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Screening of some anti-androgenic endocrine disruptors using a recombinant cell-based in vitro bioassay

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Abstract

The present work describes the development and optimization of a cell-based androgen reporter assay using the Chinese hamster ovarian cell line (CHO K1) in the 96-well format. The recent reports on increasing exposure of humans and wild-life to environmental endocrine disrupting chemicals (ED) prompt the need for high throughput screening systems for such compounds in environmental and biological samples. To this end, CHO cells were cotransfected with plasmids encoding mouse mammary tumour virus-neomycin-luciferase and human androgen receptor (hAR), and a stable cell line was established. After selection with neomycin, a highly active clone was obtained which stably expressed both the hAR and the androgen-responsive luciferase reporter. Stimulation of the cells with androgens for 24 h resulted in about 15-fold stimulation of luciferase activity, with the minimum effective dose of testosterone being 0.1 nmol/l. Potent steroidal and non-steroidal anti-androgens, such as hydroxyflutamide and cyproterone acetate, significantly inhibited the androgen-induced transactivation. Non-androgenic steroids like estradiol, progesterone, dexamethasone and cortisol showed weak activity at high concentrations. RT-PCR and western blot confirmed proper transcription and translation as well as stable expression of the AR gene in the cells. About 60 different chemicals (mostly pesticides or their metabolites, and common industrial chemicals) were screened with the cell line for their ability to stimulate luciferase activity or inhibit that evoked by 0.1 nmol/l R1881, used as a positive androgenic control. About 10 highly potent anti-androgenic chemicals were identified. The most potent anti-androgenic compounds identified in our assay included bisphenol A, α-hexachlorocyclohexane, vinclozolin and 4,4-DDE. These compounds had alone either no effect or were weak agonists (with cytotoxic effects at very high concentrations), but none showed any significant agonistic activity. In conclusion, we demonstrate that the bioassay based on this cell line provides a reliable test for detecting androgenic and anti-androgenic compounds. The 96-well plate format makes the assay suitable for high throughput screening.

Keywords: Human androgen receptor; CHO cells; Endocrine screening; Stable cell line; Bisphenol A; α-Hexachlorocyclohexane; Vinclozolin; 4,4-DDE

1. Introduction

Androgens are the hormones that play a pivotal role in the development and maintenance of the male sex characteristic. Their biological effects are mediated by the ubiquitously expressed androgen receptor (AR). The levels of AR change in different pathological conditions such as malignancies or in response to physiological changes of the endocrine system. Upon ligand binding, the cytosolic AR translocates to the nucleus where it binds to the regulatory regions of androgen-responsive genes and subsequently stimulates their transcription [1,2]. Anti-androgenic compounds, respectively, bind to the AR, but block its transcriptional activity.

In addition to endogenous steroid hormones, an increasing number of natural products and industrial chemicals, such as pesticides and fungicides, have been identified as AR agonists and antagonists. These compounds have the capability to alter male and female sexual functions, are generally considered as endocrine disruptors, and have become an important environmental concern [3–5]. Numerous reports exists on testing of the estrogenic activity

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of these chemicals, but limited information is available on their androgenic or anti-androgenic nature. Recently, Sohini and Sumpter [6] showed that environmental estrogens also possess anti-androgenic activity. A similar study was performed by Sultan et al. [7] using an in vitro bioassay where a line of AR-deficient PC-3 cells was stably transfected with a human AR expression vector and the reporter gene MMTV-luciferase. The cell line was characterized by its response to androgens and anti-androgens, as reflected by the expression of luciferase activity. Numerous studies have been made for AR activation using the human prostate carcinoma cell lines LNCaP [8] and DU-145 [9], but neither is able to discriminate between androgenic and anti-androgenic compounds. Therefore, the latter cell line needs to be cotransfected with AR and androgen-responsive reporter gene. AR co-transfection assays have also been performed in naïve cells (COS 1, CHO, COS 7, CV 1 [10–13]). Raivio et al. [10] in their in vitro bioassay cotransfected the COS 1 cells with AR-interacting protein 3 (ARIP3) to increase the sensitivity of the assay. These transient transfections do not reflect physiological conditions because the target DNA sequences are overexpressed and maintain their responsiveness only for a limited time. Hence, the most convenient approach would be to create cell lines stably transfected with androgen-responsive reporter genes. Until now, three such reporter systems have been described for screening of environmental chemicals. All of them employ cell lines lacking the endogenous AR and were stably cotransfected with either human or rat AR expression plasmid in combination with a reporter plasmid containing either chloramphenicol acetyl transferase (CAT) or a luciferase genes under transcriptional control of the MMTV promoter [13–15]. Recently Blankvoort et al. [2] developed a stably AR reporter gene expressing cell line, the androgen receptor-mediated luciferase expression assay (AR-LUX) system, utilizing the endogenous AR of these cells [2].

The main aim of the present study was to develop a robust cell-based in vitro bioassay to screen different environmental chemicals for their androgenic and anti-androgenic activities in a 96-well format. A stable cell line was made with CHO K1 cells, stably transfected with AR and the androgen dependent promoter driven luciferase reporter gene. Using this recombinant cell line about 60 environmental chemicals were tested for their anti-androgenic activities.

