Endocrine Toxicology – Contributions of *In Vitro* Methods to the 3R Concept

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Summary

Concerns have been raised about the potential of natural and man-made chemicals to interfere with the endocrine system of humans and wildlife adversely. This issue has been addressed by intensive research over the past years. Most studies focused on interactions with sex hormone receptors. An estrogenic or (anti)androgenic potential was demonstrated for a number of natural and anthropogenic compounds. This work focused on the detection of compounds with affinity to the androgen receptor (AR). An AR binding assay based on a recombinant rat AR (fusion protein to thioredoxin) properly ranked strong and weak (anti)androgens and other compounds with affinity to the AR. The data correlated excellently with that obtained in binding studies employing a cytosolic AR preparation from rat prostate. Studies with detergents indicated that these are confounders of the assay. They most likely disturb ligand receptor interactions and/or modify of the receptor protein. A transactivation assay based on the androgen-sensitive PALM cell line detected strong and weak androgens, and was able to detect antiandrogenicity when cells had been stimulated with an androgen. These findings indicate that in vitro methods can contribute successfully to the characterisation of compounds with affinity to sex hormone receptors. Whereas binding assays provide the basic information on affinity to the receptors, transactivation assays can discriminate agonists and antagonists and also provide information on cytotoxicity. These in vitro assays could also reduce animal use in endocrine toxicity testing (a) by using recombinant sex hormone receptors instead of cytosolic receptor preparations, (b) by applying intelligent screening strategies that include (potential) metabolites, as these assays probably have only little or no metabolic capacity and by combining several in vitro systems should equivocal results be obtained in one system, and (c) by using in vitro methods to identify mechanisms of action of effects observed in animal studies. In order to support the acceptance of these methods, a thorough validation and the characterisation of confounders is desirable. Whether receptor binding and transactivation assays can predict the outcome of in vivo screening assays reliably remains to be established.

Keywords: endocrine toxicology, in vitro methods, sex hormone receptor binding assay, transactivation assay, PALM cells

Zusammenfassung: Endokrine Toxikologie: 3R Beitrag von *in vitro* Methoden

Vor einiger Zeit wurde die Frage aufgeworfen, ob natürliche und anthropogene Substanzen mit dem Hormonsystem von Mensch und Tier interagieren und so adverse Effekte auslösen könnten. Diese Fragestellung wurde in den vergangenen Jahren intensiv beforscht. Die meisten Untersuchungen konzentrierten sich dabei auf Interaktionen mit Sexualhormonrezeptoren, da für eine Reihe von natürlichen und anthropogenen Substanzen ein östrogenes oder (anti)androgenes Potenzial gezeigt werden konnte. Diese Untersuchung hat den Schwerpunkt auf der Detektion von Verbindungen mit Affinität zum Androgenrezeptor (AR). Ein AR-Bindingsassay auf der Basis eines rekombinanten AR (Fusionsprotein mit Thioredoxin) klassifizierte korrekt starke und schwache (Anti)androgene und andere Substanzen mit Affinität zum AR. Die Daten korrelierten exzellent mit solchen aus Bindungsstudien, bei denen zytosolische AR-Präparationen aus Rattenprostata verwendet wurden. Untersuchungen mit Detergenzien zeigten auf, daß diese Substanzen mit dem Assay interferieren, vermutlich durch Störung der Ligand-Rezeptorinteraktionen und/oder durch Modifikation des Rezeptorproteins. Ein Transaktivierungsassay auf der Basis der androgen-sensitiven PALM-Zelllinie detektierte starke und schwache Androgene, war aber auch in der Lage, Antiandrogenität nachzuweisen, sofern die Zellen mit einem Androgen stimuliert waren. Diese Ergebnisse zeigen auf, daß in vitro Methoden erfolgreich zur Charakterisierung von Verbindungen mit Affinität zu Sexualhormonrezeptoren beitragen können. Während Bindingsassays Basisinformation über Affinität zum Rezeptor liefern, können Transaktivierungsassays zwischen Agonisten und Antagonisten unterscheiden und auch Hinweise zur Zytotoxizität liefern. Diese in vitro Assays können auch den Einsatz von Labortieren im Bereich der endokrinen Toxikologie reduzieren (a) durch den Einsatz rekombinanter Sexualhormonrezeptoren an Stelle von zytosolischen Rezeptorpräparationen, (b) durch Anwendung intelligenter Screeningstrategien, welche die Testung (potenzieller) Metaboliten mit beinhalten, da diese Assays vermutlich nur geringe oder keine metabolische Kompetenz haben, und welche mehrere in vitro Testsysteme kombinieren, sofern fragwürdige Ergebnisse in einem einzelnen Testsystem erhalten werden, und (c) durch den Einsatz von in vitro Methoden zur Identifizierung von Wirkmechanismen, die tierexperimentell erhobenen Befunden zugrunde liegen. Um die Akzeptanz dieser Methoden zu stärken, ist eine umfassende Validierung und die Charakterisierung von Substanzen, welche die Assays stören und so zu falschen Ergebnissen führen, wünschenswert. Inwiefern Rezeptorbindings- und Transaktivierungsassays verläßlich das Ergebnis von in vivo Screeningassays vorhersagen können, muß erst noch gezeigt werden.

