Interlaboratory Comparison of Four *in Vitro* Assays for Assessing Androgenic and Antiandrogenic Activity of Environmental Chemicals

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We evaluated and compared four *in vitro* assays to detect androgen agonists and antagonists in an international interlaboratory study. Laboratory 1 used a cell proliferation assay (assay 1) with human mammary carcinoma cells stably transfected with human androgen receptor. The other laboratories used reporter gene assays, two based on stably transfected human prostate carcinoma cells (assay 2) or human mammary carcinoma cells (assay 4), and the third based on transient transfection of Chinese hamster ovary cells (assay 3). Four laboratories received four coded compounds and two controls: two steroidal androgens, two antiandrogens, an androgenic control, 5α-dihydrotestosterone (DHT), and an antiandrogenic control, bicalutamide (ICI 176,334). All laboratories correctly detected the androgenic activity of 4-androsten-3,17-dione and 17α-methyltestosterone. For both compounds, the calculated androgenic potencies relative to the positive control (RAPs) remained within one order of magnitude. However, laboratory 3 calculated a 50-fold higher RAP for 4-androsten-3,17-dione. All assays detected and quantified the antiandrogenic effect of vinclozolin [median inhibitory concentration (IC_{50}) values ranging from 1.1×10^{-7} M to 4.7×10^{-7} M]. In assays 2 and 3, vinclozolin showed partial androgenic activity at the highest concentrations tested. For vinclozolin, calculated antiandrogenic potencies relative to bicalutamide (RAAPs) differed no more than a factor of 10, and IC_{50} values matched those of bicalutamide. Similarly, we found antiandrogenic activity for tris-(4-chlorophenyl)methanol. RAAP values were between 0.086 and 0.37. Three assays showed cytotoxicity for this compound at or above 1×10^{-5} M. In summary, all assays proved sensitive screening tools to detect and quantify androgen receptor–mediated androgenic and antiandrogenic effects of these chemicals accurately, with coefficients of variation between 8 and 90%. *Key words:* androgenicity, 4-androsten-3,17-dione, antiandrogenicity, A-SCREEN, bicalutamide, tris-(4-chlorophenyl)methanol (TCPM), vinclozolin. *Environ Health Perspect* 112:695–702 (2004). doi:10.1289/ehp.6715 available via *http://dx.doi.org/* [Online 22 January 2004]

In recent years, researchers have shown that a large and continuously growing number of man-made chemicals and naturally occurring compounds can mimic endogenous estrogens of vertebrates including humans (Colborn et al. 1993; Schlumpf et al. 2001; Soto et al. 1995). In addition to compounds naturally occurring in plants and some mycotoxins (Breithofer et al. 1998; Miksicek 1994), various pesticides and industrial chemicals have shown estrogenic effects *in vitro* (Jobling et al. 1995; Klotz et al. 1996; Körner et al. 1998; Miller et al. 2001; Soto et al. 1991; Soto et al. 1995) and *in vivo* in mammals (Dodds and Lawson 1936; Milligan et al. 1998; Nagel et al. 1997; Soto et al. 1991) as well as fish (Christiansen et al. 2000; Donohoe and Curtis 1996; Gimeno et al. 1996; Jobling et al. 1996). However, we have not identified a common chemical substructure responsible for the estrogenic activity of the so-called xenoestrogens and phytoestrogens. The elucidation of the crystal structure of the ligand-binding domain of the human estrogen receptor (ER)-α (Brzozowski et al. 1997) explained, in part, this surprising structural diversity. It revealed a ligand-binding domain gap almost twice as large as that required by 17β-estradiol (E_2), allowing space for a variety of other molecules to interact with the ER. Therefore, we must assume that more xenobiotics with unknown estrogenic activity exist, requiring specific, sensitive, and practical experimental screening systems for detection.

In contrast, we know comparatively little about the interference of chemicals with the human androgen receptor (hAR). A few derivatives of the known nonsteroidal antiandrogen bicalutamide (ICI 176,334) have shown androgenic effects *in vitro* (Dalton et al. 1998), and the bioaccumulating DDT metabolite *p,p′*-DDE (dichlorodiphenyldichloroethylene), the fungicides vinclozolin, procymidone, and prochloraz, and the herbicide linuron (Cook et al. 1993; Fail et al. 1995; Gray et al. 1994; Kelce et al. 1995; Vinggaard et al. 2002) have demonstrated androgen receptor (AR)-mediated antiandrogenic activities *in vitro* and *in vivo*. The two vinclozolin metabolites, M1 and M2, cause antiandrogenic effects in male rats (Kelce et al. 1994). These metabolites (Figure 1) and linuron, but not *p,p′*-DDE, relate structurally to the therapeutic antiandrogen flutamide. The natural insecticide pyrethrin and some synthetic pyrethroids act as competitive AR antagonists in human fibroblasts (Eil and Nisula 1990). Tyler et al. identified 3-phenoxybenzylalcohol, a metabolite of the pyrethroid permethrin, as an antiandrogen in genetically modified yeast cells (Tyler et al. 2000). In addition, polycyclic aromatic hydrocarbons block AR activation *in vitro* (Vinggaard et al. 2000) and suppress androgen-dependent growth of accessory sex organs in juvenile male rats (Chang and Liao 1987). Recently, several phenolic chemicals demonstrated antiandrogenic activity in a reporter cell line (Paris et al. 2002).

These examples indicate that, analogous to xenoestrogens and phytoestrogens, a common chemical substructure may not exist for AR-mediated antiandrogenic activity. This stresses the need for adequate screening systems. Although many *in vitro* and *in vivo* bioassays can detect ER-mediated activity, relatively few test systems measure androgen agonist and antagonist effects *in vitro*. To devise strategies for screening new and existing chemicals, we must test the accuracy and comparability of existing assays. While recent comparison studies of bioassays for detection of estrogenic activity of pure substances have revealed comparable results in most cases (Andersen et al. 1999; Fang et al. 2000), this work presents, for the first time, an interlaboratory comparison of *in vitro* assays for measuring AR-mediated androgenic and antiandrogenic activity.