2. Materials and methods

2.1. Chemicals

All the chemicals were purchased from Sigma (St. Louis, MO), unless otherwise stated. The steroids (R1881, progesterone, dexamethasone, estradiol) and RU486 were kind gifts of Dr. Willem Schoonen of N.V. Organon (Oss, The Netherlands). The 57 different compounds tested in this bioassay were obtained from commercial sources.

2.2. Cell lines

CHO cells were obtained from American type culture collection (ATCC, Bethesda, MD). The cells were grown in 75 cm^2 culture flask using DMEM:F12 (1:1) mixture without phenol red (GIBCO, BRL, Inchinnan, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS, Autogen Bioclear, UK), penicillin (10^5 U/l) and streptomycin (100 mg/ml) (GIBCO, BRL) in a humidified 5% CO_2 incubator.

All the treatments of the cells were performed with charcoal-stripped FCS to reduce the contaminating steroids from the serum.

2.3. Stable transfection

Stable transfections were performed by the standard calcium phosphate method. About 16 h before transfection the cells were plated on a 100 mm diameter plate. A 1 ml aliquot of 2 M calcium phosphate solution mixed with 20 μg of pMMTV-neomycin-luciferase and 5 μg of pSG5-hAR-puro (both kindly provided by Dr. Charles Sultan, University of Montpellier, France) was added to the culture dish in 10 ml of culture medium. After 6 h, the precipitate was removed and the cells were supplemented with DMEM:F12 medium with 20% FCS. After 48 h, the cells were trypsinized and fresh cells were plated on a 100 mm diameter plate and selected in medium containing 1 g/l of neomycin (G418) (Promega, Southampton, UK) and 1 mg/l puromycin (Sigma, St. Louis, MO). The medium was changed two times a week. After about 3 weeks, some neomycin and puromycin resistant clones appeared in the plates. They were picked using cloning rings and replated in 24-well culture plates. Confluent cells were split into triplicate in 24-well plates. When these cells were confluent, one plate was treated with 1 nmol/l of R1881, and the incubation continued for 24 h. Thereafter, the cells in the treated plate and in one untreated plate were assayed for luciferase activity using the luminescence kit (Roche, East Sussex, UK). The clones showing highest activity in the presence of R1881 and with low background were selected from the remaining untreated plate. The three clones picked were named as the CHO-AR-Luc cell line (clones 1, 12 and 15).

During the assay, about 20,000 cells per well were plated on 96-well plate in 200 μl of DMEM-F12 medium without phenol red and with 10% FCS. The next day the cells were washed with PBS and the medium was changed to 200 μl DMEM-F12 with 10% charcoal-stripped FCS. After about 3 h, the test compounds (see Table 1) were added on the cells in a volume of 10 μl in medium. The concentrations of the stock solutions of all the test compounds were 1 mmol/l in ethanol. They were then further diluted in the medium, resulting in the final concentration of ethanol in the incubations of 0.01%. The cells were incubated with the compounds for another 24 h. Luciferase activity was measured

Table 1
The chemical compounds tested for anti-androgenic effects in the CHO-AR-Luc cells

Compound name	Exposure route/biological actions	Anti-androgenic activity
• 2-Naphtol	Pesticide	–[a]
Alkylphenols:		
• 4-Nonylphenol	Industrial chemical/degradation product	–
• 4-*n*-Octylphenol	Industrial chemical/degradation product	–
• 4-Tert-octylphenol	Industrial chemical/degradation product	–
• 4-Tert-butylphenol	Industrial chemical (resins, detergents)	–
• 4-Sec-butylphenol	Industrial chemical	–
• 4-Tert-pentylphenol	Industrial chemical (resins, detergents)	–
• 2-Tert-Butyl-4-hydroxyanisole	Industrial chemical (food antioxidant)	+
• Bisphenol-A	Industrial chemical (plastic monomer)	++
• 4-Nonylphenol diethoxylate	Degradation product of alkylphenol polyethoxylates	–
• 4-Nonylphenol dodecyloxylate	Industrial chemical (surfactant)	–
• 4-Octylphenol diethoxylate	Degradation product of alkylphenol polyethoxylates	–
Hydroxybiphenyls:		
• 4,4′-Dihydroxybiphenyl	Industrial chemical	–
• 2-Hydroxybiphenyl	Industrial chemical (rubber additive)/fungicide	–
• 4-Hydroxybiphenyl	Industrial chemical (rubber additive resins)	–
Halogenated organics:		
• α-Hexachlorocyclo hexane	Impurity in the pesticide lindane	++
• β-Hexachloro cyclohexane	Impurity in the pesticide lindane	–
• γ-Hexachloro cyclohexane	Active ingredient in lindane	–
• Vinclozolin	Fungicide	++
• Perthane	Pesticide	–
• Kepone	Pesticide	++
• Aldrin	Pesticide	–
• Heptachlor	Pesticide	–
• Methoxychlor	Pesticide	++
• Atrazine	Pesticide	–
• Dieldrin	Pesticide	++
• Dicofol	Pesticide	+
• Hexachlorobenzene	Pesticide/fungicide/byproduct in industrial processes	–
• Pentachlorobenzene	Pesticide/metabolite of hexachloro benzene	–
• 2,4′-DDT	Impurity of the pesticide 4,4′-DDT	+
• 4,4′-DDT	Pesticide	?
• 2,4′-DDE	Metabolite of 2,4′-DDT	+
• 4,4′-DDE	Metabolite of 4,4′-DDT	++
• 2,4′-DDD	Metabolite of 2,4′-DDT	–
• 4,4′-DDD	Metabolite of 4,4′-DDT	–
• α-Endosulfan	Pesticide	+
• β-Endosulfan	Pesticide	?
• 4,4′-Dichlorodiphenyl sulphone	Industrial chemical/byproduct in manufacture of pesticides	–
• Chlorendic anhydride	Flame retardant	–
• Tetrabromobisphenol-A	Flame retardant	–
• Tetrabromophtalic anhydride	Flame retardant	–
• Hexabromobenzene	Flame retardant	–
• 2,2′4,4′,5,5′-Hexabromobiphenyl	Flame retardant	–
• Octabromobiphenyl	Flame retardant	–
• Decabromobiphenyl	Flame retardant	–
• Hexachlorocyclopenta-diene	Flame retardant	–
• Pentabromochlorocyclo-hexane	Flame retardant	–
• 2,4,6-Tribromoaniline	Flame retardant	–
• 1,2,5,9,6,10-Hexabromocyclododecane	Flame retardant	–
• 2,4-Dibromophenol	Flame retardant	–
• 2,4,6-Tribromophenol	Flame retardant	–
• Pentabromophenol	Flame retardant	–
• Pentachlorophenol	Wood preservative/herbicide	?
Phtalates:		
• Diethylphtalate	Industrial chemical (solvent)	–
• Di-*n*-butylphtalate	Insect repellent	–
• Di-(2-ethylhexyl)phtalate	Industrial chemical	–
• *n*-Butylbenzylphtalate	Industrial chemical	–