Received 2 December 2003; received in final form and accepted for publication 12 February 2004

1 Introduction

Concerns have been raised that man-made and natural compounds may interfere with the endocrine system and thus may adversely affect wildlife and humans and/or their progeny. In response to these concerns, US EPA established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), and – following EDSTAC's recommendations (EDSTAC, 1998) – set up a programme with a tiered approach to evaluate potential endocrine activity of chemicals. OECD developed the conceptual framework for the testing and assessment of endocrine disrupting chemicals consisting of several levels of screening and testing (OECD, 2003). Both in the EPA programme and the OECD conceptual framework, receptor binding assays as well as transactivation assays (reporter gene assays) for sex hormones represent important components, which are part of the US EPA tier 1 screening battery and of level 2 of the conceptual frame work. Endocrine active compounds frequently act through interaction with steroid receptors. Initial studies, focused on interactions with estrogen receptor (ER)-mediated signalling, date back to the 1980's (McLachlan et al., 1984) and have been intensified over the years. A number of chemicals and natural products were characterised as weak estrogen receptor agonists (Fig. 1). More recently, interactions with androgen receptor (AR)-mediated signalling gained attention, as androgenic activity was detected in feedlot and pulp mill effluents (Parks et al., 2001; Wilson et al., 2002) and weak antiandrogenic properties of the DDT metabolite p, p'-DDE and some agrochemicals were demonstrated *in vitro* and *in vivo* at high dosages (Kelce et al., 1994, 1995; Lambright et al., 2000; see Fig. 2). Accordingly, receptor binding and transactivation assays may represent basic tools to characterise receptor-mediated endocrine activity, but may also be important tools to screen for endocrine active compounds.

These techniques may also contribute to aspects of animal welfare. Recombinant estrogen and androgen receptors have entered the market and may be able to replace cytosolic receptor preparations from uterus and prostate of ovarectomised or castrated rats as sources of ER and AR receptors. Similarly, corresponding transactivation assays have become available and could be used to assess affinity of chemicals to hormone receptors without using animals. In this work, we focus on the detection of compounds with affinity to the androgen receptor using a recently developed binding assay employing a recombinant receptor (Freyberger and Ahr, 2004) and using a transactivation assay based on the androgen-sensitive PALM cell line, an initially AR-deficient prostate PC-3 cell line stably transfected with the human AR and an androgen-sensitive response element coupled to the luciferase reporter gene (Terouanne et al., 2000). Contributions of such methods to the 3R concept (reduction, refinement, replacement) of decreasing animal experiments are discussed.

2 Material and methods

2.1 Chemicals, media and receptors

DL-dithiothreitol (DTT, > 99%), dextran-coated charcoal, dihydrotestosterone (DHT, > 99%), 17α-methyldihydrotestosterone (≥ 97%), androstenedione (98%), flutamide (≥ 99%), cyproteroneacetate (> 98%), progesterone (≥ 99%), diethylstilbestrol (DES, ≥ 99%), estradiol (≥ 98%), estrone (≥ 99%), corticosterone (≥ 95%), aldosterone (≥ 90%), methoxychlor (95%), cimetidine (≥ 98%), Triton-x-100 (≥ 99%), benzalkonium chloride (≥ 95% C_{12} and C_{14} monomers), Tween 20

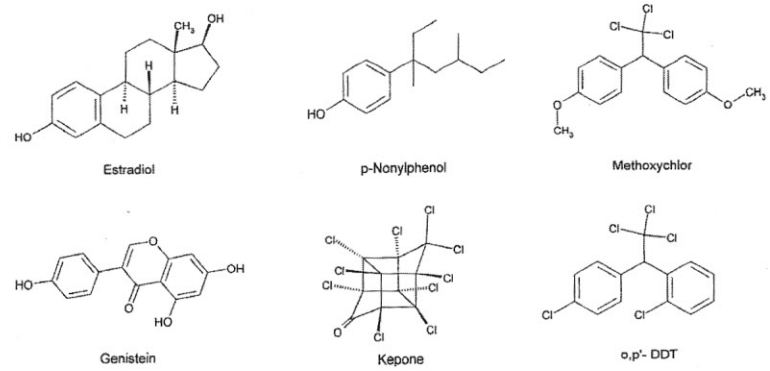

Fig. 1: Chemical structures of the physiological estrogen estradiol and chemicals with estrogenic potential. Note that methoxychlor must be metabolically activated by O-demethylation to 1, 1-bis-(4 hydroxyphenyl)-2, 2, 2-trichloroethane (HPTE) and also has antiandrogenic potential.

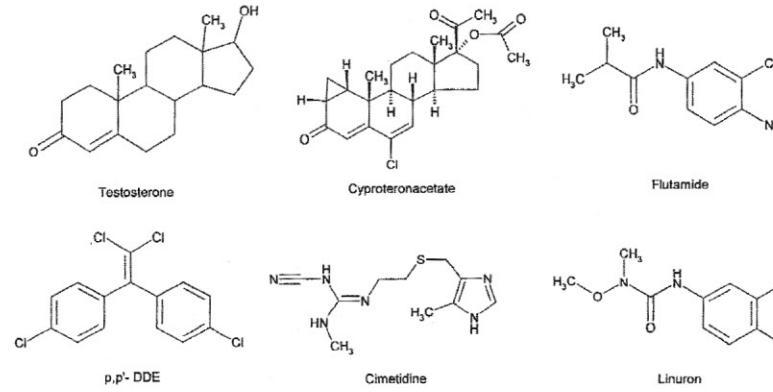

Fig. 2: Chemical structures of the physiological androgen testosterone, the therapeutically used antiandrogens cyproteronacetate and flutamide, and of chemicals with antiandrogenic potential.

