DOI 10.1002/mnfr.200400023 Mol. Nutr. Food Res. 2004, *48*, 282 – 291

Sensitive *in vitro* test systems to determine androgenic/antiandrogenic activity

Sabine E. Guth, Sonja Böhm, Bernd H. Mußler and Gerhard Eisenbrand

Department of Chemistry, Division of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, Kaiserslautern, Germany

We report on the establishment of one transgenic and two endogenous reporter gene assays to determine androgenic/antiandrogenic activity. A transient transactivation assay was developed in COS-7 African green monkey kidney cells. Three plasmids were co-transfected by electroporation: the human androgen receptor expression vector pSG5AR, the reporter gene vector pMamneoLuc, expressing luciferase under the control of the mouse mammary tumor virus (MMTV) promotor containing 4 hormone responsive elements (HREs), and the control plasmid pSVβ. Transcriptional activation was measured by luciferase-mediated chemoluminescence. In T47D human breast cancer cells two endogenous reporter gene systems were established: one based on the androgen-induced expression of prostate-specific antigen (PSA), the other based on the androgen-repressed expression of testosterone repressed message 2 (TRPM-2). PSA protein was measured by enzyme-linked immunosorbent assay (ELISA), TRPM-2 m-RNA by reverse transcriptase polymerase chain reaction (RT-PCR). All three test systems were validated using the physiological androgen dihydrotestosterone (DHT) as agonist and the established antiandrogens hydroxyflutamide and p,p′-dichlorodiphenylethene (p,p′-DDE) as antagonists. The PSA assay was the most sensitive test system with an EC_{50} of 0.7 nM for DHT-induced response. The transient transactivation assay in COS-7 cells was less sensitive with an EC_{50} of 9.7 nM DHT. In the PSA assay hydroxyflutamide was a more potent antagonist (IC_{30} = 0.02 μM) than p,p′-DDE (IC_{30} = 0.9 μM). In the transient transactivation assay in COS-7 cells, both compounds elicited about 30% of the agonistic response induced by 100 nM DHT, thus showing partial agonistic properties. In summary, three highly sensitive and complementary *in vitro* test systems, together achieving enhanced specificity for detection and assessment of androgenic/antiandrogenic activity have been established and validated.

Keywords: Androgens / Antiandrogens / Dihydrotestosterone / Reporter gene systems

Received: March 1, 2004; revised: April 29, 2004; accepted: April 30, 2004

1 Introduction

Worldwide scientific and public awareness of the potential endocrine effects of certain environmental chemicals has been triggered by observations about disturbances of wildlife fertility as a consequence of anthropogenic contamination of ecosystems by certain pesticides [1, 2]. Hints from epidemiological studies have further stimulated the scientific and public debate on whether human exposure to such environmental contaminants might also adversely influence the hormonal balance of humans and hence might have detrimental health effects. Especially critical periods of urogenital tract and nervous system development *in utero* and during early post-natal life might be sensitive to hormonal disruption [3, 4]. For example, the reported increase in testicular cancer [5] and other disorders of the male reproductive tract like chryptorchidism and hypospadia [6] have been discussed within this context.

At an early stage, attention has primarily been focused on environmental estrogens. More recent investigations have shown, however, that some pesticides and/or their metabolites, such as, *e.g.*, the DDT metabolite p,p′-dichlorodiphenylethylene (p,p′-DDE) [7], metabolites of the fungizides

Correspondence: Dr. Gerhard Eisenbrand, Department of Chemistry, Division of Food and Environmental Toxicology, University of Kaiserslautern, Erwin-Schroedinger-Straße, D-67663 Kaiserslautern, Germany
E-mail: eisenbra@rhrk.uni-kl.de
Fax: +49-(0)631-205-3085

Abbreviations: AR, androgen receptor; **ARE**, androgen response element; **CDFCS**, charcoal-dextran-stripped fetal calf serum; **DHT**, dihydrotestosterone; **EC_{50}**, effective concentration 50%; **GR**, glucocorticoid receptor; **HRE**, hormone responsive elements, **IC_{50}**, inhibition concentration 50%; **MMTV**, mouse mammary tumor virus; **p,p′-DDE**, p,p′-dichlorodiphenylethene; **PR**, progesterone receptor; **PSA**, prostate-specific antigen; **TRPM-2**, testosterone repressed prostate message 2