Materials and Methods

Participants and test systems. All four laboratories participating in this study applied cellular

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test systems. Three of the four laboratories worked with reporter gene assays with different human and mammalian cell lines. These assays rely on the expression of the firefly luciferase gene under control of the hAR. Laboratory 1 used the A-SCREEN assay, which measures androgen-dependent inhibition of proliferation of an AR-positive human mammary carcinoma cell line (Szelei et al. 1997). Table 1 lists an overview of the four participating laboratories and their respective test systems. All participants have previously described their assays in the literature (Szelei et al. 1997; Térouanne et al. 2000; Vinggaard et al. 1999; Wilson et al. 2002) and performed them in this study with the following modifications:

Laboratory 1 used the A-SCREEN assay. This assay can be run in serumless or serum-supplemented medium. The serum-free medium does not require E_2 to achieve maximal cell yield, and the serum-supplemented medium does. Both methods give comparable results (Szelei et al. 1997), so in conjunction with the other assays, laboratory 1 used serum-supplemented medium. They seeded 25,000–35,000 AR-positive MCF-7-AR1 cells in Dulbecco's modification of Eagle's medium (DMEM) with phenol red and 5% fetal calf serum (FCS) into each well of 24-well plates. Twenty-four hours later, they changed the medium to phenol red–free DMEM supplemented with 5% charcoal–dextran-treated FCS (CDFCS). Because these cells express ER as original MCF-7 cells do, E_2 was added to each well, except for a control row of four wells, to yield a final concentration of 0.1 nM, allowing maximal proliferation of MCF-7-AR1 cells. Androgens inhibit proliferation in a concentration-dependent manner to about 30% of the maximal cell count (Figure 2A). Under the conditions of the A-SCREEN assay, the cells arrest in G_0/G_1 phase (Soto et al. 1999; Szelei et al. 1997). Coincubation with an antiandrogen blocks that inhibition of cell proliferation. Laboratory 1 ran an extended dose–response curve to 5α-dihydrotestosterone (DHT) with each experiment and added DHT (to yield a concentration of 0.1 nM) to the bottom two rows of each plate with test compounds and solvent controls to test for antiandrogenicity. They left the second column in each plate as an androgenless control. After 5 days of incubation, they fixed the cells and stained them with sulforhodamine B (SRB). They resuspended the dye in 10 mM Tris base (pH 10.5), transferred aliquots from each well into a 96-well plate, then calculated cell numbers from the optical densities measured at 515 nm.

Laboratory 2 seeded stably transfected human prostate adenocarcinoma (PC-3) cells (PALM cells) in white opaque tissue culture 96-well plates (Becton Dickinson, Le Pont de Claix, France). They used 20,000 cells in 150 µL HAM-F12 medium supplemented with 3% CDFCS in each well. After an 8-hr incubation, they added the compounds in 50 µL of the same medium without replacement of the seeding medium. After 30 hr, they removed the culture medium with the tested compounds and replaced it with luminescent buffer (50 µL/well phenol red–free DMEM, 3×10^{-4} M luciferin). They measured luciferase activity in intact cells with a luminometer (Trilux Wallac; PerkinElmer, Courtaboeuf Cedex, France).

Laboratory 3 tested the compounds in a reporter gene assay based on transient transfections as originally described (Vinggaard et al. 1999), but with major modifications. They maintained Chinese hamster ovary cells (CHO K1) in DMEM/F12 (Gibco, Paisley, U.K.) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) and 10% FCS (BioWhitaker, Walkersville, MD, USA). They seeded cells in white 96-well plates (PerkinElmer Life Sciences, Packard, Groningen, the Netherlands) at a density of 7,000 cells/well in DMEM/F12 medium containing 10% charcoal-treated FCS