(≥ 97%), sodium dodecyl sulphate (SDS), and human γ-globulin (99%) were supplied by Sigma (Taufkirchen, Germany), linurone (99.5%), vinclozolin (99.4%), and 4-nonylphenol (99.6%) were obtained from Riedel de Haen (Seelze, Germany). 1,1-Bis-(4-chlorophenyl)-2,2-dichloroethylene (p, p'-DDE, 99%) was purchased from Aldrich (Steinheim, Germany). 1, 1-bis-(4 hydroxyphenyl)-2, 2, 2-trichloroethane (HPTE, > 99%) was supplied by Cedra Corp. (Austin, Texas, USA), 17α-methyltestosterone (97-103%) was purchased from Arcos Organics (Pittsburgh, PA, USA). Testosterone (> 99%) was bought from Fluka Chemie AG (Buchs, Switzerland). Unlabelled methyltrienolone (R 1881, ≥ 97%) and tritiated R 1881 (17α-methyl-^{3}H, specific activity 3089.5 GBq/mmol, radiochemical purity > 97%) were obtained from NEN Du Pont (Dreieich, Germany).

Ham's F12 medium and geneticine were purchased from Gibco-Invitrogen (Karlsruhe, Germany), fetal calf serum, penicillin and streptomycin were provided by PAA Laboratories (Linz, Austria), puromycin, coenzyme A, and ATP were obtained from Sigma, and P.J.K. (Kleinblittersdorf, Germany) and Fluka Chemie supplied luciferine and tricine, respectively.

Recombinant rat AR was purchased from PanVera (Madison, WI, USA) through MoBiTec (Göttingen, Germany). The ligand-binding domain of this receptor is identical to that of the human AR. The receptor has a molecular weight of 48.4 kDa and contains both the hinge region and the ligand-binding domain, which is fused to thioredoxin.

2.2 Determination of binding to the recombinant androgen receptor

The method described by Freyberger and Ahr (2004) was used. Briefly, test compounds added in 1% DMSO as indicated, human γ-globulin, recombinant AR and tritiated R 1881 were incubated in assay buffer [50 mM Tris-HCl pH 7.5, 0.8 M sodium chloride, 2 mM DDT, 10% glycerol (v/v)]. Experiments were performed at least in triplicate incubations on 96-well microtitre plates in a total volume of 0.2 ml. Final concentrations were 10 mg/ml γ-globulin, 2 nM radiolabelled R 1881 and nominally 2 nM AR. Following incubation at 4°C overnight under continuous shaking, 50 µl of a 5% charcoal suspension in assay buffer was added. After mixing the samples at 4°C for 10 min, charcoal was sedimented by centrifugation at 4000 rpm for 5 min and radioactivity in 50 µl aliquots of the clear supernatant containing the AR-ligand complex was determined in a liquid scintillation counter (1450 Microbeta™ Trilux, Wallac, Freiburg, Germany). Receptor binding was corrected for unspecific binding obtained in the presence of excess (5 µM) unlabelled R 1881. Displacement curves were constructed by relating specific AR binding in the presence of the test compound to that in its absence. At least two independent experiments were performed for each test compound. IC_{50} values representing 50% ligand displacement from the receptor were read from displacement curves whenever possible and IC_{50} values were used to calculate binding affinities relative to DHT.

Tab. 1: Relative androgen receptor binding affinities obtained for two different receptor preparations.

Compound	Relative binding affinity (%)	
	Cytosolic AR[a]	Recombinant AR[b]
Natural and synthetic androgens		
Dihydrotestosterone (DHT)[c]	100	100
R 1881	500	221
17α-Methyl-DHT	Not done	76
Testosterone	33	58
17α-Methyltestosterone	Not done	33
Androstenedione	2	0.25
Antiandrogens in therapeutic use		
Flutamide	Not done	0.016
Cyproteroneacetate	Not done	5
Compounds with weak antiandrogenic properties		
p, p'-DDE	0.17	0.31
Methoxychlor	0.001	- (100 µM)[d]
HPTE	0.17	0.13
Procymidone	Not done	0.0067
Linuron	0.005	0.0038
Vinclozolin	0.001	Non-binder (300 µM)[e]
Vinclozolin metabolite 1	0.014	Not done
Vinclozolin metabolite 2	0.32	Not done
Progestins and Corticoids		
Progesterone	0.002	0.48
Corticosterone	0.001	0.017
Aldosterone	Not done	- (300 µM)
Synthetic and steroid estrogens		
DES	0.11	0.19
Estradiol	4.5	3.7
Estrone	Not done	- (100 µM)

a) Calculated from K_i-values published by Waller et al. (1996)
b) In this investigation, values are means of at least two independent experiments.
c) Results observed for cytosolic and recombinant AR of the underlined compounds were correlated with each other (Fig. 7).
d) A dash indicates that some affinity for the AR was present, but an IC_{50}-value was not reached. The maximal employed concentration is given in brackets.
e) Neither an IC_{50}-value nor a characteristic displacement curve was achieved. Accordingly vinclozolin was considered a non-binder. The maximal employed concentration is given in brackets.