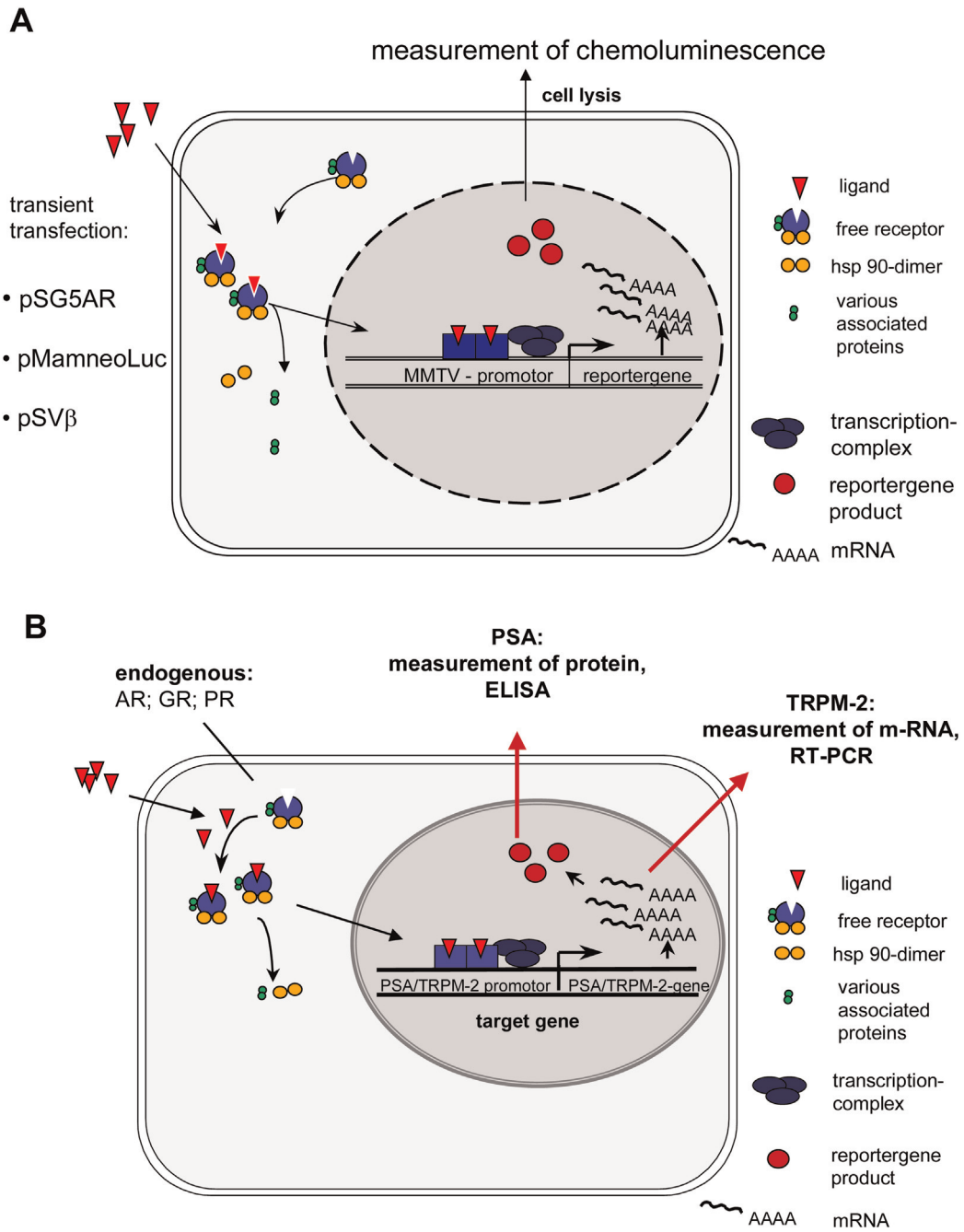

Figure 1. (A) Transient transactivation assay in COS-7 cells, (B) PSA/TRPM-2 assay in T47D cells. (A) The plasmids pMamneoLuc, pSG5AR and pSVβ were cotransfected by electroporation in COS-7 cells. (B) A transfection is not necessary: AR is expressed by T47D cells. (A + B) Ligands enter the cell and bind to the free receptor. The ligand activated receptor dimerizes, is translocated to the cell nucleus and binds to HREs in the promotor upstream of the target gene. Transcription is induced. (A) After cell lysis, the luciferase activity can be measured by chemiluminescence detection. (B) PSA protein is secreted into the culture medium and measured by ELISA. TRPM-2 m-RNA is measured by RT-PCR.

vinclozolin [8] and procymidone [9], the fungizide prochloraz [10] or the herbizide linuron [11] display antiandrogenic properties. These compounds have been found to compete with androgens for the androgen receptor (AR) and thereby to alter androgen-dependent gene expression *in vitro* and *in vivo*. Exposure to environmental androgen antagonists during critical windows of fetal and postnatal development has been discussed to inhibit male sexual development by blocking expression of androgen-dependent genes [12].

Therefore, in addition to rapid screening systems to detect estrogenic/antiestrogenic effects, reliable functional test systems to identify androgenic/antiandrogenic activities also are urgently needed. Estrogenic activity has been assayed earlier *in vitro* using proliferation assays in hormone-dependent cell lines, such as the so-called E-screen assay carried out in the human mammary carcinoma line MCF-7, as a measure for estrogenic/antiestrogenic potential [13]. However, assays measuring just effects on proliferation of hormone-dependent cells do not provide the functional specificity displayed by reporter gene systems. Moreover, for the detection of potentially androgenic/antiandrogenic effects no cell system is available yet based on androgen dependent proliferation. Prostate cell lines such as the established line LNCaP grow rather slowly and express a mutated form of the androgen receptor. In these cells established antiandrogens were found to induce androgenic rather than antiandrogenic effects [14].

In recent years, the development of assay systems to measure androgenic/antiandrogenic activity based on transgenic or endogenous androgen-regulated reporter gene systems has been pursued by several groups [15 – 19]. We here report the establishment of one transgenic and two endogenous reporter gene assays that can be used in parallel as highly sensitive, complementary *in vitro* systems to identify androgens/antiandroges thereby achieving enhanced specificity (Figs. 1 A and B).

The transient reporter gene assay was developed in COS-7 African green monkey kidney cells that do not contain receptors for steroid hormones [20]. For transfection with a steroid hormone receptor expression vector, we used the human expression vector pSG5AR, constitutively expressing the AR under the control of the SV-40 promotor. The androgen-dependent reporter gene vector pMamneoLuc and the control vector pSVβ were cotransfected. The pMamneoLuc contains the mouse mammary tumor virus (MMTV) promotor with four androgen-, corticoid-, and progestin-dependent hormone responsive elements (HREs) [21, 22]. In the cotransfected control vector pSVβ, β-galactosidase is constitutively expressed under the control of the SV-40 promotor. This allows correction for differences in transfection efficiency and cell preparation.

Furthermore, two endogenous reporter gene assays were established in T47D mammary cancer cells. One assay measures androgen stimulated expression of prostate-specific antigen (PSA). The second assay is based on measuring androgenic effects on testosterone repressed prostate message 2 (TRPM-2). By virtue of their endogenous expression, these test systems do not require transfection. They therefore more closely reflect the normal physiological background situation. Moreover, they also offer the perspective of being utilized *in vivo* and therefore allow direct *in vitro, versus in vivo,* comparisons. PSA is a serine protease expressed in epithelium cells of the prostate and in the breast glands [23, 24] under the control of the physiological human PSA promotor. This promotor contains two androgen-responsive elements and an androgen-responsive region [25, 26]. One physiological role that is known for PSA is the liquefaction of semen [24]. PSA is expressed by T47D cells in the presence of androgens and progestins and is secreted into culture medium [15, 27 – 29]. Antiandrogenic activity therefore can be determined by inhibition of androgen-induced PSA expression.

