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Expression of recombinant human androgen receptor and its use for screening methods

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17α-TbOH	17α-trenbolone
17β-TbOH	17β-trenbolone
19-NT	19-nortestosterone
3,4-DCA	3,4-dichloroaniline
3,4-DCAc	3,4-dichloroacetanilid
3,4-DCPU	3,4-dichlorophenylurea
^{3}H-DHT	tritium labelled DHT
ABP	androgen binding protein
ADI	acceptable daily intake
AR	androgen receptor
b	bovine
BPA	bisphenol A
cAMP	cyclic adenosine monophosphate
cDNA	complementary desoxyribonuclein acid
d	day
DDT	(1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethan)
DHT	dihydrotestosterone
DNA	desoxyribonuclein acid
E. coli	escherichia coli
ED	endocrine disrupter
ES	endocrine system
GC	gas chromatography
GR	glucocorticoid receptor
hAR	human AR
HPLC	high-performance liquid chromatography
HRP	hormone responsive element
HSP	heat shock protein
IC_{50}	inhibition concentration 50%
IRA	immuno-immobilised receptor assay
K_D	dissociation constant
kDa	kilo Dalton
K_i	inhibition concentration
LC	liquid chromatography
M	molar
MGA	melengestrol acetate
mRNA	messenger ribonucleic acid
MS	mass spectroscopy
OH-flutamide	hydroxy-flutamide
op'-DDT	(1,1,1-trichloro-2,2-bis(o,p-chlorophenyl)ethan)
ORG	organon
PCBs	polychlorinated biphenyl's
pp'-DDE	1,1-dichloro-2,2-bis(p-cholophenyl)ethylen)
ppt	parts per trillion
PR	progestin receptor
R_{ABP}	ABP receptor
RBA	relative binding affinity
rhAR	recombinant human AR
R_{SHBG}	SHBG receptor
S. pombe	shizosaccharomyces cerevisae
SHBG	sex hormone binding globulin
TBA	trenbolone acetate
TbO	triendione
TBT	tributyltin
TPT	triphenyltin
WHO	World Health Organisation

1. Introduction

1.1. Endocrine disrupters

During the last few decades considerable attention has been paid to the possibility that man-made chemicals (xenobiotics) in the environment may constitute a hazard to human and animal reproductive health. Today, it is generally agreed that the endocrine system (ES) of vertebrates is indeed influenced by different xenobiotics (Colborn 1995, Colborn and Clemmens 1992, Colborn et al. 1993, Cooper and Kavlock 1997, Toppari et al. 1996). As early as 1926, the estrogenic effects of different plant compounds (phytoestrogens) were recognised (Dohrn et al. 1926). A few years later, the first report about a chemical with estrogenic effects was published, viz. the uterotropic effects of bisphenol A (BPA) (Dodds and Lawson 1936). In the sixties it was recognised that further synthetic substances such as methoxychlor, DDT and polychlorinated biphenyl's (PCBs) exhibit estrogenic effects in laboratory animals (Tullner 1961, Bitman et al. 1968, Bitman and Cecil 1970).

The concept that environmental pollutants might have harmful effects on reproduction is not based on theory, but is rather derived from the observations of wildlife biologists in the field. For different wildlife species alterations in male reproduction, issues such as feminisation and demasculinisation, reduced fertility, reduced hatchability, reduced viability of offspring, impaired hormone secretion or activity, and altered sexual behaviour were reported (Colborn and Clemmens 1992), e.g. in bald eagles (Broley 1952, Grier 1982), otters (Mason et al. 1986, Mason and MacDonald 1993), minks (Aulerich et al. 1973), alligators (Jennigs et al. 1988, Guillette et al. 1994, Guillette 1995) or fish (Leatherland and Sonsteyard 1982, Morrison et al. 1985, Sumpter and Jobling 1995).

Even changes in sexual development and human reproduction were proposed. Although the reports on the decrease of sperm counts during the past 50 years (Carlsen et al. 1992) could not be confirmed for the whole world by further analyses (Giwercman and Bonde 1998), the increase of reproductive disorders is commonly accepted, e.g. cryptorchidism, hypospadias, testicular cancer, prostate cancer and breast cancer in the last few decades (Forman and Møller 1994, Czeizel 1985, Møller 2000, Sasco 2000). In the case of PCBs and dibenzofuranes, the connection to

reproductive disorders could be demonstrated by medical examination of prenatally exposed boys (Guo et al. 2000). These diseases might be hormone dependent, but hitherto a causal relationship could not be generally established between xenohormones and disorders observed in man.

All these examples point to the possibility that there might be some xenobiotics interacting with the endocrine system. In 1996 at the European workshop on the impact of endocrine disrupters (EDs) on human health and wildlife, the term ED was defined as follows:

> "An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function" (EUR 1996, 17549).

Disruptions of hormonal co-ordination can be induced by xenobiotics at various levels of the hierarchically organised endocrine system (ES) of vertebrates (Stahlschmidt-Allner et al. 1997). One marked difference between exposure to EDs during critical periods in development versus adulthood is the irreversibility of an effect established during development (Gray and Kelce 1996, Toppari and Skakkebaek 1998). Here the basic femaleness of mammalia provides the explanation that development depends on steroidal environment during foetal development. Hence most attention has been paid to direct steroid agonist or antagonist actions of environmental chemicals.