Compounds considered to be confounders are not listed. The IC_{50} value of DHT of the corresponding experiment was divided by the indicated chemical's IC_{50} value and multiplied by 100 in order to calculate relative binding affinities.

2.3 Determination of (anti)androgenicity by means of a PALM cell-based transactivation assay

PALM cells obtained from INSERM (Montpellier, France) were cultivated in Ham's F12 medium supplemented with 7% heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 µg/ml puromycin and 1 mg/ml geneticine in a humidified incubator at 37°C and 5% CO_2. Three days before seeding, culture medium was removed and replaced with medium supplemented with 7% FCS that was stripped twice with dextran-coated charcoal (DCC-FCS). Antibiotics were excluded during the assay. Cells were seeded at a density of 2 x 10^3 cells per well in 60 µl DCC-FCS-supplemented medium into tissue culture treated 384-well plates. Plates were seeded in duplicate for determination of luciferase activity (in white plates, Greiner Bio-One, Frickenhausen, Germany) and cytotoxicity (in clear plates, Greiner Bio-One). After three days, test compounds were added in 20 µl medium to yield androgen concentrations in the range of 0.01 pM to 10 µM and flutamide concentrations in the range of 0.1 µM to 100 µM. For assessment of antiandrogenicity, cells were co-incubated with R 1881 at concentrations of 0.1 nM and 1 nM. Each concentration was tested at least in quadruplicate. Control incubations without test compounds and blanks without cells (medium only) were also included. Measurements were performed after a treatment period of 24 h. Cell viability was determined using the CellTiter©, AQueous One Solution Cell Proliferation Assay (MTS test, Promega, Mannheim, Germany) according to the manufacturer's instructions. 15 µl reagent was added to each well and the optical density was read at 492 nm using a reference wavelength of 630 nm in a microplate spectrophotometer (Tecan ULTRA, Crailsheim, Germany) after 80 min incubation. Optical density was corrected by subtracting the reagent blank value observed in wells containing only medium. Corrected optical density observed for untreated cells was used as a measure for 100% viable cells. To measure luciferase activity, medium was removed and 20 µl luciferase reaction solution (LRS) was added. LRS consists of two volumes of substrate buffer (20 mM tricine, 2.67 mM Mg_2SO_4, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 µM coenzyme A, 470 µM luciferine and 530 µM ATP, pH 7.8) and one volume of Triton-x-100 buffer (3% Triton-x-100, 11.5% glycerol, 2 mM dithiothreitol, 25 mM Na_2HPO_4 in 20 mM Tris-HCl buffer, pH 7.8) mixed immediately before use. After 5 min at room temperature, luminescence was measured in a microplate luminometer (Tecan ULTRA). Luminescence was corrected by subtracting the reagent blank value observed in wells containing only medium. Corrected luminescence observed for untreated cells was used as a measure for basal transactivation activity and was set to 100%.

3 Results

3.1 Androgen receptor binding studies

A broad range of compounds with affinity for the AR, non-binders as well as potentially confounding chemicals was used to explore the properties of the assay. Compounds with affinity for the AR included natural and synthetic androgens, therapeutically used antiandrogens, corticoids, progesterone, certain estrogens and chemicals with known affinity for the AR. The synthetic androgen methyltrienolone (R 1881) and dihydrotestosterone (DHT) most effectively displaced labelled R 1881 from the AR. Testosterone was slightly less active than DHT, likewise the 17α-methyl derivatives of DHT and testosterone were slightly less effective than their parent compounds. Cyproteronacetate, estradiol, androstenedione, p, p'-DDE, the methoxychlor metabolite (HPTE) and diethylstilbestrol (DES) showed intermediate affinity to the AR. Flutamide, linurone, procymidone and cimetidine displaced the ligand from the AR only at very high concentrations, and binding affinity of vinclozolin could not be established (Fig. 3, for relative binding affinities see Tab. 1).

Although receptor binding studies have been used for decades, hardly any work has been published on compounds that might confound the assay system.