TRPM-2 or clusterin is a multifunctional protein that has been implicated in homeostatic control of lipoprotein metabolism, tissue repair and remodeling, sperm maturation, inhibition of complement-mediated cell lysis, and epithelial cell differentiation. TRPM-2 is induced *de novo* during the regression of the prostate and other hormone-dependent tissues after hormone ablation. Furthermore, it is overexpressed in several human neurodegenerative diseases, including Alzheimer's disease and epilepsy [30, 31]. A number of potential regulatory sequences is present in the TRPM-2 gene, including four androgen response element (ARE) half-sites. These regions either alone or in combination appear of relevance in androgen-induced repression of TRPM-2 gene expression [32]. In T47D cells TRPM-2 is constitutively produced in the absence of the physiological hormone dihydrotestosterone (DHT), whereas in the presence of androgen, the expression of TRPM-2 is abrogated. In contrast, antiandrogens inhibit the repression and, therefore, increase TRPM-2 concentration. This test system therefore allows detection of a positive response induced by antiandrogens. We demonstrate here that all three test systems represent highly sensitive and reliable tools for identifying androgens/antiandrogens *in vitro*.

2 Materials and methods

2.1 Materials

DHT and p,p′-DDE were from Sigma Chemicals (Deisenhofen, Germany), hydroxyflutamide was obtained as a generous gift from Schering AG (Berlin, Germany). The human androgen receptor expression vector pSG5-AR was kindly provided by Dr. Andrew Cato, Forschungszentrum Karlsruhe, Institute of Toxicology and Genetics, Germany. The reporter-gene vector pMamneoLuc and the β-galactosidase control vector pSVβ were from Clontech (Palo Alto, CA, USA).

2.2 Cell culture

COS-7 cells (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and T47D cells (ATCC,

American Type Culture Collection) were grown in RPMI 1640 medium with phenol red, supplemented with 10% fetal calf serum (FCS) and 100 U/mL penicillin/streptomycin (Gibco, Life Technologies, Grand Island, NY, USA). Cells were grown at 37°C with 5% CO_2 in a humidified incubator. For all hormone treatments, cultures were grown in Dulbecco's medium without phenol red, containing 10% charcoal-dextran-stripped fetal calf serum (CDFCS), supplemented with 1.6 mM L-glutamine and 100 U/mL penicillin/streptomycin.

2.3 Transient transactivation assay in COS-7 cells

Confluent 80% COS-7 cells were harvested by trypsinization, centrifuged (1500 rpm about 5 min) and resuspended in ice-cold PBS (pH 7.4). The cell suspension was counted and diluted to 8000 cells/μL. In each of three sterile genepulse chambers, 4×10^6 cells were mixed with 10 μg each of the plasmids pSG5-AR, pMamneoLuc, and pSVβ. Cells were exposed to a controlled electrical field of 1000 μF at 330 V in an Invitrogen electroporator. Each electroporation sample was diluted in 1 mL of serum containing medium. Contents of the three genepulse chambers were mixed and evenly distributed into 58 mm culture dishes. Cells were incubated in normal medium for *ca*. 18 h, then hormones were added for 24 h in CDFCS medium. After an additional period of growth (*ca*. 24 h) in CDFCS medium, cells were lysed in lysis buffer (Promega, Madison, WI, USA). Luciferase and β-galactosidase activities of the lysate were measured in a plate luminometer (BMG LABTECH GmbH, Offenburg, Germany) using luciferase assay reagent (Promega) or, respectively, β-gal substrate (Clontech), as indicated by the manufacturer. Values obtained for luciferase activity were normalized to β-gal activity.

2.4 PSA assay in T47D cells

The assay according to Rosenberg *et al.* [15] was slightly modified. In brief, T47D cells were seeded into 24-well tissue culture plates (50 000 cells/well), using CDFCS medium and were grown for 24 h. Cells were stimulated by adding DHT in DMSO (1 μL) at various concentrations so as to provide a concentration range of 100 nM to 0.01 nM DHT in 0.1% DMSO per well. After 5 days incubation, the tissue culture supernatants were removed for PSA analysis. For detection of antiandrogenic activity, cells were incubated (i) with DHT alone at a final concentration of 1 nM and (ii) first with the test compound for 4 h at final concentrations of 10 μM to 10 nM before DHT (1 nM) was added. Appropriate multiple positive controls (with stimulating steroids) and negative controls (only 0.1% DMSO) were included in each experiment. PSA was measured in the tissue culture supernatants (undiluted) with the PSA-Immulite third generation Assay (DPC Biermann, Bad Nauheim, Germany) on an Immulite Analyzer (DPC Biermann), as indicated by the manufacturer. The determination limit of this assay is 0.003 ng/mL and higher. To control for cytotoxicity, effects on cell growth were measured by the sulforhodamin B (SRB) test [33]. Briefly, after incubation for 5 days, cells were fixed with trichloroacetic acid (50%) and cellular protein was stained with sulforhodamin B (0.4% in 1% acetic acid). After resolubilization with Tris buffer (10 mM, pH 10.5), absorption was measured at 564 nm in an UV-Vis-photometer thus providing a direct measure of cell numbers.

2.5 TRPM-2 assay in T47D cells

2.5.1 Generation of the exogenic internal standard

A 556 bp PCR amplification strand of the TRPM-2 gene (primer: 5′-AAT CCC TCC AGG TCG CTG AGA-3′ and 5′-TTC CCA TGA GCA GAG TCG-3′) was amplified from T47D cells. The amplification strand was again amplified to generate *Hin*dIII and *Xba*I restriction-interfaces (primer: 5′-ATC AAG CTT AAT CCC TCC AGG TCG CTG AGA-3′ and 5′-TTC CCA TGA GCA GAG TCG TCT AGA GGA-3′). These amplification strands and the pUC18-vector (Amersham Pharmacia Biotech, Uppsala, Sweden) were restricted by *Hin*dIII and *Xba*I (Amersham Pharmacia Biotech) and ligated using T 4 DNA Ligase (MBI Fermentas). This modified Vector was transformed by heat shock into the *Escherichia coli* DH5α strain and amplified. The obtained plasmid was digested using the restriction enzyme *PstI* (Amersham Pharmacia Biotech) that provided two cuts within a distance of 96 bp. The shortened DNA-construct was ligated, transformed into *E. coli* and amplified. The linearized shortened plasmid-DNA was used as exogenic internal standard.