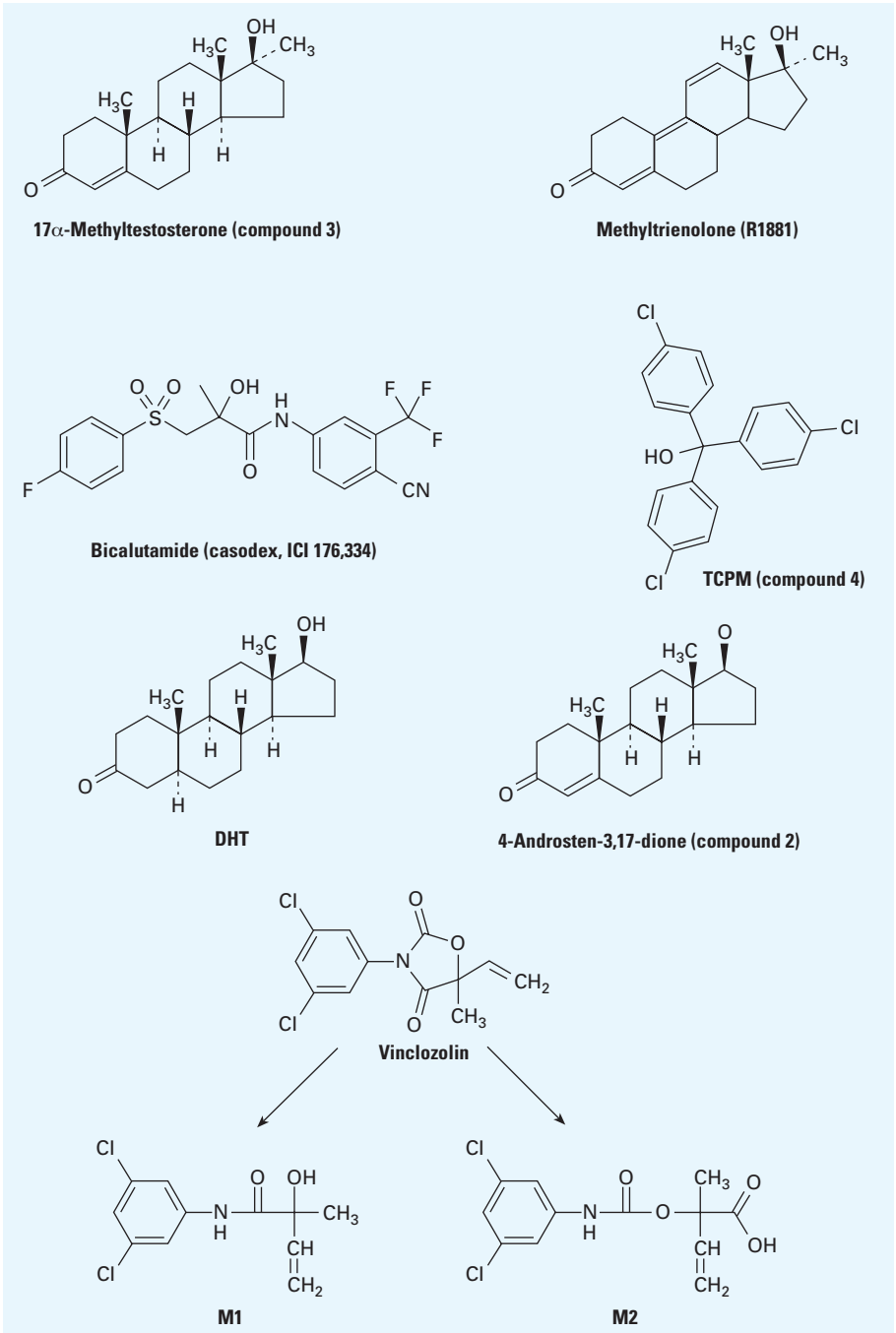

Figure 1. Structures of the test compounds and the positive controls. Abbreviations: DHT, 5α-dihydrotestosterone; TCPM, tris-(4-chlorophenyl)methanol.

(BioWhitaker) and incubated the plates at 37°C in a humidified atmosphere of 5% CO_2/air. After 24 hr, they transfected the cells for 5 hr with a total of 75 ng DNA/well consisting of the AR expression vector pSVAR0 and the mouse mammary tumor virus-luciferase (MMTV-LUC) reporter plasmid (both provided by A. Brinkmann, Erasmus University, Rotterdam, the Netherlands) in a ratio of 1:100 using 0.30 µL of the transfection reagent FuGene (Boehringer, Mannheim, Germany). They kept the ratio of DNA (micrograms) to FuGene (microliters) at 0.25. After removing the transfection medium, they added the test compounds. Laboratory 3 tested all concentrations in quadruplicate. After incubation for 20 hr, they aspirated the medium and lysed the cells by adding 20 µL/well of a lysis buffer containing 25 mM trisphosphate, pH 7.8, 15% glycerol, 1% Triton X-100, 1 mM dithiothreitol, and 8 mM $MgCl_2$, followed by shaking at room temperature for 10 min. They measured luciferase activity directly in the culture plates using a BioOrbit Galaxy luminometer (Anthos Labtec Instruments, Wals, Austria).

Laboratory 4 carried out a test according to the protocol previously described by Wilson et al. (2002) with some modifications and simplifications. They routinely maintained MDA-kb2 human breast carcinoma cells in Leibowitz's L-15 medium supplemented with 10% heat-inactivated (56°C, 30 min) FCS and 1% antibiotic–antimycotic (all from Gibco) at 37°C in a humidified incubator without CO_2. They detached cells by trypsinization and seeded them into 96-well plates at a density of about 10,000 cells/well in 100 µL medium. After cells had attached for 24 hr, they replaced the medium with a total of 100 µL/well of medium containing various dilutions of test chemicals. In each plate they filled four wells in one column with medium only as the negative control, four wells in another column with 1×10^{-10} M DHT (final concentration) as the androgen control, and four wells in a third column with 1×10^{-6} M bicalutamide (final concentration) as an antiandrogen control. They assayed each concentration of each test compound in at least four wells. After incubation for 24 hr, they removed the medium and washed the cells gently twice with phosphate-buffered saline. To measure luciferase activity, they added 25 µL/well lysis buffer (Promega, Wallisellen, Switzerland), transferred these into a microtiter plate with a multichannel pipettor after 30 min, and read them on a luminometer (ML 1000; Dynex, Frankfurt/Main, Germany).

Test compounds, controls, and solvent. Each laboratory received four commercially available test compounds, all sent in a coded manner, plus two control compounds. Each laboratory used the following controls and test compounds: androgen control, DHT (Sigma-Aldrich,) [laboratory 2 used methyltrienolone (R1881; Perkin-Elmer)]; antiandrogen control, bicalutamide (Casodex, ICI 176,334), a gift from Zeneca Pharmaceuticals (Macclesfield, U.K.); compound 1, vinclozolin (Dr. Ehrenstorfer, Augsburg, Germany); compound 2, 4-androsten-3,17-dione (Sigma-Aldrich, Taufkirchen, Germany); compound 3, 17α-methyltestosterone (Fluka, Taufkirchen, Germany); and compound 4, tris-(4-chlorophenyl)methanol (TCPM) (Lancaster, Mühlheim, Germany).

Figure 1 illustrates the structures of the assayed substances. Each laboratory received the solid substances in amber glass vials with screw caps and Teflon and parafilm sealings. Each vial contained the amount required to obtain 4 mL of a 0.01 M stock solution (weighed on microscales with an uncertainty of ± 0.05 mg). Participants stored all substances at 4°C. The vials of the androgen and antiandrogen controls contained a minimum of 30 mg, which was enough for 10 mL and 5 mL of 0.01 M stock solution, respectively. Laboratory 2 used the synthetic androgen R1881 instead of DHT as an androgen control; the PALM assay has a