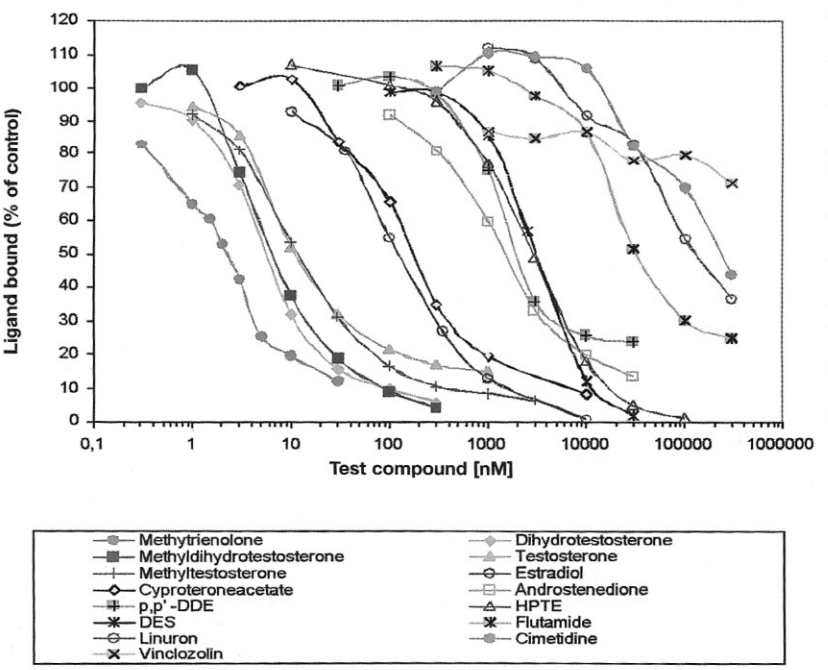

Fig. 3: Displacement of radiolabelled R 1881 from the androgen receptor by natural or synthetic androgens, antiandrogens in therapeutic use, estrogens and by compounds with reported weak antiandrogenicity. Test compounds were added in the concentrations indicated up to maximal employable concentrations or 1 mM.

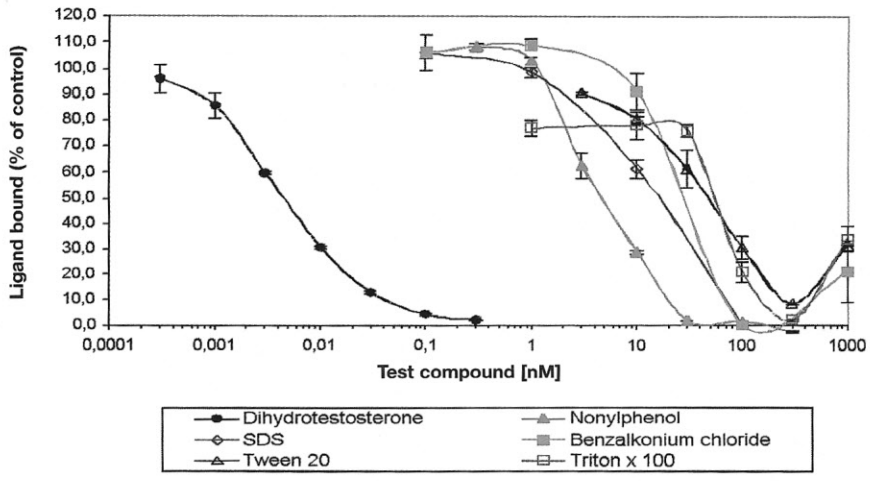

Fig. 4: Effects of various detergents on the binding of radiolabelled R 1881 to the androgen receptor.

We studied this issue by testing detergents of different chemistry. At concentrations of 10 µM and above, all tested detergents, namely nonylphenol, Triton-x-100, Tween 20, benzalkonium chloride, and sodium dodecylsulphate decreased ligand binding. The slope in the linear range of the displacement curve was similar to that of DHT for most of the detergents (Fig. 4). At a concentration of 1 mM, the effect of Triton-x-100, benzalkonium chloride and Tween 20 seemed to be reversed. The apparent displacement of the ligand was less effective than the displacement observed at lower concentrations (Fig. 4).

3.2 Detection of androgenic and antiandrogenic compounds using a PALM cell-based transactivation assay

Strong and weak synthetic and natural androgens tested at non-cytotoxic concentrations were detected sensitively using this cellular system. Methyltrienolone and 17α-methyldihydrotestosterone most effectively induced the expression of the reporter gene luciferase. Dihydrotestosterone, 17α-methyltestosterone and testosterone were less active in the induction of luciferase activity, and androstenedione was the least active compound. Typically, the maximal induction of luciferase activity by all these compounds was eight-fold (Fig. 5).

Provided that the assay is properly conducted, i.e. PALM cells are stimulated with an androgen, the transactivation assay can also be used to detect antiandrogens. Demonstration of the antiandrogenic properties of flutamide is shown in Figure 6A, both in the presence of a marginal and a clearly stimulatory concentration of the androgen methyltrienolone (R 1881). Performing a cytotoxicity assay in parallel to transactivation can serve as a control to discriminate true displacement of the androgen from the receptor and subsequent reduced luciferase expression from decreased luciferase expression as a consequence of a cytotoxic effect. Results of cytotoxicity testing given in Figure 6B indicate that the strongly reduced luciferase induction in the presence of 10 µM flutamide was not the result of cytotoxicity.

4 Discussion

The value of receptor binding assays both as mechanistic tools to characterise receptor-mediated endocrine activity, but also as important screening assays for endocrine active compounds, is well recognised. The androgen receptor binding assay described readily detected and ranked the AR binding properties of androgens and therapeutically used antiandrogens. Likewise, weak binding properties of estrogens and structurally diverse chemicals with antiandrogenic activity, such as p, p'-DDE, linurone, procymidone, and cimetidine and a methoxychlor metabolite were detected (Fig. 3, Tab. 1) and results were in agreement with previously published data (Gaido et al., 2000; Kelce et al., 1994, 1995; Lambright et al., 2000; Sivelle et al., 1982). Likewise, weak binding properties of corticoids (Waller at al., 1996) could be detected (Tab. 1). Vinclozolin, which shows no relevant AR binding unless it is metabolised to metabolites M1 and M2 (Kelce et al., 1994), turned out to be a non-binder in our hands (Fig. 3). As the AR binding assay is devoid of a metabolising system and thus cannot metabolically activate vinclozolin, this outcome was expected. Unfortunately, the active metabolites of vinclozolin were not available for testing.