2.5.2 Isolation and amplification of mRNA

T47D cells were incubated for 20 h in serum free medium with DHT and the test compounds. RNA was isolated using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction and stored at −80°C. RT-PCRs were performed using the Qiagen® OneStep RT-PCR Kit. TRPM-2 was measured by competitive RT-PCR using 1 μg template mRNA, 40 ng of the linearized deletion mutant described above as a synthetic internal standard, and the following oligonucleotides as primers: 5′-AAT CCC TCC AGG TCG CTG AGA-3′ and 5′-TTC CCA TGA GCA GAG TCG-3′. The conditions of the Qiagen® OneStep RT-PCR Kit were adapted as follows: After a reverse transcription step at 50°C for 30 min, the resultant cDNA underwent an initial PCR step at 95°C for 15 min, followed by 10 cycles consisting of 30 s denaturing at 94°C, 1 min annealing at 55°C and 1 min extension at 74°C. The next 15 cycles

consisted of 30 s denaturing at 94°C, 1 min annealing at 55°C and 1 min + 5 s per cycle extension at 72°C. A final extension for 10 min completed the amplification. Amplification products were detected using agarose gel electrophoresis and ethidium-bromide staining. Intensities of the ethidium-bromide stains at 556 bp (sample) and 460 bp (standard) were quantified using a Lumi-Imager (Boehringer, Mannheim, Germany). Results were normalized by dividing light units detected for the sample by light units detected for the standard. The value obtained for control samples was set as reference point (value 1.0). All other values were evaluated relative to the reference point.

2.6 Evaluation

Dose-response curves: Data were plotted in a semilogarithmic scale. The sigmoidal dose-response curve was fitted to the experimental data using

$$Y = \frac{A1 - A2}{1 + (X/X_0)^P} + A2 \quad (1)$$

where Y equals the term for the sigmoidal dose response. $A1$ is the lower response plateau (receptor not activated), $A2$ is the upper response plateau (saturation of the receptor), P is the power (slope), X_0 is the x-value at 50% activity (inflection point), and X is the mean value of luciferase activity at a given concentration ± standard deviation (SD).

3 Results

3.1 Transient transactivation assay in COS-7 cells

African green monkey kidney COS-7 cells do not express steroid hormone receptors. Cells were therefore transiently cotransfected with an expression vector for the human androgen receptor (pSG5AR), the MMTV-promotor controlled luciferase reporter vector (pMamneoLuc), and a β-gal control vector (pSVβ). The test system allows for the determination of both, androgenic and antiandrogenic activity. The physiological ligand DHT induced a dose-dependent reporter response with an EC_{50} of 9.7 nM (Fig. 2).

To validate the assay for the detection of antiandrogenic effects, the established antiandrogens p,p′-DDE and hydroxyflutamide were used. AR-mediated transcriptional activity induced by 100 nM DHT was significantly inhibited to about 50% inhibition at 1 μM p,p′-DDE and down to about 70% at 10 μM (Fig. 3). At 10 μM, the onset of an androgenic response by p,p′-DDE became apparent reaching about 30% of the activity induced by 100 nM DHT (Fig. 3). This is indicative that p,p′-DDE acts as a partial AR-agonist.

The active metabolite of flutamide, hydroxyflutamide, exhibited slightly less potent antagonistic efficacy as compared to p,p′-DDE, reducing DHT-induced response by about 40% at 1 μM and by about 50% at 10 μM (Fig. 4). Again, partial agonistic activity, comparable to that of p,p′-DDE, became apparent at rather high concentration (Fig. 4).

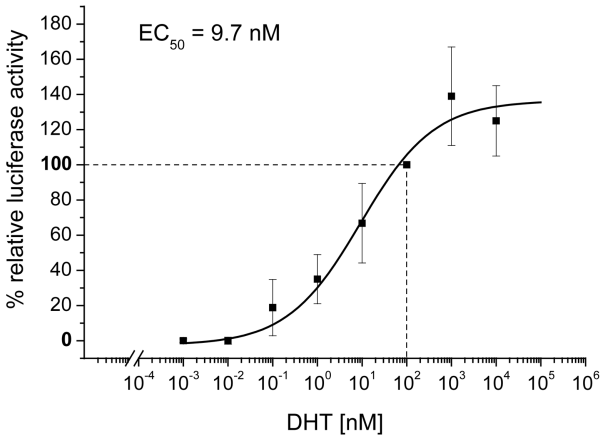

Figure 2. Transient transactivation assay in COS-7 cells. Androgen (DHT) induced reporter gene response: normalized graph (n = 6, mean ±SD; 100 nM DHT was the reference point for each experiment and set as 100%). The EC_{50} value was 9.7 nM. SD, standard deviation.

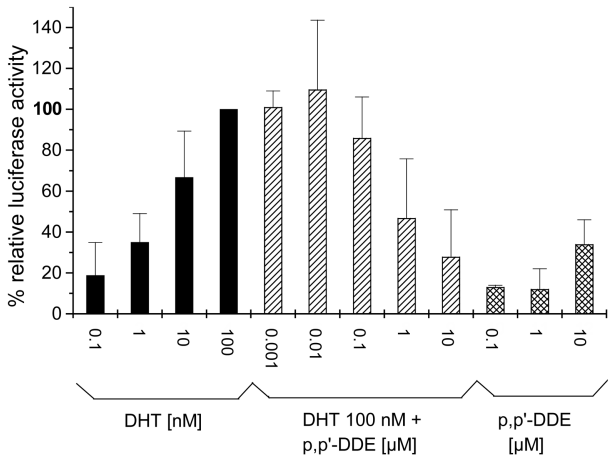

Figure 3. Assay of antiandrogenic efficacy of p,p′-DDE in COS-AR-Luc cells. p,p′-DDE acts as a potent antiandrogen, abrogating concentration-dependently DHT-induced reporter gene activity and showing partial agonism at high concentration (n = 3, mean ± SD, values for p,p′-DDE alone: n=4, mean ± SD).