[a] No effect; +, weak anti-androgen; ++, strong anti-androgen; ?, unclear response.

using the luciferase reaction kit from Roche according to the manufacturer's instruction.

2.4. Isolation of RNA and RT-PCR

CHO cells were left untreated or treated as described previously, and were grown on 100 mm dishes until 80% confluency. The cells were washed with PBS twice, total RNA was isolated using the single step method of Chomczynski and Sacchi [16]. The concentration of total RNA was determined by reading the O.D. at 260 nm. One microgram of total RNA was used as template for reverse transcription by using the MMLV reverse transcriptase and oligo-dT primers (Promega, Madison, WI). Oligonucleotide primers (18–24-mers) were designed from areas conserved in the published sequences of the human AR and rat glucocorticoid receptor (GR) cDNA sequences, respectively. Primers used in the PCR were as follows: for human AR forward, 5′-AGATGGGCTGATTTCCCAGAAAG-3′; reverse, 5-ATGCTGTCATTCAGTACTCCTGGA-3′; product size 204 bp; for rat GR forward, 5′-GCCTTGGGTTGGAGATCATA-3′, reverse, 5′-TCATGCATGG AGTCCAGAAG-3′; product size 344 bp. Amplification was achieved using 25 PCR cycles with *Taq* polymerase (94 °C for 30 s, 50 °C for 75 s and 72 °C for 90 s). Each PCR product was separated on 1.8% agarose gel in 0.045 M Tris-borate-EDTA buffer (pH 8.0) and visualized by ethidium bromide staining. PCR amplification of the housekeeping gene, β-actin (product size 256 bp), was performed as control.

2.5. Western blot analysis

Immunoblotting was performed as previously described [17,18]. Briefly CHO cells were cultured on 10 mm dishes and after pre-incubation for 16 h with 10 nM R1881, the cells were lysed directly in the dish with 50 μl of lysis buffer (160 mM Tris, pH 6.9, 200 mM DTT, 4% SDS, 20% glycerol, 0.004% bromophenol blue) in the presence of a protease inhibitor cocktail (Sigma). The lysate was boiled for 10 min and cellular debris pelletted at 13, 000 × *g* for 10 min. The extracts were immunoblotted with a polyclonal antibody for AR (kindly donated by Dr. Jorma Palvimo, University of Helsinki, Finland). The signal was detected by using the ECL kit from Amersham.

3. Results

3.1. Levels of expression of AR in stable cell line

A single-step transfection procedure using calcium phosphate was used to obtain a CHO cell line stably overexpressing AR and the androgen-responsive reporter gene. About 10 neomycin and puromycin-resistant clones were obtained after selection with incubation in the presence of 10 nmol/l of R1881. Some of the clones were found to be highly active in the presence of androgen. Clones 1, 12 and 15 showed the highest luminescence response to R1881 (Fig. 1). Of these three clones, number 12 was slightly more active than the other two, and it was therefore selected for further studies.