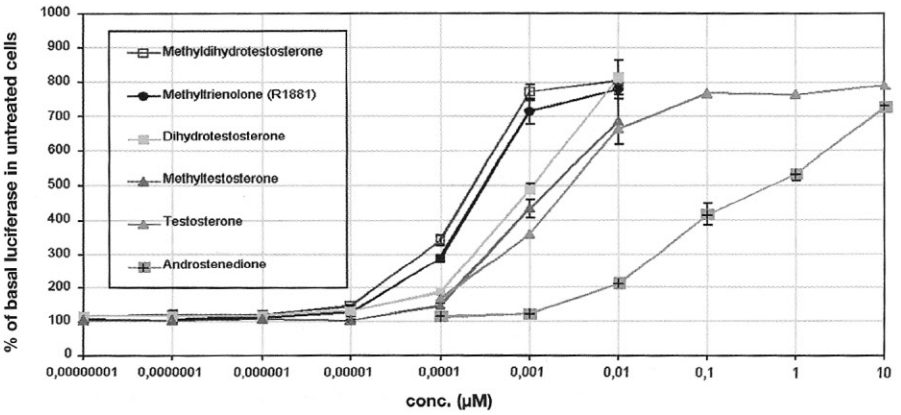

Fig. 5: Induction of luciferase expression by various androgens in PALM cells. Basal expression of luciferase activity in untreated cells is set to 100%.

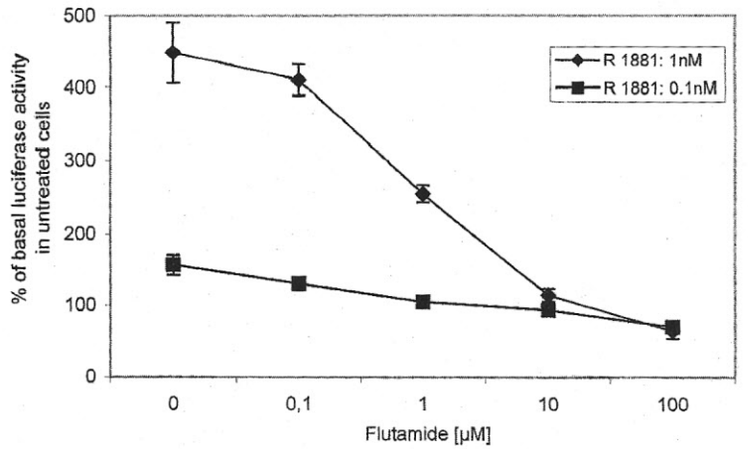

Fig. 6A: Suppression by flutamide of androgen (R 1881)-induced expression of luciferase activity in PALM cells. Basal expression of luciferase activity in untreated cells is set to 100%.

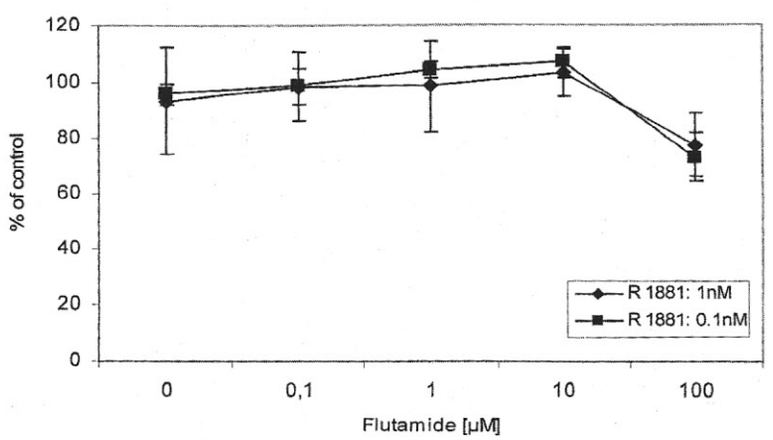

Fig. 6B: Determination of flutamide cytotoxicity in PALM cells by means of the MTS assay.

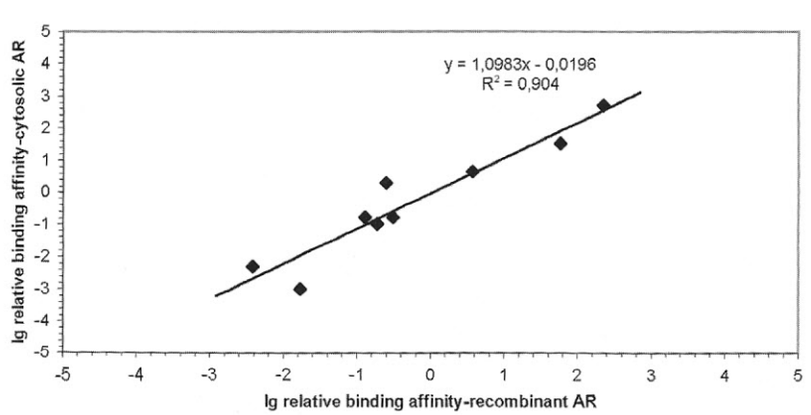

Fig. 7: Correlation between the results of receptor binding assays using two different AR sources, namely the recombinant rat AR reported in this paper versus rat prostate cytosol published by Waller et al. (1996). Relative binding affinities of underlined compounds from Table 1 were used.