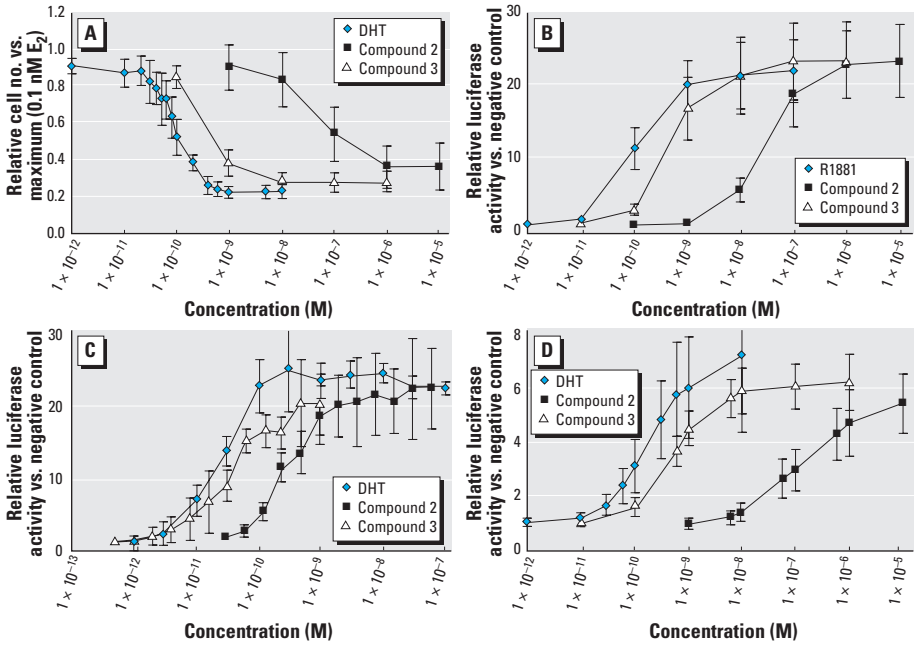

Figure 2. Concentration–response curves of androgen control, 4-androsten-3,17-dione (compound 2), and 17α-methyltestosterone (compound 3) for all four assays. (*A*) Assay 1. (*B*) Assay 2. (*C*) Assay 3. (*D*) Assay 4. Values represent means ± SD of at least three independent experiments.

Table 1. Participating laboratories and test systems.

Laboratory	Cell type	End point	Exposure time (hr)	Statistical program[a]	Reference
1 (Tufts University School of Medicine)	MCF-7-AR1 human mammary adenocarcinoma cells expressing hAR	Cell number (determined with SRB assay)	120	Lotus 1-2-3 (logit)	Szelei et al. 1997
2 (INSERM U 439, Pathologie Moléculaire des Récepteurs Nucléaires)	Human prostate adenocarcinoma PC-3 cells stably transfected with pSG_5-puro-hAR and pMMTV-neo-Luc	Luciferase activity	30	VBA program for EXCEL 5[b] (log-probit)	Térouanne et al. 2000
3 (Danish Veterinary and Food Administration, Institute of Food Safety and Nutrition)	Chinese hamster ovary cells transiently transfected with pSVAR0 expression vector and MMTV-LUC reporter plasmid	Luciferase activity	20	SigmaPlot [Chapman (three parameter); four parameter logistic (for antagonists)]	Vinggaard et al. 1999
4 (University of Zürich, Institute of Pharmacology and Toxicology)	MDA-MB-453-KB2 human mammary carcinoma cells, endogenously expressing hAR and stably transfected with pMMTV-neo-Luc	Luciferase activity	24	GraphPad Prism (variable slope)	Wilson et al. 2002

[a]Type of sigmoid regression. [b]Program designed by J. Greve, Fraunhofer-Institute of Environmental Chemistry and Ecotoxicology, Schmallenberg, Germany.

lower sensitivity for DHT than for R1881 because of rapid metabolism of natural androgens in PC-3 cells, as described by Térouanne et al. (2000). These cells metabolize testosterone and DHT quickly, in less than 2 hr, whereas detection of luciferase activity requires at least 24 hr incubation with compounds. Castagnetta et al. (1994, 1997) have reported the ability of PC-3 cells to metabolize androgens in addition to a high expression of 17β-hydroxysteroid dehydrogenase. All laboratories used high-purity DMSO for dissolving test and control compounds.

All test systems showed that bicalutamide and hydroxyflutamide act as potent androgen antagonists (Ma et al. 2003; Szelei et al. 1997; Térouanne et al. 2000; Vinggaard et al. 1999). Although hydroxyflutamide proved more potent than bicalutamide in assays 2 and 3 (Térouanne et al. 2000; Vinggaard et al. 1999), we selected bicalutamide as the antiandrogen control. We used the two steroidal androgens (compounds 2 and 3) because of their expected different potencies. Because Kelce et al. (1994) detected the two metabolites of vinclozolin, M1 and M2, as AR antagonists *in vivo,* we expected the results on vinclozolin would provide information on the metabolic capacities of the *in vitro* systems. The persistent, bioaccumulating environmental contaminant TCPM (DeBoer 1997) showed antiandrogenic activity in a cell proliferation assay (Körner et al. 1997, 2000). Therefore, because TCPM has high resistance to metabolic conversion, its use offered the opportunity to confirm these results.

Performance of experiments. The laboratories received instructions that each single experiment must contain *a*) a negative control; *b*) a solvent control; *c*) at least five appropriate concentrations of the androgen control DHT that encompass the whole range of the concentration–response curve; *d*) one concentration of DHT that gives nearly maximum response (0.1 nM) together with the following five concentrations of the antiandrogen control bicalutamide: 0.0001, 0.001, 0.01, 0.1, and 1 μM; *e*) 1 μM bicalutamide; *f*) five (or more) concentrations of each test compound covering the whole range of the concentration–response curve (the highest concentration tested should equal 10 μM for compounds 1, 2 and 4, and 1 μM for compound 3); and *g*) five (or more) concentrations of each test compound together with 0.1 nM DHT covering the whole range of the concentration–response curve.

Each laboratory tested the compounds in at least three independent experiments alone and together with 0.1 nM DHT.

Quantitative evaluation. Laboratory 1 used cell number as the end point. They used raw cell numbers for quantitative evaluation, but for graph representation, they normalized the experiments, set the maximum cell number to 1, and reported ratios in relationship to this.

The three reporter gene assays (assays 2–4) used luciferase activity relative to the hormone-free negative control as the basic end point. For quantitative evaluation of antiandrogenic activity, all laboratories set the luciferase activity of the androgen control (0.1 nM DHT or R1881) to 100%.