3.2 PSA assay in T47D cells

In T47D mammary carcinoma cells the physiological androgen DHT induced PSA expression in a concentration-dependent manner (Fig. 5). Compared to the transient trans-

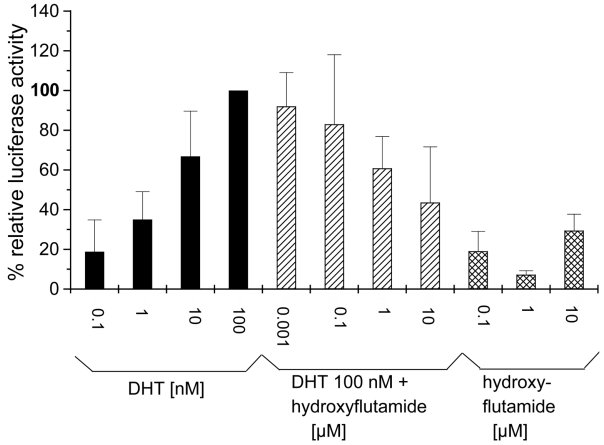

Figure 4. Assay of antiandrogenic efficacy of hydroxyflutamide in COS-AR-Luc cells. Hydroxyflutamide acts as a potent antiandrogen, concentration-dependently abrogating DHT-induced reporter gene activity and showing partial agonism at high concentration (n = 3, mean ± SD, values for hydroxyflutamide alone: n = 4, mean ± SD).

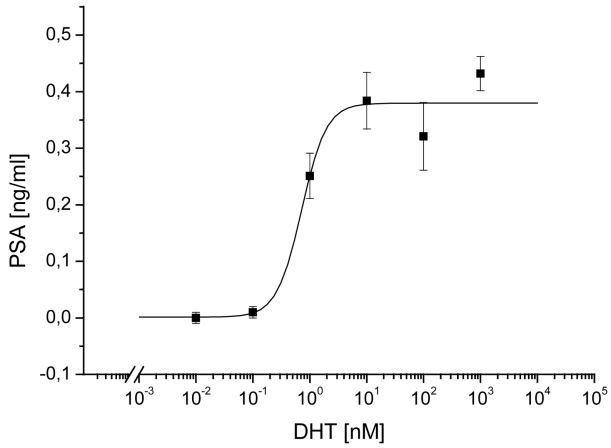

Figure 5. PSA assay for antiandrogenic activity: validation with DHT. DHT induced PSA expression: normalized graph (n = 4, mean ± SD). The EC_{50} value was 0.7 nM.

activation assay in COS-7 cells the PSA assay is considerably more sensitive, resulting in an EC_{50} value at 0.7 nM DHT, as compared to 9.7 nM in the former system. Since the concentration-response curve is shifted to a lower concentration range, a DHT concentration of 1 nM, about equivalent to the EC_{50} value of 0.7 nM, was chosen for inhibition experiments.

p,p′-DDE and hydroxyflutamide inhibited DHT-induced PSA expression in a concentration-dependent manner. However, in this test system, p,p′-DDE was found to be a less potent antiandrogen than hydroxyflutamide, both in terms of potency (IC_{30}) and efficiency of inhibition (Fig. 6).

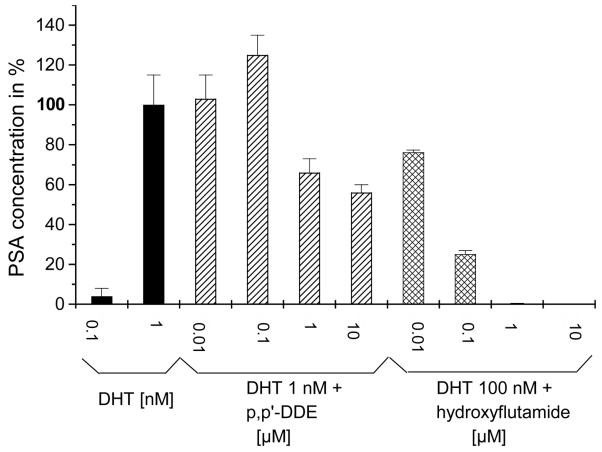

Figure 6. PSA assay for antiandrogenic activity. Concentration-dependent inhibition of DHT (1 nM) induced PSA expression by p,p′-DDE and hydroxyflutamide (n = 3, mean ± SD).

In contrast, PSA expression was completely inhibited at 1 and 10 µM concentrations by hydroxyflutamide (Fig. 6). No cytotoxic effects were detected by the SRB test for both compounds (data not shown).

The response to a steroidal gestagen and to a corticoid was also tested. The progestin norgestimate displayed an agonistic response in a dose-dependent manner with an EC_{50} at 5 nM, reaching a plateau at 60% of DHT-induced response. Dexamethasone, a glucocorticoid receptor (GR)-agonist, induced weak response in a dose-dependent manner. Cotreatment with hydroxyflutamide reduced norgestimate-induced PSA expression to some extent, however, no complete reduction could be achieved (data not shown).

3.3 TRPM-2 assay

In T47D mammary carcinoma cells DHT was found to inhibit the expression of TRPM-2 m-RNA in a concentration-dependent manner. Figure 7 shows the stains for samples and standards (A) and the normalized results (B). Evaluation of an IC_{50} value by logistic fit was not possible in this case because the upper and lower response plateaus were not reached. However, the data clearly indicate a dose-dependent repression of TRPM-2 m-RNA. The antiandrogens p,p′-DDE and hydroxyflutamide indeed induced a dose-related increase of TRPM-2 m-RNA (Fig. 8).