An important criterion for an appropriate substitute for the cytosolic AR assay is the degree of correlation between the assays using the recombinant and the cytosolic AR. Waller et al. (1996) tested a broad variety of compounds for AR binding using the cytosolic AR assay. We calculated the corresponding relative binding affinities from the K_i-values reported by Waller et al. (1996). These relative binding affinities generally corresponded well with our data (see Tab. 1). When the relative binding affinities observed in both investigations were plotted against one another (progesterone not included) in a double logarithmic manner (Fig. 7), a correlation of 0.904 was observed. This result is excellent, especially if it is taken into consideration that different methodologies were used to separate the unbound ligand from the ligand-hormone complex (hydroxylapatite versus charcoal). A larger discrepancy was only observed for progesterone. This may be due to methodological differences: Waller et al. (1996) had to add triamcinolone in order to block the binding of the ligand to progesterone receptors contained in their cytosolic AR preparation (Kelce et al., 1994), a procedure that is not necessary when using the recombinant receptor. Accordingly, the recombinant AR receptor represents an appropriate alternative to the use of prostate cytosol from castrated rats as a source of AR. Just published data by Fang et al. (2003) also support this statement. These authors also used the recombinant AR and the more laborious hydroxyapatite method to separate the unbound ligand from the ligand-hormone complex. A comparison of their data with that of Waller et al. (1996) provided a correlation of 0.92 for relative binding affinities, when progesterone was not included.

The set of strong and weak androgens investigated in the transactivation assay clearly tested positive for androgenicity (Fig. 5), however, the ranking of androgenic potency differed from that observed in receptor binding: In the PALM cell line 17α-methyltestosterone and 17α-methyldihydrotestosterone were slightly or pronouncedly more active than their unmethylated parent compounds, whereas in the binding studies

the 17α-methylated derivatives were slightly less active than their parent compounds. These differences are most plausibly explained by intracellular metabolism of the non-methylated compounds. The 17ß-hydroxyl group of androgens is readily subjected to metabolism by 17ß-hydroxysteroid dehydrogenase, whereas methylation or ethinylation at the 17α position prevents metabolism by the dehydrogenase. It remains open whether the intracellular dehydrogenase level of PALM cells corresponds with the *in vivo* situation. In contrast to receptor binding assays, transactivation assays can differentiate between agonists and antagonists. To assess antiandrogenicity in PALM cells, induction of luciferase expression is necessary. As is evident from Figure 6A, the antiandrogenic properties of flutamide, which has a rather low AR binding affinity, is readily detected if proper stimulation with an androgen (i.e. 1 nM R 1881) is provided. The absence of cytotoxicity assessed in a parallel experiment (Fig. 6B) further supports an effect at the receptor level at flutamide concentrations of ≤ 10 µM.

Receptor binding assays have been basic tools in pharmacology for decades and, more recently, were introduced in toxicology. Similarly, in recent years transactivation assays have been used increasingly to characterise interactions with hormone receptors. However, few efforts have been made so far to identify and characterise compounds that might confound these assay systems. We approached this issue by testing a set of detergents as potential confounders in the androgen receptor binding assay. Indeed, all of the detergents tested reduced ligand binding in a concentration-dependent manner (Fig. 4). Both ionic and non-ionic detergents of different chemical structures acted in the same manner, implying that detergents can interfere with receptor binding and that there is a risk that detergent effects may be interpreted as true binding to the AR. Although a more in-depth evaluation provided evidence that displacement curves of detergents differed from those of true binders with respect to their steeper slope, the absence of a decreasing slope when approaching 100% ligand displacement, and a pretended reduced displacement of the ligand at very high detergent concentrations, these findings indicate the need to carefully assess confounding factors in such assay systems.

It is worthwhile to mention that the bioavailable detergent nonylphenol, which clearly displaced the radiolabelled ligand from the AR, tested negative for (anti)androgenicity in the sensitive Hershberger *in vivo* assay even at the maximum tolerated dose (Yamasaki et al., 2003). Detergents may interact and disturb receptor binding in various ways: by binding to and thus modifying the receptor's properties, by denaturing the receptor or by including receptor or ligand in micelles formed at higher concentrations. Any of these events could reduce ligand binding and thus pretend true affinity to the androgen receptor. Decreased displacement of ligand in the presence of very high detergent concentrations obviously indicates a collapse of the assay system due to an impairment of the absorption of unbound radiolabelled ligand to charcoal. In the absence of AR, absorption of radiolabel to charcoal was clearly reduced in the presence of 1 mM detergent (Freyberger, unpublished). Thus, the applicability of receptor binding assays to detergents is obviously limited, especially when relatively high concentrations are used.

Whereas confounding reactions in receptor binding appear to be limited to modification/denaturation of the receptor and interactions with the binding of the ligand to receptor or charcoal at the physico-chemical level, even more confounding reactions are conceivable for transactivation assays such as toxicities that are not readily detected by the applied cytotoxicity test, stimulation or suppression of the transactivating machinery at the DNA, mRNA or protein level or interactions with (unknown) pathways that target the transactivating machinery. Accordingly, the properties of the cell-based assays and their potential confounders should be assessed thoroughly.