Each laboratory used different statistical software (Table 1) for performance of sigmoid regression of concentration–response curves and calculation of median effective concentration (EC_{50}) values for androgens and IC_{50} values for antiandrogens, respectively.

The androgenic potency of a test compound relative to the positive control (RAP) equals the quotient of the EC_{50} values of DHT (or R1881) and the compound:

$$\text{RAP} = EC_{50}\ (\text{DHT or R1881}) \div EC_{50}\ (\text{test compound}).$$

The antiandrogenic potency of a test compound relative to the positive control bicalutamide (RAAP) equals the quotient of the IC_{50} values of bicalutamide and the compound. The IC_{50} equals the concentration required for the compensation of half of the androgenic effect of 0.1 nM DHT (or R1881):

$$\text{RAAP} = IC_{50}\ (\text{bicalutamide}) \div IC_{50}\ (\text{test compound}).$$

RAP and RAAP have no dimension.

Results and Discussion

Overview. The solvent control (laboratories 1–3: 0.1% DMSO; laboratory 3 also tested 0.25 and 0.5%; laboratory 4: 0.2%) showed no significant effect in any assay; for example, in assay 2 the luciferase activity relative to the negative control was 0.97 ± 0.37 (n = 3). All four laboratories correctly detected the androgenic activity of the testosterone metabolite 4-androsten-3,17-dione and of 17α-methyltestosterone. All laboratories calculated similar androgenic potencies relative to the positive control (RAP) for both test substances and, with the exception of 4-androsten-3,17-dione in assay 3, fell within one order of magnitude.

Each test system detected and quantified the antiandrogenic effect of the fungicide vinclozolin. Moreover, this compound showed slight androgenic activity at the highest tested concentrations with assays 2 and 3. Calculated antiandrogenic potencies relative to the positive control bicalutamide (RAAP) differed less than a factor of 10. Similarly, all assays found antiandrogenic activity for TCPM. This chemical showed cytotoxic effects at $\geq 1 \times 10^{-5}$ M in the assays of laboratories 1, 2, and 3. Nevertheless, all laboratories calculated RAAP values well within one order of magnitude.

Androgens. Table 2 summarizes data on the performance of the assays and the quantitative results for the androgen control DHT (or R1881). Assays 1 and 3 obtained EC_{50} values for DHT that were 3- and 10-fold lower, respectively, than those obtained with assay 4. In general, assay 3 resulted in the lowest EC_{50} values for all three androgens tested.

One might question the comparability of the results of assay 2 and those of the others because of the use of two different positive controls. However, the potency of the synthetic androgen R1881 in assay 2 fell within the range found for DHT in the other three test systems. In all other assays, R1881 gives comparable results, but we preferred DHT because it is a natural androgen.

Table 3 summarizes information on the performance of the tests and the quantitative results for 4-androsten-3,17-dione and 17α-methyltestosterone. Figure 2 illustrates the concentration–response curves of both androgens and DHT (or R1881) for all four test systems. The coefficients of variation (CVs) of the EC_{50} values of DHT (or R1881) fell within a reasonable range (12–57%) for all test systems (Table 2). All assays showed acceptable repeatability for the RAP values of the two androgenic test compounds, with CVs between 8 and 73% (Table 3).

The cell proliferation assay showed a 3-fold dynamic range. The three reporter gene assays revealed differences in the magnitude of the fold induction obtained after androgen exposure. In assays 2 and 3 the androgen con-

Table 2. Overview of the results for the androgen control DHT.

Laboratory	No. of assays	Replicates per assay	Concentrations tested	Lowest concentration (M)	Highest concentration (M)	EC_{50} mean (M)	SD (M)	CV (%)	Percent induction caused by 0.1 nM (mean ± SD)
1	5	4	15	1.0×10^{-12}	1.0×10^{-8}	7.8×10^{-11}	9.3×10^{-12}	12	69 ± 12
2	6	4	7	1.0×10^{-12}	1.0×10^{-7}	1.1×10^{-10}[a]	2.3×10^{-11}	22	53 ± 11
3	4	4	11	1.0×10^{-12}	1.0×10^{-7}	2.2×10^{-11}	6.7×10^{-12}	30	85 ± 11
4	5	4	9	1.0×10^{-12}	1.0×10^{-8}	2.1×10^{-10}	1.2×10^{-10}	57	35 ± 8

[a]Laboratory 2 used R1881.

trol induced a luciferase activity of > 20-fold of that of the negative control, whereas laboratory 4 obtained a 7-fold induction. However, none of the parameters measured covaried with the dynamic range.

With all assays 17α-methyltestosterone induced about the same maximal effect as the androgen control, thereby confirming this compound as a full AR agonist. Assays 1–4 produced EC_{50} values of 4.3×10^{-10}M, 7.4×10^{-10} M, 3.3×10^{-11} M, and 5.3×10^{-10}M, respectively. 4-Androsten-3,17-dione also induced about the same maximal effect as the androgen control with assays 1, 2, and 3. This compound induced only 72% of the maximal effect of DHT with assay 4. Assays 1–4 produced EC_{50} values of 5.9×10^{-8}M, 3.5×10^{-8}M, 2.7×10^{-10}M, and 1.4×10^{-7}M, respectively. Compared with other test systems, assay 3 yielded lower EC_{50} values of both compounds, as well as DHT. Because DHT and 17α-methyltestosterone varied in the same way, the resulting RAP value compared with those of the other assays. However, laboratory 3 found a 100-fold lower EC_{50} for 4-androsten-3,17-dione than the other groups. As this compound was relatively more potent compared with DHT in this assay, a 30- to 60-fold higher RAP than that obtained in the other assays resulted. The possibility that the cells used in these assays may have different metabolic capacities may partly explain this discrepancy. Generally, synthetic androgens such as 17α-methyltestosterone resist metabolism better than natural androgens. For example, Koh et al. (2001) recently demonstrated that PC-3 cells rapidly convert 4-androsten-3,17-dione predominantly to the inactive dehydroepiandrosterone and to a lesser extent to testosterone and subsequently to DHT.