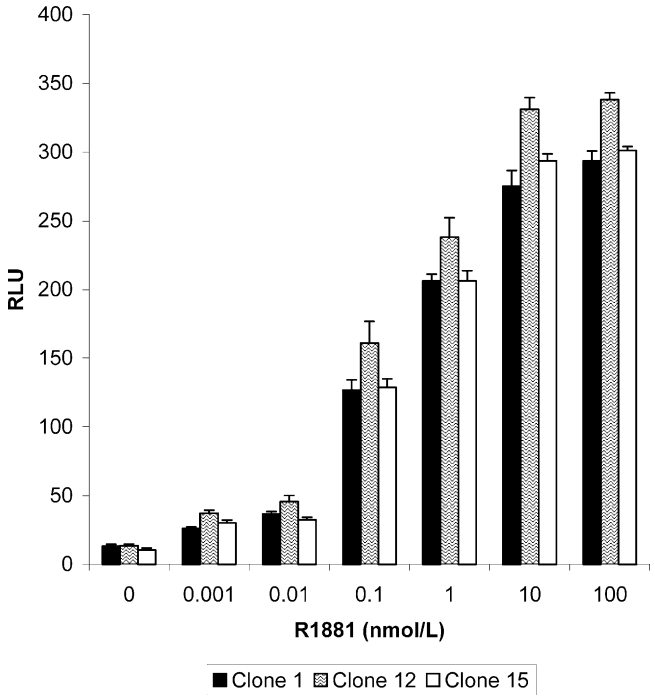

Fig. 1. Functional characterization of the stably transfected CHO-AR-Luc cell lines. Three most active clones (no. 1, 12 and 15) were selected and incubated at increasing concentrations of R1881. The values represent the mean ± S.D. of one of three similar experiments, each performed in quadruplicates. RLU denotes the relative luminescence units.

The expression levels of AR were analysed until passage 20, and they remained stable, even after freezing. Thereafter, the cells showed gradual reduction in AR activity (result not shown).

Fig. 2A shows the transcriptional activity of the transfected AR cDNA, following isolation of total RNA and RT-PCR amplification. The finding indicated stable AR expression in clone 12 of the cells, while no AR was expressed in untransfected CHO cells.

Fig. 2B shows a western blot of four different positive clones of CHO-AR-Luc cells. Proteins isolated from clones were blotted using an AR antibody. An immunoreactive protein band with apparent molecular weight of 110 kDa was observed in all clones tested, with slight variation in their levels of expression. This band was absent in untransfected CHO cell. Another band of approximate size of 72 kDa was observed in all the clones, and it is apparently a degradation product of the larger immunoreactive AR protein. This band was also absent in untransfected CHO cells. Treatment of the cells with testosterone resulted in slight elevation of its expression, probably due to stabilization of the receptor following ligand binding (data not shown).

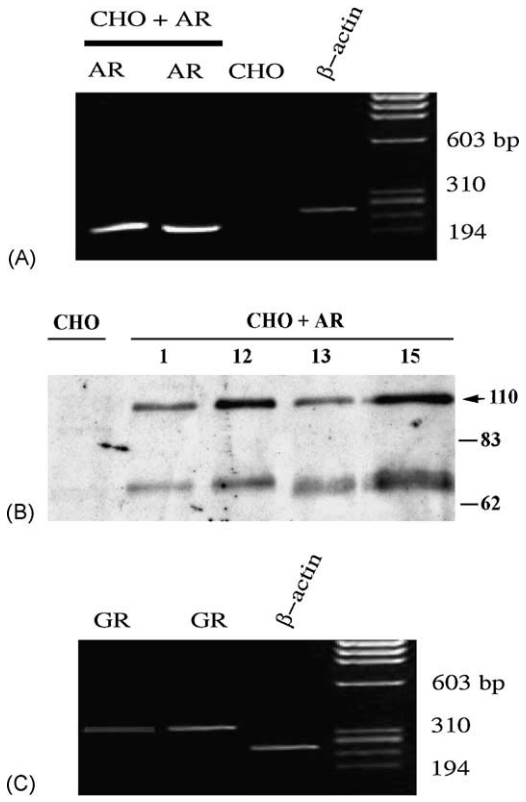

Fig. 2. (Panel A) RT-PCR analysis of AR expression in the stably transfected CHO-AR-Luc cell line (clone 12, in duplicate). The 194 bp amplicon corresponds to the expected size of cDNA (lanes AR). β-Actin was amplified as control of quality of RNA. (Panel B) Immunoblot analysis of expression of AR in CHO-AR-Luc cell lines. The four lanes show the blots of proteins isolated from four different positive clones (1, 12, 13 and 15) out of selected ten clones. The expected size of the AR protein is about 110 kDa. The additional band of about 72 kDa may be a degradation product of full length AR. Untransfected CHO cells (left lane) shown as negative control. (Panel C) RT-PCR analysis of GR expression in CHO-AR-Luc cell line. A 310 bp amplicon corresponds with expected size of cDNA (lanes GR). β-Actin was amplified as control of quality of the RNA.

3.2. Optimization of cell number for androgen bioassay

The effect of cell number per well in the 96-well format assay was determined in the CHO-AR-Luc cell line. Fig. 3 shows that an increase in the cell number from 2500 to 40,000 per well resulted in a progressive rise in luciferase activity. Further increase in cell number beyond 30,000 did not increase the luciferase activity. We therefore optimized the cell number to 30,000 cells per well of the 96-well plate.