4 Discussion

In vitro test systems to identify androgenic and, especially, antiandrogenic activity have been established and compared using transgenic and endogenous reporter gene sys-

Table 1. Comparison of the established *in vitro* test systems

	Advantages	Disadvantages
Transient transactivation assay (COS-AR-Luc)	● Specific ● Selective expression of AR	● Monkey kidney cells ● Transfection necessary: – time consuming, – more artificial
PSA assay	● Human mammary carcinoma cells (T47D) ● PSA is secreted into the culture medium ● Determination at protein level ● Antiandrogenic effects specifically measured	● Coexpression of other steroid hormone receptors ● Specific measurement of androgenic effects may require additional refinement by appropriate receptor blocking agents to correct for potential cross sensitivity
TRPM-2 assay	● Human mammary carcinoma cells (T47D) ● Positive response indicated by antiandrogens	● Coexpression of other steroid hormone receptors ● Specific measurement of androgenic effects may require additional refinement by appropriate receptor blocking agents to correct for potential cross sensitivity ● Determination at m-RNA level, not at protein level

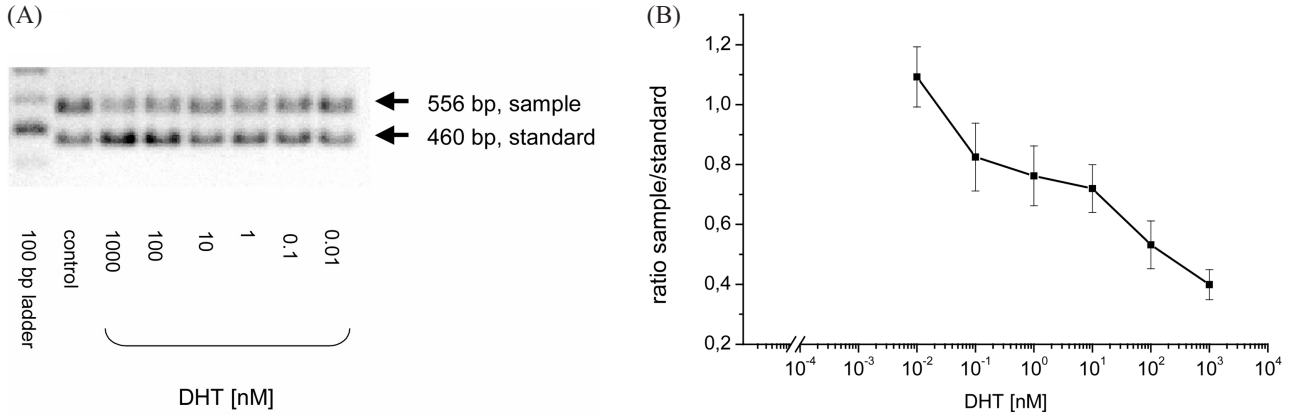

Figure 7. TRPM-2 assay in T47D cells. (A) Results of agarose gel electrophoresis after ethidium bromide staining. Stains of samples (556 bp) and standard (460 bp) are indicated. Intensities of the ethidium-bromide stains were quantified using a Lumi-Imager (Boehringer). Results were normalized by dividing light units detected for the sample by light units detected for the standard. (B) DHT induced the reduction of TRPM-2 m-RNA in a dose-dependent manner (n = 5, mean ± SD). The value obtained for control samples was set as reference point (value 1.0). All other values were evaluated relative to the reference point.

tems. The respective advantages and disadvantages of the test systems are summarized in Table 1.

Specific identification of androgenic potential can be achieved when using a cell line which exclusively expresses the androgen receptor. In this situation, other steroid hormone receptors, such as glucocorticoid and progesterone receptors, are absent. Those receptors are known also to bind to HREs in the respective promotors and, thereby, to cause transcriptional activation [34]. We have achieved this aim of selective response by transient transfection of the steroidhormone receptor negative African green monkey kidney cell line COS-7 with a human androgen receptor expression vector (pSG5AR). As a reporter gene vector the androgen-controlled pMamneoLuc was cotransfected together with a control vector (pSVβ). The pMamneoLuc vector contains the MMTV promotor including 4 HREs which can bind androgen-activated AR. A constitutively expressed androgen-independent control vector (pSVβ) was cointroduced to obtain a measure for transfection efficiency and for variations in individual preparation. Our results show that this transient transactivation assay in COS-7 cells allows the determination of both androgenic and antiandrogenic potential at high sensitivity. However, one disadvantage of this transient transactivation system lies in the fact that for every experiment it requires a new transfection. Furthermore, as a consequence of transfection with three vectors and the usage of monkey kidney cells, the system is relatively artificial. Test systems that more closely simulate the physiological situation make use of endogen-

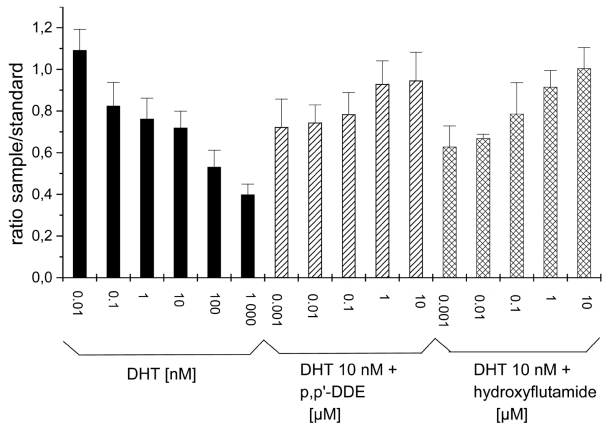

Figure 8. TRPM-2 assay in T47D cells. p,p′-DDE and hydroxyflutamide inhibited the DHT-induced reduction of TRPM-2 in a concentration-dependent manner (p,p′-DDE: $n = 6$, hydroxyflutamide: $n = 4$, means ± SD).

ous reporter gene systems in human cell lines expressing the androgen receptor. When using this approach one needs to take into consideration that the response will not be totally androgen specific, because available cell lines express not only the AR.

