrations of testosterone, which was measured at 0.097 nM in a stream receiving runoff from a field utilizing poultry litter as a source of fertilization [41]. Methyltestosterone is commonly used in aquaculture in food pellets to promote efficient growth and has been detected in the pond water after removal of fish at around 0.023 nM [42], a concentration within detection limits of the assay for methyltestosterone. The assay may not detect low potency androgens at the low concentrations detected thus far in the environment. For example, androstenedione concentrations reported in a pulp mill effluent and at a WWTP would be below the detection limit of the assay [13,15,43]. Trendione, with an EC5.7 at 0.20, was detected at a concentration of 0.059 nM in surface water near a beef feedlot [44], which would not induce luciferase in the cells. But environmental sample preparation techniques such as whole sample and extraction/concentration of sample would need to be taken into account when considering the above detection limits.

Several of the androgens used for our study also have been evaluated by others using different types of in vitro systems. To compare our data to past work in this area, the relative potency of each androgen was calculated using dihydrotestosterone instead of 17β-trenbolone, because all studies used dihydrotestosterone in common. In most cases, 17β-trenbolone, dihydrotestosterone, testosterone, and methyltestosterone were the most potent androgens, while trendione, 17α-trenbolone, and androstenedione had lower potency relative to dihydrotestosterone (Table 3). The order of potency varied slightly in the different assays, possibly due to a number of variables including analysis methodology; androgen receptors from different sources; transient versus stable insertion of the receptor, reporter and/or response elements; and assessment of competitive receptor binding versus transactivation endpoints. Overall, however, rank potency of the seven androgens across the relatively varied studies was remarkably consistent.

In our seven androgen mixture experiment, the derived concentration addition model accurately predicted observed responses over most of the range of the dose–response curve. However, the two curves did not have a maximum response of 100%. This is probably due to 17α-trenbolone, methyltestosterone, and androstenedione not producing a maximal response in the cell assay individually and is consistent with the conjecture of Thorpe et al. [45], who predicted that the response of a mixture cannot exceed the response of the compounds with the lowest maximum response. Our observation of additivity for the androgen mixture is consistent with other in vitro and in vivo experiments with mixtures of endocrine-active chemicals including estrogens, antiandrogens and thyroid disrupting compounds [21,46–48]. Although no previous studies have been conducted to demonstrate the additive nature of mixtures of androgens in vitro or in vivo, our in vitro data suggest that it is reasonable to assume additivity for this mechanism of action when assessing the risk of complex environmental mixtures in vivo.

Our experiments with E2 and a binary mixture of E2 and 17β-trenbolone indicate that the estrogen affects luciferase activity in the MDA-kb2 cells, but only at very high concentrations relative to environmental concentrations. When E2 was tested alone, it acted as a partial agonist in the MDA-kb2 cells at concentrations greater than 2.5 nM. Wilson et al. [49] also learned that, at comparatively high concentrations, E2 activated the androgen receptor in MDA-kb2 cells, and hypothesized this to be due to the significant structural homology between the estrogen and androgen receptors. An earlier study from that laboratory demonstrated that E2 had 1/40th of the binding affinity of 17β-trenbolone for the human androgen receptor in a whole cell binding assay [3]. Consistent with weak binding of E2 to the androgen receptor, experiments with binary mixtures of 17β-trenbolone and the estrogen showed that at the high concentration of 250 nM E2 also acts as a receptor antagonist. However, to put the agonistic and antagonistic properties of E2 into perspective relative to environmental samples, concentrations of E2/estrogen equivalents in environmental samples from livestock waste are on the order of 0.004 nM [24,50,51], suggesting that under normal circumstances, estrogenic chemicals should not cause false-positive (i.e., androgen-like) responses or block the activity of androgens in the MDA-kb2 cell assay.

A new, currently undefined, endocrine mechanism of action is enhancement of receptor-mediated endocrine responses by some types of chemicals present in the environment. Triclocarban has been used for the past five decades in a wide variety

Table 3. A comparison of data found in the literature to the present study in terms of potencies of seven androgens

	MDA-kb2 cells, STR,[a] EhAR[b]	MDA-kb2 cells, STR, EhAR	AR-Eco screen cells, STR, SThAR[c]	TARM-Luc cells, STR, SThAR	AR CALUX cells, STR, SThAR	β-galactosidase yeast assay, TTR[d], TThAR[e]	Green fluorescent protein yeast assay, TTR, TThAR	Human androgen receptor binding assay
Reference	This Study	[49]	[61]	[62]	[63]	[32]	[32]	[31]
Compound								
17β-Trenbolone	180	120	NT[f]	35	NT	NT	NT	109
Dihydrotestosterone	100	100	100	100	100	100	100	100
Methyltestosterone	46	NT	31	0.87	17	60	84	36
Testosterone	35	NT	21	9.3	21	60	70	31
Trendione	9.0	NT	NT	NT	NT	NT	NT	0.36
17α-Trenbolone	3.7	NT	NT	NT	NT	NT	NT	4.5
Androstenedione	0.32	NT	22	NT	3.3	0.47	12	NT
				EC50 (nM), used to calculate relative potency[g]				
Dihydrotestosterone	0.19	0.14	0.22	0.07	0.13	16	16	2.5