3.3. Kinetics of the luciferase response to androgen

The cells were incubated with 1 nmol/l of R1881 up to 36 h, and the luciferase activity was measured in cell lysates. Fig. 4 shows that the luciferase activity was clearly detectable at 4 h and increased in a progressive fashion about five-fold when the incubation was continued up to 36 h. This finding demonstrated that the cell line has the potential to

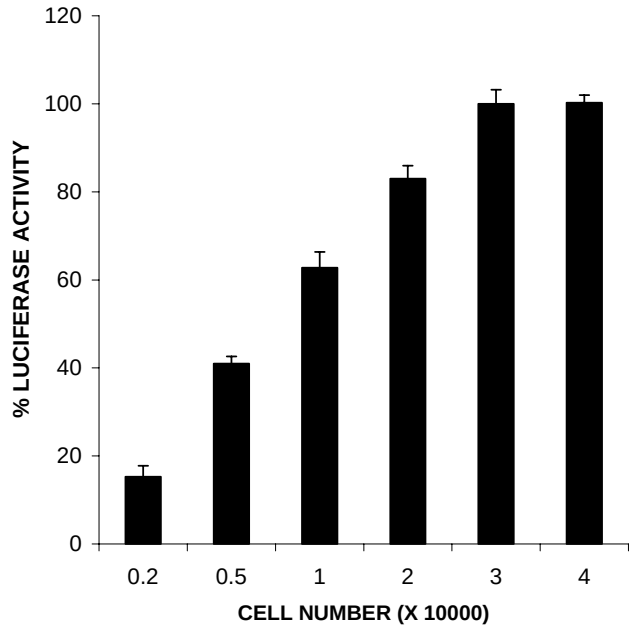

Fig. 3. Optimization of the number of cells for luciferase induction in cell clone 12 on stimulation with R1881. The cells were stimulated with 1 nmol/l R1881 for 24 h. The results are expressed as percentage of luciferase activity, measured with 30,000 cells (100%). The results represent the mean ± S.D. of three similar experiments each performed in quadruplicate.

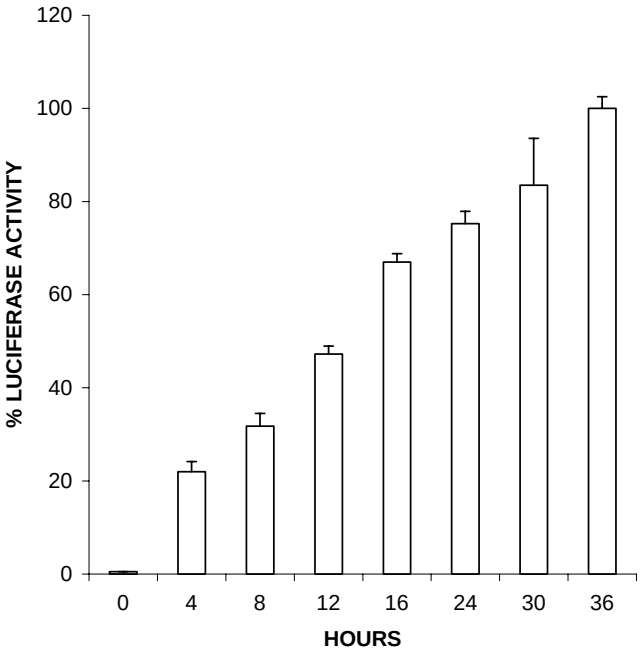

Fig. 4. Time course of luciferase induction in CHO-AR-Luc cells by R1881. The cells were incubated with 1 nmol/l R1881 for 0–36 h. The results are expressed as percentage of luciferase activity, taken the mean activity at 36 h as 100%. The results represent the mean ± S.D. of three similar experiments performed in quadruplicate.

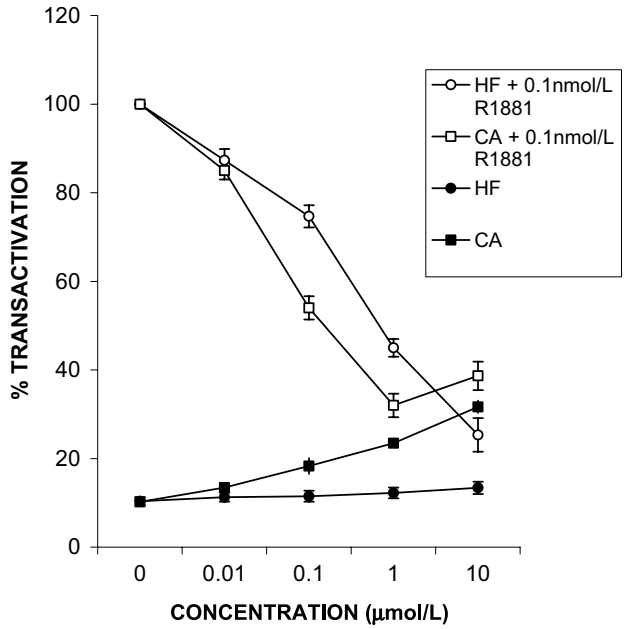

Fig. 5. Demonstration of antagonistic activities of selected anti-androgens in CHO-AR-Luc cells. Cells were incubated at increasing concentration of anti-androgens alone or in the presence of 0.1 nmol/l R1881. The mean transactivation obtained with 0.1 nmol/l R1881 was given a value of 100%. The values represent the mean ± S.D. of three similar experiments each performed in quadruplicates. CA, cyproterone acetate; HF hydroxyflutamide.

be used for measuring androgenic activities of compounds that are stable even for short duration.