Antiandrogens. Table 4 summarizes data on the performance of the assays and the quantitative results for the antiandrogen control bicalutamide. In all reporter gene assays, bicalutamide showed slight but measurable androgenic activity at the highest concentration of 1×10^{-6} M. However, compared with the activity induced by 0.1 nM DHT (or R1881), we found the androgenic effect of bicalutamide negligible (Figure 3). Laboratory 2 tested bicalutamide concentrations up to 1×10^{-5} M. They found the androgenic effect low compared with 0.1 nM R1881 but higher than that of 1×10^{-6}M. Laboratory 1 did not detect

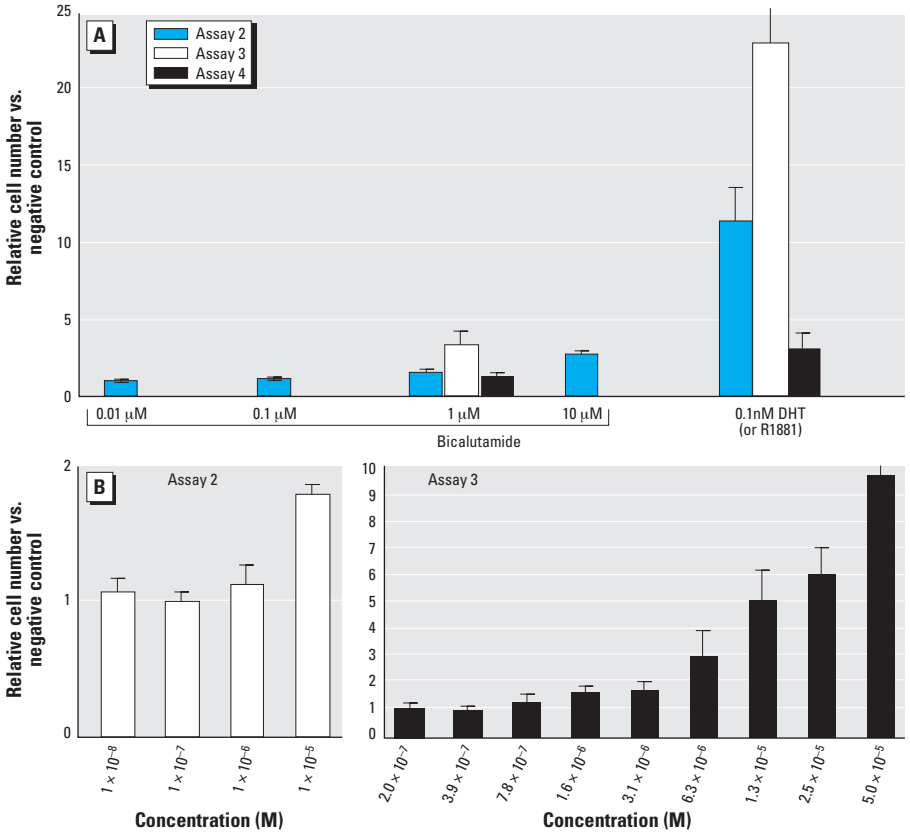

Figure 3. Partial androgenic effects of antiandrogens in reporter gene assays. (*A*) Bicalutamide (mean ± SD of three to five independent experiments); the effects of 0.1 nM DHT (or R1881) are also shown for comparison. (*B*) Vinclozolin (assay 2 and assay 3; mean ± SD of three and four independent experiments, respectively). Figure 2B and 2C show the maximal effect of the androgen control.

Table 3. Overview of the results for the androgenic compounds 4-androsten-3,17-dione (compound 2) and 17α-methyltestosterone (compound 3).

Compound, laboratory	No. of assays	Concentrations tested	Lowest concentration (M)	Highest concentration (M)	EC_{50} mean (M)	SD (M)	RAP mean	SD	CV (%)
4-Androsten-3,17-dione									
1	4	5	1.0×10^{-9}	1.0×10^{-5}	5.9×10^{-8}	2.1×10^{-8}	1.4×10^{-3}	5.4×10^{-4}	38
2	3	6	1.0×10^{-10}	1.0×10^{-5}	3.5×10^{-8}	7.9×10^{-9}	3.1×10^{-3}	2.5×10^{-4}	8
3	4	12	3.0×10^{-11}	6.0×10^{-8}	2.7×10^{-10}	6.4×10^{-11}	8.7×10^{-2}	2.1×10^{-2}	24
4	5	8	1.0×10^{-9}	1.0×10^{-5}	1.4×10^{-7}	4.0×10^{-8}	1.5×10^{-3}	7.7×10^{-4}	50
17α-Methyltestosterone									
1	4	5	1.0×10^{-10}	1.0×10^{-6}	4.3×10^{-10}	1.8×10^{-10}	0.23	0.16	70
2	3	6	1.0×10^{-11}	1.0×10^{-6}	7.4×10^{-10}	1.3×10^{-10}	0.15	0.04	27
3	4	12	5.0×10^{-13}	9.4×10^{-10}	3.3×10^{-11}	2.2×10^{-11}	1.03	0.75	73
4	5	8	1.0×10^{-11}	1.0×10^{-6}	5.3×10^{-10}	1.4×10^{-10}	0.44	0.32	73

Table 4. Overview of the results for the antiandrogen control bicalutamide.

Laboratory	No. of assays	Replicates per assay	Concentrations together with 0.1 nM DHT[a]	Lowest concentration (M)	Highest concentration (M)	IC_{50} mean (M)	SD (M)	CV (%)
1	4	2	5	1.0×10^{-10}	1.0×10^{-6}	1.3×10^{-7}	1.6×10^{-8}	12
2	3	4	5	1.0×10^{-9}	1.0×10^{-5}	1.7×10^{-7}	2.7×10^{-8}	16
3	3	4	10	1.0×10^{-10}	5.0×10^{-6}	2.4×10^{-7}	1.0×10^{-7}	42
4	5	4	8	1.0×10^{-10}	1.0×10^{-6}	3.8×10^{-7}	3.2×10^{-7}	85

[a]Laboratory 2 used 0.1 nM R1881.

an androgen-like decrease of cell count for bicalutamide.