Receptor binding and transactivation assays probably have no, limited or possibly selective (e.g. for the inactivation of androgens) metabolic capacity. Accordingly, not only the parent compounds, but also known and anticipated metabolites should be included in the testing routine in order to optimally use these assay systems (compare vinclozolin and its metabolites and methoxychlor and its metabolite HPTE in Tab. 1). Although the inclusion of a metabolic activating system in these assays appears desirable, efforts to achieve this were unsuccessful so far.

In conclusion, *in vitro* methods can successfully contribute to the characterisation of compounds with affinity to sex hormone receptors. Whereas binding assays provide basic information on affinity to the receptors, transactivation assays can identify agonists and antagonists and can also provide information on cytotoxicity. In order to obtain optimal and reliable results using these assays, a comprehensive standardisation/validation of the assay systems is essential. Likewise, characterisation of potential confounders that may limit the use of such assays is mandatory. Most appropriate in this respect would be a validation study at the international level. *In vitro* assays can reduce animal use in endocrine toxicity (a) by using recombinant sex hormone receptors instead of cytosolic receptor preparation from uterus and prostate of ovarectomised or castrated rats, (b) by intelligent screening strategies that include (potential) metabolites and combine several *in vitro* systems should equivocal results be obtained in one system, and (c) by using *in vitro* methods to identify mechanisms of action if changes in endocrine organs and hormone-sensitive tissues are observed in animal studies. Over the last years we experienced a high degree of acceptance from authorities when results from additional well-designed and well-conducted *in vitro* studies performed in order to further characterise *in vivo* findings were provided along with the *in vivo* guideline studies. In these cases there was never a request for additional *in vivo* mechanistic work. Whether receptor binding and transactivation assays, possibly after inclusion of a metabolic activation system, can reliably predict the outcome of *in vivo* screening assays for sex hormone-mediated effects such as the rat uterotrophic,

rat Hershberger and fish screening assays presently being validated under the umbrella of OECD, remains to be established.

References

EDSTAC, 1998. Final report. Available via EPA http://www.epa.gov/scipoly/oscpendo/history/finalrpt.htm.

Fang, H., Tong, W., Branham, W. S. et al. (2003). Study of 202 natural, synthetic and environmental chemicals for binding to the androgen receptor. *Chem. Res. Toxicol. 16*, 1338-1358.

Freyberger, A. and Ahr, H.-J. (2004). Development and standardization of a simple binding assay for the detection of compounds with affinity for the androgen receptor. *Toxicology 195*, 113-126.

Gaido, K. W., Maness, S. C., McDonnell, D. P. et al. (2000). Interaction of methoxychlor and related compounds with estrogen receptor α and ß, and androgen receptor: structure activity studies. *Mol. Pharmacol. 58*, 852-858.

Kelce, W. R., Monosson, E., Gamcsik, M. P. et al. (1994). Environmental hormone disruptors: Evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. *Toxicol. Appl. Pharmacol. 126*, 276-285.

Kelce, W. R., Stone, C. R., Laws, S. C. et al. (1995). Persistent DDT metabolite p, p'-DDE is a potent androgen receptor antagonist. *Nature 375*, 581-585.

Lambright, C. R., Ostby, J., Bobseine, K. et al. (2000). Cellular and molecular mechanisms of action of linurone: An antiandrogenic herbicide that produces reproductive malformations in male rats. *Toxicol. Sci. 56*, 389-399.

McLachlan, J. A., Korach, K. S., Newbold, R. R. and Degen, G. H. (1984). Diethylstilbestrol and other estrogens in the environment. *Fundam. Appl. Toxicology 4*, 686-691.

OECD, 2003. Draft summary report of the sixth meeting of the task force on endocrine disrupters testing and assessment (EDTA 6). Draft as of March 12, 2003.

Parks, L. G., Lambright, C. S., Orlando, E. F. et al. (2001). Masculinization of female mosquitofish in kraft mill effluent-contaminated Fenholloway river water is associated with androgen receptor agonist activity. *Toxicol. Sci. 62*, 257-267.

Satoh, K., Nagai, F. and Aoki, N. (2001). Several environmental pollutants have binding affinities for both androgen receptor and estrogen receptor α. *J. Health Sci. 47*, 495-501.

Sivelle, P. C., Underwood, A. H. and Jelly, J. A. (1982). The effects of histamine H_2 receptor antagonists on androgen action in vivo and dihydrotestosterone binding to the rat prostate androgen receptor in vitro. *Biochem. Pharmacol. 5*, 677-684.

Terouanne, B., Tahiri, B., Georget, V. et al. (2000). A stable prostatic bioluminescent cell line to investigate androgen and antiandrogen effects. *Mol. Cell. Endocrinol. 160*, 39-49.

Waller, C. L., Juma, B. W., Gray, L. E. and Kelce, W. R. (1996). Three-dimensional quantitative structure-activity relationships for androgen receptor ligands. *Toxicol. Appl. Pharmacol. 137*, 219-227.

Wilson, V. S., Lambright, C., Ostby, J. and Gray, L. E. jr. (2002). In vitro and in vivo effects of 17ß-trenbolone: a feedlot effluent contaminant. *Toxicol. Sci. 70*, 202-211.

Yamasaki, K., Takeyoshi, M., Sawaki, M. et al. (2003). Immature rat uterotrophic assay of 18 chemicals and Hershberger assay of 30 chemicals. *Toxicology 183*, 93-115.

Acknowledgement

The authors wish to thank W. Lofink and U. Krella for excellent technical assistance.

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