3.4. The effect of known potent anti-androgens on luciferase activity of CHO-AR-Luc cells

The effects of two potent anti-androgenic compounds, one steroidal (cyproterone acetate, CA) and one non-steroidal (hydroxyflutamide, HF) were tested in the cell line (Fig. 5). The anti-androgenic activity was checked by treating the cells with increasing concentrations of anti-androgens in the presence of the half-maximally stimulating concentration of 0.1 nmol/l R1881. In this cell line, CA was found to be slightly more potent an anti-androgen than HF. However, at 1 μmol/l concentration, CA alone showed a slight agonistic activity by displaying a mild elevation in the luciferase activity. A further increase in concentration of CA resulted in a 40–50% elevation of transcriptional activity (data not shown), which finding proves that CA is a partial AR agonist and antagonist.

3.5. Specificity of the CHO-AR-Luc cell line to stimulation with other steroids

We next examined several non-androgenic steroids for their eventual stimulating effects on the cell line. The cells were stimulated with estradiol, progesterone, dexamethasone and cortisol. All compounds were devoid of activity below the concentration of 1 nmol/l. As shown in Fig. 6, estradiol and progesterone did not have significant activity until 100 nmol/l concentration, which is 1000-fold higher than obtained with the reference compound, R1881. At 1 μmol/l concentration, both estradiol and progesterone resulted in about 5 and 8% transactivation, respectively, as compared to maximum effect of the reference compound. A surprising finding was the significant transactivation by cortisol and dexamethasone, up to 13 and 22% of the reference compound, respectively. The specificity of these steroidal activities was checked by using their antagonist, RU486. When the cell lines were treated with this potent anti-glucocorticoid, in the presence or absence of the corresponding agonist, it did not have any significant agonistic activity, as reported in some other cell lines, even at 1 μmol/l concentration [17] (result not shown). Instead, RU486 inhibited the stimulatory response to dexamethasone (Fig. 6), which implied that the dexamethasone effect occurred through activation of the glucocorticoid receptor (GR) in this cell line. Indeed, Fig. 2C demonstrates by RT-PCR that GR mRNA is expressed in the CHO-AR-Luc cell line. This suggests that the presence of GR in the cell line explains the 25% elevation of transactivation, since both GR and AR act through the same MMTV promoter.

3.6. Screening of activity of various androgenic and anti-androgenic compounds using the CHO-AR-Luc cell line

Several compounds were tested for their androgenic and anti-androgenic activities (Fig. 7, Table 1). The activities of all the compounds tested were measured against that of 0.1 nmol/l of R1881. The compounds used were of various classes and they can in general be called anthropogenic chemicals showing endocrine disrupting activities. Vinclozolin, a potent fungicide, inhibited the action of 0.1 nmol/l of R1881 at a concentration of 1 μmol/l. As a whole, out of about 60 different compounds tested, the compounds 4,4-DDE, bisphenol A, hexachlorocyclehexane (HCHα), dieldrin, vinclozolin and methoxychlor showed very significant anti-androgenic activities (IC_{50} values 20.9, 19.6, 7.7, 62.8, 3.9 and 34.2 μmol/l, respectively) whereas HCH β and γ were weaker anti-androgens on the basis of our assay, with IC_{50} values above the detectable range. For most of the compounds, the lowest concentration of maximum effect was between 1 and 10 μmol/l. At this concentration for most of the compounds tested, the percentage transactivation was between 40 and 60, as compared to 100% transactivation with 0.1 nmol/l R1881. Some of the compounds also seemed to show significant inhibition at about 50 μmol/l concentration (data not shown). No cytotoxic effects were observed even at this concentration, which confirms that the compounds specifically inhibited the transactivation of AR. However, none of the compounds appeared to have any androgenic activity in our cell line up to the

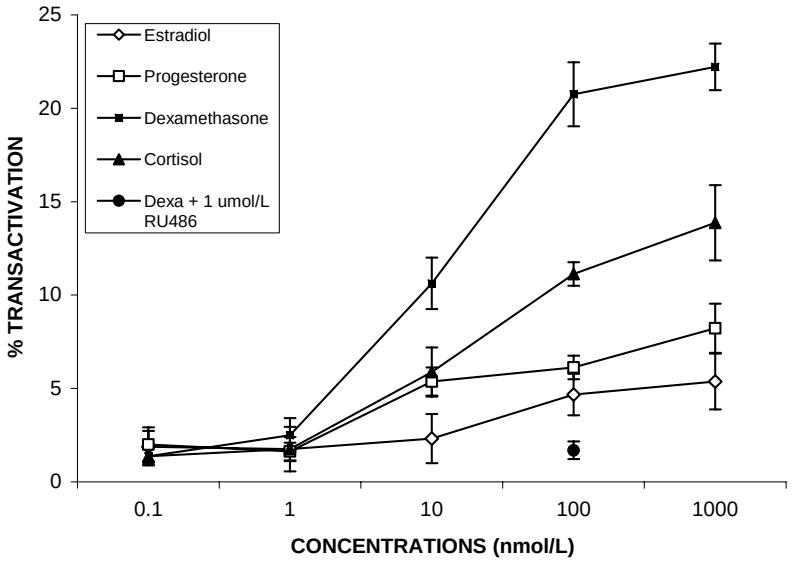

Fig. 6. Demonstration of estrogen, progesterone, glucocorticoid effects in the CHO-AR-Luc cells and inhibition of glucocorticoid effects by RU486. The luciferase activity was expressed as percentage of mean response obtained in the presence of 0.1 nmol/l R1881. The values represent the mean ± S.D. of three similar experiments performed in quadruplicates.