As an example, the T47D human breast cancer cell line which was utilized in our experiments contains not only ARs but also PRs and GRs [35]. In our feasibility experiments, we established two endogenous reporter gene systems, one utilizing the androgen-inducible protein PSA, and the other the androgen-repressed protein, TRPM 2. These systems need validation of agonistic response, *e.g.*, by using appropriate receptor blocking agents in order to correct for potential agonistic cross sensitivity mediated by progesterone or glucocorticoid receptors. Norgestimate and dexamethasone also were found to induce PSA expression in T47D cells. Attenuation of norgestimate-induced response by the antiandrogen hydroxyflutamide indicates that this gestagen behaves as a partial AR-agonist in this system. Other progestins such as medroxyprogesterone acetate (MPA) have also been reported to have some AR-agonist activity [19]. Studies with selective PR antagonists will be needed for further mechanistic insight.

In the present study antagonistic activity towards the androgen receptor was selectively assayed by measuring test compound mediated reduction of DHT-induced PSA protein and induction of DHT-reduced TRPM-2 message. An advantage of the PSA assay lies in the fact that PSA is secreted into the culture medium, and thus can be determined directly in the medium. Furthermore, PSA is determined at the protein level *via* ELISA, whereas TRPM-2 is to be measured *via* RT-PCR at the m-RNA level. Thus, potential post-transcriptional modifications would not be detected in the TRPM-2 assay. At present, however, the

Table 2. Comparison of the effects of DHT, p,p′-DDE, and hydroxyflutamide in the different test systems

	DHT EC_{50} (nM)[a)]	p,p′-DDE IC_{30} (µM)[b)]	hydroxy-flutamide IC_{30} (µM)[b)]
COS-AR-Luc	9.7	0.47	0.62
PSA assay	0.7	0.9	0.02
TRPM-2 assay	–	$EC_{30} = 4.8$[b)]	$EC_{30} = 3.3$[b)]

a) Evaluated by logistic fit
b) Evaluated by linear regression between two values

TRPM-2 assay, to our knowledge, is the only approach allowing detection of a positive response induced by antiandrogens.

All three test systems were validated with the physiological hormone dihydrotestosterone and with the established antiandrogens p,p′-DDE and hydroxyflutamide. The EC_{50} and respective IC_{30} values are summarized in Table 2. The PSA assay represents the most sensitive assay with an EC_{50} value obtained from the DHT concentration-response curve about one order of magnitude lower than the EC_{50} value measured in the transient transactivation assay (0.7 nM *versus* 9.7 nM). In the PSA assay, which is considered to more closely reflect the physiological situation, hydroxyflutamide revealed more potent antiandrogenic activity than p,p′-DDE, as reflected by IC_{30} values differing by about one order of magnitude. This contrasts with the findings in the transient transactivation assay and in the TRPM-2 assay, where both established antiandrogens elicited about equivalent antiandrogenic activity with similar IC_{30} values.

Androgenic activity was determined using the transient transactivation assay in COS-7 cells. Both, p,p′-DDE and hydroxyflutamide, clearly displayed partial androgenic potential at higher concentrations. Thus, according to results of our *in vitro* test system, they appear to represent partial agonists. It has previously been reported that some androgen antagonists like hydroxyflutamide or the vinclozolin metabolite M2 can act as agonists in a concentration dependent manner, depending on the presence of competing natural ligands [8, 19]. Our results are in good agreement with these studies.

In conclusion, three *in vitro* test systems, one transgenic and two endogenous test systems, have been established and validated. The three approaches complement each other and together offer highly useful and sensitive tools for identifying potential androgens/antiandrogens. Studies with a spectrum of recently identified antiandrogens are planned to further establish specificity and sensitivity of these assays.

We wish to thank A. Cato for providing the androgen receptor expression vector pSG5AR; Schering AG, Berlin, for

providing hydroxyflutamide and the laboratory of the Westpfalz Hospital, Kaiserslautern, for providing the Immulite unit. This investigation was supported by Baden-Württemberg, a federal state of Germany, project life foundation of the environment and its preservation, grant BWB 99002.

5 References

[1] Colborn, T., vom Saal, F. S., Soto, A. M., Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* 1993, *101*, 378 – 384.

[2] Toppari, J., Larsen, J. C., Christiansen, P., Giwercman, A., Grandjean, P., Guilette, L. J. Jr., *et al.,* Male reproductive health and environmental xenoestrogens. *Environ. Health Perspect.* 1996, *104*, *Suppl. 4*, 741 – 803.

[3] Vollmer, G., Starcke, S., Wober, J., Zierau, O., Endocrine modulation and the fragile balance of homeostasis – an overview. *Neuroendocrinol. Lett.* 2002, *23*, *Suppl. 2*, 37 – 42.

[4] Singleton, D. W., Khan, S. A., Xenoestrogen exposure and mechanisms of endocrine disruption. *Front Biosci.* 2003, *8*, 110 – 118.

[5] Bergstrom, R., Adami, H. O., Mohner, M., Zatonski, W., Strom, H., Ekbom, A., *et al.*, Increase in testicular cancer incidence in six European countries: a birth cohort phenomen. *J. Natl. Cancer Inst.* 1996, *88*, 727 – 733.

[6] Paulozzi, L. J., International trends in rates of hypospadias and cryptorchidism. *Environ. Health Perspect.* 1999, *107*, 297 – 302.

[7] Kelce, W. R., Stone, C. S., Laws, S. C., Gray, L. E., Kemppainen, J. A., Wilson, E. M., Persistent DDT metabolite p,p′-DDE is a potent androgen receptor antagonist. *Nature* 1995, *375*, 581 – 585.

[8] Wong, C., Kelce, W. R., Sar, M., Wilson, E. M., Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *J. Biol. Chem.* 1995, *270*, 19998 – 20003.

[9] Ostby, J., Kelce, W. R., Lambright, C., Wolf, C. J., Mann, P., Gray, L. E., The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist *in vivo* and *in vitro*. *Toxicol. Indust. Health* 1999, *15*, 80 – 93.

[10] Vinggaard, A. M., Nellemann, C., Dalgaard, M., Jorgensen, E. B., Andersen, H. R., Antiandrogenic effects *in vitro* and *in vivo* of the fungizide prochloraz. *Toxicol. Sci.* 2002, *69*, 344 – 353.