Figure 4 illustrates the concentration–response curves for all four assays of bicalutamide and the antiandrogenic chemicals vinclozolin and TCPM. Each laboratory derived the IC_{50} values from the coincubation of cells with 0.1 nM DHT (or R1881) and different concentrations of antiandrogen, leading to a subsequent increasing inhibition of the androgenic effect of DHT (or R1881). A concentration of 1×10^{-6}M bicalutamide did not lead to complete inhibition of the androgenic effect in any of the assays. For calculation of IC_{50} values, the laboratories extrapolated the concentration–response curves to maximal effect.

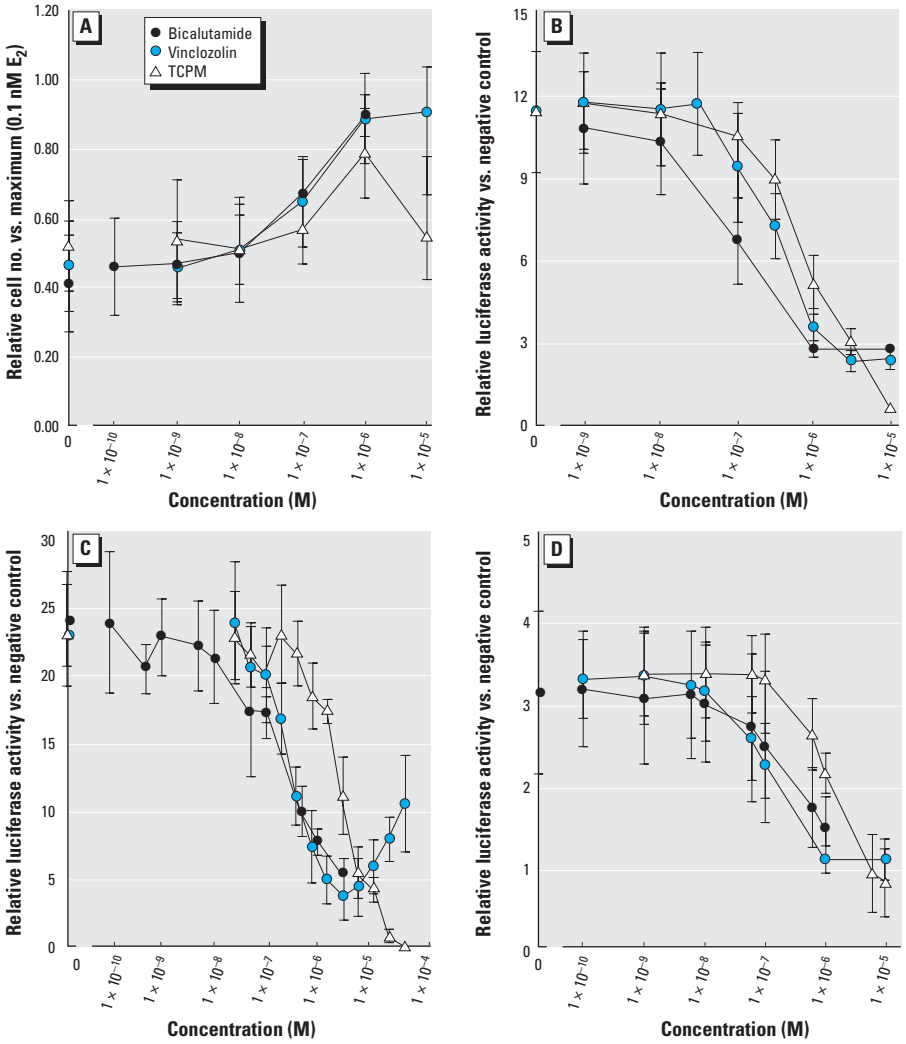

Figure 4. Concentration–response curves of the antiandrogen control bicalutamide, vinclozolin (compound 1), and TCPM (compound 4) for all four assays. (*A*) Assay 1: antiandrogens plus 0.1 nM DHT. (*B*) Assay 2: antiandrogens plus 0.1 nM R1881. (*C*) Assay 3: antiandrogens plus 0.1 nM DHT. (*D*) Assay 4: antiandrogens plus 0.1 nM DHT. Values represent mean ± SD of at least three independent experiments.

Laboratories 2 and 3 disregarded the values measured for coincubation of androgen with 1×10^{-5} M bicalutamide and > 3.0×10^{-6}M vinclozolin, respectively, for IC_{50} calculation because of the slight androgenic activity of these compounds at higher concentrations. All four laboratories calculated comparable IC_{50} values, and the difference between the highest and the lowest value was a factor of 3.

Table 5 and Figure 4 summarize and illustrate the results on the activity of vinclozolin and TCPM. With the exception of assay 4, neither compound inhibited the androgenic effect of 0.1 nM DHT (or R1881) completely. Therefore, for calculation of IC_{50} values, all laboratories extrapolated the concentration–response curves to maximal effect as for bicalutamide.

Table 2 shows the percentage of maximal effect induced by 0.1 nM of the androgen control. Because the response to antiandrogens depends on the magnitude of this response (the stronger the effect of the androgen control, the greater competition of the antiandrogen required), the different experimental conditions used in the assays may explain some of the observed differences in IC_{50} values of the antiandrogens. Thus, laboratories 1–4 tested the antiandrogens at 69, 53, 85, and 35% induction of maximum androgen response, respectively, resulting in the most favorable experimental conditions in terms of sensitivity for detecting antiandrogens for assay 4 and the least favorable conditions for assay 3.

In assays 2 and 3, vinclozolin showed slight and moderate androgenic activity at the highest concentrations, respectively (Figure 3B). These results agree with results obtained by Wong et al. (1995), who found agonistic activity of the vinclozolin metabolite M2 at 10 µM, and by Nellemann et al. (2003), who observed agonistic activity of vinclozolin itself at concentrations ≥ 3 µM. TCPM showed cytotoxic effects ≥ 10 µM in test systems 1, 2, and 3 (Figure 4).

For both chemicals, IC_{50} values as well as calculated RAAP values remained similar among all assays, although the percentage of the maximal effect induced by 0.1 nM DHT (or R1881) differed (Table 2). The highest and

Table 5. Overview of the results for antiandrogenic compounds vinclozolin (compound 1) and TCPM (compound 4).