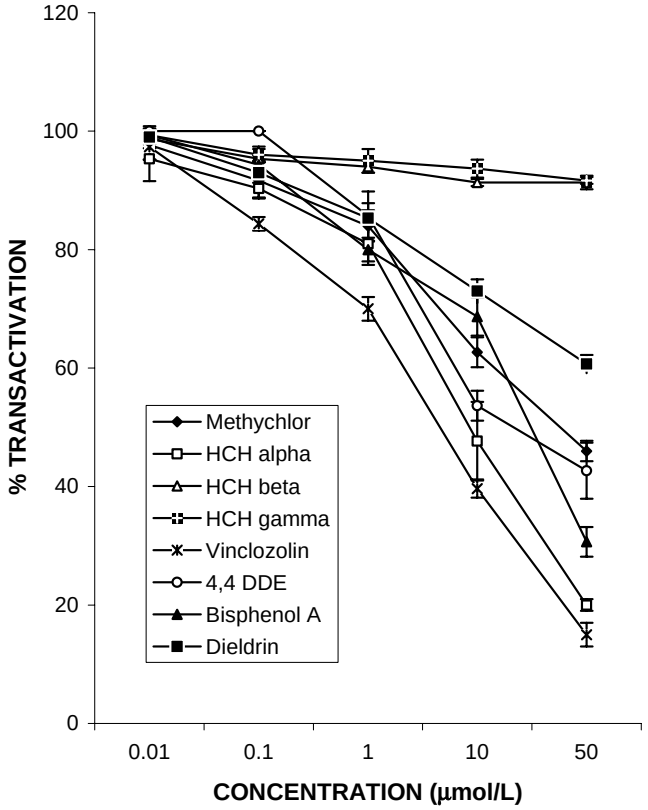

Fig. 7. Determination of anti-androgenic activities of some endocrine disrupting chemicals with clear endocrine disrupting nature. Cells were treated with various concentrations of the chemicals (0.1–50 μmol/l) in the presence of 0.1 nmol/l R1881. Luciferase activities were expressed as percentage of that obtained with 0.1 nmol/l of R1881 which was given the value of 100%. The values represent the mean ± S.D. of three similar experiments performed in quadruplicates (HCH, hexachlorocyclohexane).

concentration of 50 μmol/l, when tested alone (results not shown).

4. Discussion

Many endocrine disrupting compounds identified so far are persistent organochlorine pesticides (e.g. DDT, methoxychlor and dieldrin). In addition, the endocrine activity of numerous commonly used compounds like industrial wastes, fungicides, pesticides has not been studied. Therefore, good and user friendly assay systems are needed for the screening of any potentially androgenic or anti-androgenic compounds. Numerous methods are available for this screening, each with some advantages and disadvantages. For example, in in vivo screening, castrated and intact animals have been used [18–21]. The anti-androgenic activities in vitro have been assessed using cellular models in the same fashion as has been developed earlier for studying agonistic and antagonistic properties of ligands in other steroid receptors. These models usually use cells expressing endogenously the hormone receptor of interest, like MCF 7 cells with estrogen receptor (ER) [22] and T47 D cells with progesterone receptor (PR) [23]. Besides the endogenous ER and PR, respectively, these cells were stably transfected with a steroid-responsive reporter gene.

Only few models have been developed that stably express the functional AR and an androgen-responsive reporter gene, suitable for screening of androgenic and anti-androgenic compounds. Battman et al. [24] used the breast cancer cell line T47 D, expressing endogenously the AR and stably transfected with a MMTV-CAT reporter gene construct. However, because this cell line also expresses the PR, it

is unspecific for the screening of androgenic activity. Subsequently, a number of androgen-responsive stable cell lines were created by different groups. Blankvoort et al. [2] reported the T47 D cell line utilizing endogenous AR. There are reports for using monkey kidney cell line, CV 1 [14], prostate cell lines [15,17] and very recently the use of MDA-MB-453 cell [25,26] for screening of androgenic activity. In addition to these cells, Raivio et al. [10] reported a bioassay using the COS 1 cells, where the sensitivity of the assay was improved using the AR-interacting protein 3 coactivator. However, this assay system is quite complicated and requires transient transfections. In addition, there are three very recent reports on the use of CHO cell lines for androgenic transactivation assays. Two of them utilize the transient transfection assay with hAR and ARE-Luc constructs [13,27] and the third one, is a stable CHO cell line expressing the hAR and MMTV-neomycin-luciferase constructs [28]. There is no doubt that CHO cells provide an excellent model for in vitro assays, but the transient transfections also have several disadvantages, including the need of repeated transfection in each assay, the transient cellular response and above all the inter-assay variability of results due to variation of transfection efficiency. On the other hand, the cell line developed by Paris et al. [28] was neither tested for specificity using other non-androgenic steroids, nor for suitability for testing of any EDS.