[11] Lambright, C., Ostby, J., Bobseine, K., Wilson, V., Hotchkiss, A. K., Mann, P. C., *et al.*, Cellular and molecular mechanisms of action of linuron: an antiandrogenic herbicide that produces reproductive malformations in male rats. *Toxicol. Sci.* 2000, *56*, 389 – 399.

[12] Euling, S. Y., Gennings, C., Wilson, E. M., Kemppainen, J. A., Kelce, W. R., Kimmel, C. A., Response-surface modeling of the effect of 5alpha-dihydrotestosterone and androgen receptor levels on the response to the androgen antagonist vinclozolin. *Toxicol. Sci.* 2002, *69*, 332 – 343.

[13] Soto, A. M., Sonnenschein, C., The E-Screen-Assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.* 1995, *103*, 113 – 122.

[14] Veldscholte, J., Berrevoets, C. A., Brinkmann, A. O., Grootegoed, J. A., Mulder, E., Antiandrogens and the mutated androgen receptor of LNCaP cells: differential effects on binding affinity, heat-shock protein interaction, and transcription activation. *Biochemistry* 1992, *31*, 2393 – 2399.

[15] Rosenberg, R. S., Grass, L., Jenkins, D. J. A., Kendall, C. W. C., Diamandis, E. P., Modulation of androgen and progesterone receptors by phytochemicals in breast cancer cell lines. *Biochem. Biophys. Res. Commun.* 1998, *48*, 935 – 939.

[16] Térouanne, B., Tahiri, B., Georget, V., Belon, C., Poujol, N., Avances, C., *et al.*, A stable prostatic bioluminescent cell line to investigate androgen and antiandrogen effects. *Mol. Cell. Endocrinol.* 2000, *160*, 39 – 49.

[17] Schrader, T. J., Cooke, G. M., Examination of selected food additives and organochlorine food contaminants for androgenic activity *in vitro*. *Toxicol. Sci.* 2000, *53*, 278 – 288.

[18] Blankvoort, B. M., de Groene, E. M., van Meeteren-Kreikamp, A. P., Witkamp, R. F., Rodenburg, R. J., Aarts, J. M., Development of an androgen reporter gene assay (AR-LUX) utilizing a human cell line with an endogenously regulated androgen receptor. *Anal. Biochem.* 2001, *298*, 93 – 102.

[19] Wilson, V. S., Bobseine, K., Lambright, C. R., Gray, L. E., A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. *Toxicol. Sci.* 2002, *66*, 69 – 81.

[20] Couette, B., Le Ricousse, S., Fortin, D., Rafestin-Obelin, M. E., Richard-Foy, H., The establishment of the long terminal repeat of the mouse mammary tumor virus into CV-1 cells allows a functional analysis of steroid receptors. *Biochim. Biophys. Acta* 1994, *1219*, 607 – 612.

[21] Beato, M., Hormone induces binding of receptors and transcription factors to a rearranged nucleosome on the MMTV promotor *in vivo. EMBO J.* 1995, *14*, 1737 – 1751.

[22] Beato, M., Chromatin structure and the regulation of gene expression: remodeling at the MMTV promotor. *J. Mol. Med.* 1996, *74*, 711 – 724.

[23] Diamandis, E. P., Yu, H., Sutherland, D. J. A., Detection of prostate-specific antigen immunoreactivity in breast tumors. *Breast Cancer Res. Treat.* 1994, *32*, 301 – 310.

[24] Herrala, A., Kurkela, R., Vihinen, M., Kalkkinen, N., Vihko, P., Androgen-sensitive human prostate cancer cells, LNCaP, produce both N-terminally mature and truncated prostate-specific antigen isoforms. *Eur. J. Biochem.* 1998, *255*, 329 – 335.

[25] Sadar, M. D., Androgen-independent induction of prostate-specific antigen expression *via* cross-talk between androgen receptor and protein kinase. A signal transduction pathways. *J. Biol. Chem.* 1999, *274*, 7777 – 7783.

[26] Cleutjens, K. B., van der Korput, H. A., van Eekelen, C. C., van Rooij, H. C., Faber, P. W., Trapman, J., An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promotor. *Mol. Endocrinol.* 1997, *11*, 148 – 161.

[27] Yu, H., Diamandis, E. P., Zarghami, N., Grass, L., Induction of prostate specific antigen production by steroids and tamoxifen in breast cancer cell lines. *Breast Cancer Res. Treat.* 1994, *32*, 291 – 300.

[28] Zarghami, N., Grass, L., Diamandis, E. P., Steroid hormone regulation of prostate-specific antigen gene expression in breast cancer. *Br. J. Cancer* 1997, *75*, 579 – 588.

[29] Magklara, A., Grass, L., Diamandis, E. P., Differential steroid hormone regulation of human glandular kallikrein (hK2) and prostate-specific antigen (PSA) in breast cancer cell lines. *Breast Cancer Res. Treat.* 2000, *59*, 263 – 270.

[30] Jin, G., Howe, P. H., Regulation of clusterin gene expression by transforming growth factor β. *J. Biol. Chem.* 1997, *272*, 26620 – 26626.

[31] Wong, P., Taillefer, D., Lakins, J., Pineault, J., Chader, G., Tenniswood, M., Molecular characterization of human TRPM-2/clusterin, a gene associated with sperm maturation, apoptosis and neurodegeneration. *Eur. J. Biochem.* 1994, *221* 917 – 925.

[32] Wong, P., Pineault, J., Lakins, J., Taillefer, D., Léger, J., Wang, C., *et al.*, Genomic organization and expression of the rat TRPM-2 (clusterin) gene, a gene implicated in apoptosis. *J. Biol. Chem.* 1993, *268*, 5021 – 5031.

[33] Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., *et al.*, New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* 1990, *82*, 1107 – 1112.

[34] Günzburg, W., Salomons, B., Factors controlling the expression of mouse mammary tumor virus. *Biochem. J.* 1992, *283*, 625 – 632.

[35] Keydar, I., Chen, L., Karby, S., Weiss, F. R., Delarea, J., Radu, M., *et al.*, 1979. Establishment and characterization of a cell line of human breast carcinoma origin. *Eur. J. Cancer* 1979, *15*, 659 – 670.