Compound, laboratory	No. of assays	Concentrations tested	Lowest concentration (M)	Highest concentration (M)	IC_{50} mean (M)	SD (M)	RAAP mean	SD	CV (%)
Vinclozolin									
1	4	5	1.0×10^{-9}	1.0×10^{-5}	2.1×10^{-7}	1.3×10^{-7}	1.0	0.31	31
2	3	8	1.0×10^{-9}	1.0×10^{-5}	4.7×10^{-7}	1.3×10^{-7}	0.37	0.07	18
3	4	12	2.5×10^{-8}	5.0×10^{-5}	3.7×10^{-7}	1.2×10^{-7}	1.6	0.5	32
4	5	8	1.0×10^{-10}	1.0×10^{-5}	1.1×10^{-7}	3.2×10^{-8}	3.7	3.1	83
TCPM									
1	3	5	1.0×10^{-9}	1.0×10^{-5}	5.7×10^{-7}	1.4×10^{-7}	0.22	0.04	16
2	3	7	1.0×10^{-9}	1.0×10^{-5}	8.0×10^{-7}	1.8×10^{-7}	0.22	0.04	20
3	4	12	2.5×10^{-8}	5.0×10^{-5}	3.1×10^{-6}	1.4×10^{-6}	0.086	0.03	36
4	5	8	1.0×10^{-9}	1.0×10^{-5}	1.1×10^{-6}	7.6×10^{-8}	0.37	0.33	90

the lowest IC_{50} differed by a factor of 3 for vinclozolin and a factor of 10 for TCPM. Vinclozolin and bicalutamide had similar antiandrogenic potencies, whereas TCPM had about one fifth the potency of these compounds. Two proliferation assays using AR-positive human mammary carcinoma cell lines have demonstrated the antiandrogenic effect of TCPM (Körner et al. 1997, unpublished data). The fact that all four assays unambiguously confirmed the antiandrogenic properties of TCPM implicates environmental relevance, because this compound is a ubiquitous and highly bioaccumulating chemical and we know little about its sources and toxicologic properties (DeBoer 1997).

The test systems are applicable to other environmental antiandrogens such as *p,p′*-DDE. In assay 4, DDE reduced DHT-induced luciferase activity with an IC_{50} value of $2.8 \pm 0.8 \times 10^{-6}$ M ($n = 5$), whereas cytotoxicity was observed at 1×10^{-4} M. In assay 3, *p,p′*-DDE had an IC_{50} of 1.1×10^{-6} M (Vinggaard et al., unpublished data).

When testing for agonists and antagonists of AR, it is important to consider any cytotoxicity of test compounds to avoid classification of false positives. In assay 1 cytotoxicity is assessed by inspecting the appearance of the cells using an inverted microscope before the cells are fixed. Cytotoxicity is recognized by the presence of floating (dead) cells and the presence of cytoplasmic vacuoles in those still attached to the substrate. In this assay, agonists inhibit cell proliferation and antagonists overcome this inhibition; hence, evaluation of cytotoxicity can also be made by testing whether the inhibitory response observed in the presence of a putative agonist is totally reversed by excess antiandrogen. If the antagonist does not reverse the low cell yield, the effect is considered cytotoxic. In the other three assays, agonists increase and antagonists decrease the expression of the reporter gene. In the experimental conditions of this study, in which antiandrogens were tested for both agonistic and antagonistic effects, any cytotoxicity in the reporter gene assays was indirectly revealed in the agonism test by an inhibition of the transcriptional/translational process (i.e., a decreased luciferase activity). When only antagonism is tested, a very specific cytotoxicity test has been developed for assays based on transient transfections. This method involves transfection of cells with a constitutive active AR expression vector that lacks the ligand-binding domain of the receptor (Kelce et al. 1995; Vinggaard et al. 2002). Cytotoxicity is measured directly at the transcriptional/translational level with this method.

All assays showed comparable repeatability for agonistic and antagonistic androgenic activity. The CVs of the IC_{50} values of bicalutamide ranged from 12 to 85% (Table 4), and those of the RAAP values ranged from 16 to 90% (Table 5). Comparing CVs between the four test systems, one should take into account that the different number of independent experiments (three to five) performed with the assays influence the CV values.

The various statistical programs and types of regression used for calculation of EC_{50} and IC_{50} values had little contribution to the differences of the quantitative results between the four assays.

Conclusions

We compared four different cellular *in vitro* assays for the detection of AR-mediated agonistic and antagonistic effects of chemicals in an interlaboratory study. All four test systems produced comparable quantitative results for two androgens and two antiandrogens. The EC_{50} values calculated for the androgens and the resulting androgenic potencies relative to the positive control differed by less than a factor of 10 between the assays, with one exception. We saw this exception in assay 3, which also shows a general greater sensitivity toward detection of androgens. For the antiandrogenic chemicals, differences of IC_{50} values and calculated relative antiandrogenic potencies between the assays remained well within one order of magnitude.

The CVs we obtained for the different EC_{50} and IC_{50} values stayed generally within the same range, and we found no obvious differences in performance between assays. Discrepancies among IC_{50} values may stem from the fact that all laboratories tested the antiandrogens with a fixed concentration of 0.1 nM androgen, giving rise to diverging percentages of maximum induction. Different metabolic capacities of each cell line for each compound may also play a role. Differences in RAP may reflect differences in metabolism of the test compound, DHT, or both. This could explain why 4-androsten-3,17-dione showed considerably greater differences than the other three test compounds and the positive controls.

In summary, all four cellular *in vitro* assays proved sensitive screening tools to detect and quantify AR-mediated androgenic and antiandrogenic effects of these chemicals with reasonable accuracy. We did not design this experiment to test which assay was best for screening purposes, because a number of other factors must be taken into account. All laboratories have reported specificities of the assays in the original publications, which should be considered when deciding which assay to use for screening purposes. In choosing a test system to use, the equipment of the laboratory, specific background and experience of the staff, and cost-effectiveness must also be evaluated.

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