We developed a CHO cell line stably overexpressing the hAR and MMTV-Luc reporter plasmids. This system is well suited for in vitro screening of the androgenic or anti-androgenic activity of chemical compounds due to its high sensitivity and simplicity. An additional advantage is that the assay could be adapted for the 96-well format suitable for high throughput screening, using the bioluminescent detection system. The cytotoxic effects of the compounds to be tested on the cells were examined by the use of cell proliferation reagent WST-1 (Roche). Most of the compounds were found non-toxic to cells even at the high concentration of 50 μmol/l. The same finding was made with R1881 up to the concentration of 100 nmol/l, exceeding the physiological androgen concentration. However, the reduction of serum concentration from 10 to 2% upon the stimulation increased cell death in the next 12–24 h. Therefore, all assays were performed with 10% of charcoal-stripped fetal calf serum.

The effects of synthetic anti-androgens (HF and CA) on the cell line correlated well with earlier reports of bioassay on naïve cell lines such as CV 1 [11], CHO [13,27] or prostatic cell line DU-145 [9] and PALM cell line [17]. There can definitely be variation of IC_{50} values of two different antagonists with earlier reported values in other cell lines. This discrepancy can be attributed to the presence of different coactivators in different cell lines. It is conceivable that differences in expression pattern and levels of coactivators of the AR [9,29] contribute to the differences as observed in actions of various anti-androgenic compounds in different cell lines [2,30]. Another possible explanation could be the interference through other signal transduction pathways or squelching of common transcription factors [2].

The androgen specificity of the cells was tested with some other steroids, such as progesterone, estradiol, dexamethasone and cortisol. Progesterone and estradiol were almost totally inactive up to 10 nmol/l concentration, while both dexamethasone and cortisol were active at and above 10 nmol/l concentration resulting in about five to eight-fold induction of luciferase activity as compared with the other two non-androgenic steroids, estradiol and progesterone. Previous studies have shown that the potent synthetic glucocorticoid, dexamethasone, is able to induce transactivation in CHO cells, which contain the endogenous GR, although at a relatively low level [31,32]. In addition, previous results have shown that in CHO cells transfected with MMTV-Luc plasmid, dexamethasone caused a five-fold induction of luciferase activity [27]. However, according to Vinggard et al. [27], even at a concentration of 10 μmol/l, dexamethasone showed no effect. This discrepancy may be due to a very low level of functionally active GR in their CHO cells. Those cells had gone through several passages during many months of culture and may have lost the ability to express the GR. However, as our results clearly show, it is very important to establish the response to glucocorticoids in an androgen assay based on CHO cells [27]. In our cells, RU486 (a dexamethasone and progesterone antagonist) blocked this activity specifically through GR. The RT-PCR detection of GR mRNA indicated the presence of this transcript at low level compared to AR. Our current data also showed some partial agonistic activities for estradiol and progesterone. Because it has been reported that CHO cells do not contain estrogen or progesterone receptors, the estrogen and progesterone effects are presumably mediated via the AR [27,28].

The main aim of this study was to develop a model to monitor different androgenic and anti-androgenic compounds using an in vitro cell model. We analysed about 60 different compounds of pesticide, fungicide or organochemical class in this model (Table 1). Some of them were found to be anti-androgens but none had androgenic activity. The most potent of them was vinclozolin, as was previously demonstrated both in in vitro and in vivo [13,33–37]. Also some other chemicals tested were found to be highly antagonistic to androgenic action. Most of the compounds that were found to be anti-androgenic in our assay have earlier been reported as estrogenic [7,38], vinclozolin has not yet been reported to be estrogenic. These results reconfirm the effects of these compounds in our CHO cell line transactivation assay, since it is known that most of the environmental anti-androgens are estrogenic in nature. In addition, these compounds have been analysed in other in vitro cell-based assay systems such as T 47D [2], MDA-Kb2 [25] and transiently transfected CHO cells [13,27]. HCHα seems to be strong antagonist while the β and γ forms do not seem to have any strong antagonistic activity, as has been reported before [15]. This is the first ever report of screening these

compounds in a stably transfected AR and reporter overexpressing cell line.

In summary, recombinant cell-based in vitro assays are becoming increasingly attractive as screening tools for the endocrine activity of chemicals and environmental samples. Their clear assets are the speed and sensitivity, but they also reduce the need of animals for in vivo screening. The cell lines are excellent tools in determining potential mechanism of action of endocrine disrupting compounds. Each type of assay has its own advantages and specificity. The assays using CHO-AR-Luc cell lines are sensitive and generate highly reproducible data. A main advantage of this cell line is that it is highly robust and grows very well, without the need of specified medium. Above all we have optimized the assay to the 96-well format for high throughput screening. It provides a very accurate screening tool for the determination of androgen agonists and antagonists. The only problem that remains with this cell line is that it expresses a low level of endogenous GR which might interfere with screening. To consider any compound as androgenic with this cell line, it is always recommended to check the percentage of its glucocorticoid activity in that response. This potential source of GR can be eliminated by including anti-glucocorticoids in the incubation. Naturally, this cell line can still be considered a robust model to screen androgenic and anti-androgenic compounds.

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