The relative potencies were calculated using dihydrotestosterone as a reference chemical because it was used in common in all studies considered.
[a] Stably transfected reporter/response elements (STR).
[b] Endogenous human androgen receptor (EhAR).
[c] Stably transfected human androgen receptor (SThAR).
[d] Transiently transfected reporter/response elements (TTR).
[e] Transiently transfected human androgen receptor (TThAR).
[f] Not tested.
[g] Relative potency was calculated by dividing the median effective concentration (EC50) of the individual chemical by the specified EC50 of dihydrotestosterone.

of personal care products and has been detected in U.S. waterways at 0.8 nM downstream of WWTPs or leaking sewers [52]. Recent studies have shown that this antimicrobial increases responses to testosterone both in vivo and in vitro, but the compound alone does not induce a significant androgenic response [26]. Ahn et al. [27] showed that triclocarban enhanced the effects of both testosterone and E2 using androgen- or estrogen-responsive CALUX bioassays. Similarly, in our study triclocarban increased the effect of 17β-trenbolone on luciferase activation in the MDA-kb2 cell line in a dose-dependent manner. A notable difference between previous studies and our work with triclocarban was that the antimicrobial itself seemed to cause some degree of activation of the androgen receptor (near the detection limit of the assay). Further work with triclocarban is required to determine the exact nature of this chemical's effect on androgen signaling pathways.

Our studies suggest that environmental androgen receptor antagonists, unless very potent or present at comparatively high concentrations, should not affect ability of the MDA-kb2 assay to detect androgens in environmental samples. For example, in the present study a 50% inhibition of androgenic activity by three representative antiandrogenic pesticides occurred only at concentrations much higher (greater than 10-fold) than what has been measured in different environmental samples. For example, M2 is more likely to be present in water than vinclozolin itself (http://www.epa.gov/oppsrrd1/REDs/factsheets/2740fact.pdf) [53], but M2 was not found in a river in Italy near possible agricultural sources of the pesticide [30,54]. In other studies, prochloraz was detected in surface waters at concentrations ranging from 0.0027–1.1 nM after periods of rain in Denmark and on a Brazilian rain forest reserve [55,56], while procymidone concentrations ranged from 0.18–32 nM in a river near an agricultural region in South Africa [57]. While it certainly is possible that environmental antiandrogens could interfere with responsiveness of the MDA-kb2 assay to androgens, to the extent that the pesticides we tested represent what might occur in the environment, this would not seem to be a major concern. But a recent study demonstrates the importance of testing not just a whole environmental sample (in this case river sediment), but also fractionating the sample to test for the possible presence of both antiandrogens and androgens using MDA-kb2 cells [58].

The relative potencies of the three antiandrogens evaluated in our study are comparable to reports from the literature where rank potency is consistently M2 > procymidone > prochloraz [29,59]. An interesting result from our androgen/antiandrogen mixture studies was that an antagonist in combination with the weakest androgen tested, androstenedione, required a higher concentration of the antiandrogen to produce an inhibition of luciferase induction compared to the mixtures using testosterone and 17β-trenbolone. This may be due to a relatively greater concentration of the lower potency androgen needed to achieve induction of luciferase to 50% of maximal, so that on a molar basis there is more competition for receptor binding sites with the antiandrogens.

Our androgen/antiandrogen mixture studies suggest that the MDA-kb2 assay could be used to detect androgen receptor antagonists in environmental samples by testing samples both in the presence and absence of a well-characterized androgen such as dihydrotestosterone or 17β-trenbolone. Others have used this type of cotreatment approach with in vitro systems to detect antiandrogens. For example, androgen receptor antagonist activity in concentrated river water samples was examined using an androgen-responsive yeast cell line tested in the presence and absence of methyldihydrotestosterone [54]. Another study, in which COS7 monkey kidney cells were transiently transfected with the human androgen receptor, antiandrogenic activity was detected in concentrated effluent and surface water samples from several sites in The Netherlands when the cells were concurrently tested with the synthetic androgen R1881 [60].

In conclusion, our results with defined mixtures of chemicals indicate that the androgen-responsive MDA-kb2 cell line should produce reliable estimates of androgenic activity in complex environmental samples such as CAFO wastes. The assay could be used to screen environmental samples for endocrine-active chemicals prior to (or in conjunction with) instrumental analyses and focused in vivo tests to estimate risks associated with specific classes of chemicals in complex mixtures. In addition, the assay should prove useful to toxicity-

based fractionation studies attempting to define specific chemicals responsible of androgenic/antiandrogenic activity in complex mixtures (e.g., [15]).

SUPPLEMENTAL DATA

Propagation of error. (23 KB DOC)

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