Imbalances of hormones also have consequences in adulthood, as shown by, for example an epidemic of gynaecomastia in male Haitians. Pyrethroids, used for insect control, were identified as substances which are able to interact with androgen binding sites in dispersed human gentile skin fibroblasts (Eil and Nisula 1990). For the endocrine disrupting substances first detected, the effects in animals and the affinity to the estrogen receptor were described. Many xenoestrogens are meanwhile known, but during sexual differentiation the estrogenic and androgenic properties are crucial (Toppari and Skakkebaek 1998).

During the last century it was recognised that a few of the xenoestrogenic chemicals also display androgenic or antiandrogenic effects, e.g. p,p'-DDE, o,p'-DDT, BPA, butylbenzyl phthalate, nonylphenol and methoxychlor (Gray et al. 1989, Kelce et al. 1995, Sohoni and Sumpter 1998, Hossaini et al. 2001). Screening and evidence of such substances should also aim at identifing possible hazards to human

health and nature.

1.2. Androgens and antiandrogens

1.2.1. Definitions

Androgens play a very important role in the development of males.

They are defined as:

> Substances of physiological or synthetic origin, that influence the development, morphology, function and metabolism of an organism in a way and direction typical of the male individual (translated from: Voss 1973, I).

They display long-term effects which are either organisational on specific organs, such as the sexual differentiation of external genitalia and the programming of neural functions, or they influence enzyme activities manifested in later life. Furthermore, activational effects are exhibited that are immediate, multiple, reversible and dose dependent during all stages of development. Sexual differentiation includes the development of the genital tract, external genitalia and mammary gland and also the organisational effects of androgens on the central nervous system: pituitary regulation of liver metabolism, gonadotropin secretions, sexual dimorphic behavioural patterns and “sexualisation of the brain” (Forest 1983).

Androgens do not occur exclusively in males, they are also found in females, sometimes in similar concentrations. Androgens are the substrates for estrogen synthesis, but testosterone itself is also necessary for, for example, growth and maintenance of preovulatory follicles or epithelial growth in the uterus. Further, not necessarily sex specific roles are: Enhanced neurone survival (Nordeen et al. 1985), stimulation of muscle cell proliferation (Joubert et al. 1994), alteration of Na^+ current kinetics in electrocytes (Ferrari et al. 1995) and regulation of somatostatin release (Argente et al. 1990).

The **antiandrogens** are:

> Physiological or chemical substances suitable for total or partial inhibition of androgen action. Excluded are substances influencing manifestation of the effects of androgens by toxic or other general effects, e.g. influencing regulation of androgen production by the hypothalamus (translated from: Voss 1973, II).

1.2.2. Mode of action

To be able to exert their known biological reactions, androgens require binding to AR. AR belongs to the superfamily of functionally and partly structurally related transcription factors which are hormonally regulated. This superfamily includes receptors for various hydrophobic ligands and is divided into two subfamilies on the basis of structural homologies. One subfamily contains the steroid hormone receptors and the other includes , for example, receptors for thyroid hormone, retinoic acid, Vitamin D and unknown ligands (orphan receptors) (Green and Chambon 1988, Evans 1988) .

The hAR is a single polypeptide with 917 amino acids (Lubahn et al. 1988,Tilly et al. 1989) and a molecular weight of 110 kDa. The coding sequence is located at the X chromosome and is divided into 8 exons (Kuiper et al. 1989). Furthermore, a truncated form of the AR is known, the so-called AR-A, starting with the methionine at position 188 of the full-length hAR. No sequence or affinity differences to ligands were found (Wilson and McPhaul 1994, 1996, Gao and McPhaul 1998). Two distinct isoforms (α, β) of the AR are known only for some fish species, e.g. the rainbow trout. They show 85% identity at the amino acid sequence, but AR-β exhibits no mibolerone binding and shows a lack of transactivation activity (Takeo and Yamachita 1999).

The hAR is organised in the following discrete functional domains:

> I) The A/B domain implicated in transactivation and the hinge region with the highest variability between the subfamilies.

> II) In contrast, the DNA binding domain (C) is composed of two zinc finger structures, the most conserved domain between the nuclear receptors.

III) The carboxy terminal E domain, the largest one, builds the pocket for hormone binding, dimerisation and transcription regulation

(Laudet et al. 1992).

Its natural ligands in humans are dihydrotestosterone (DHT), testosterone, androstenedione, dihydroandrosterone and dihydroandrosteron-sulfate (Forest 1983), depending on the enzymatic setting of the corresponding tissue.

In the absence of hormones or in the presence of flutamide, the androgen receptor is located as a monomer in the cytoplasm with perinuclear distribution. In the presence of androgens, active antiandrogens and even progesterone or estradiol it is found in the nucleus (Kemppainen et al. 1992, Jacobson et al. 1995, Waller et al. 2000). After binding of hormones the previous complexes with heat shock protein (HSP) are detached and the conformation of AR is altered (Veldscholte et al. 1992). Additional AR phosphorylation (van Laar et al. 1991), dimerisation and translocation into the nucleus is followed by binding to a hormone-responsive element (HRE). The HREs are often, but not always, of a palindromic nature (Chang et al. 1995), enable the receptors to bind as homodimers (Freedman 1992) and initiate specific gene transcription after conjunction with coactivators, regulators and transcription factors. Because AR, progestin receptor (PR) and glucocorticoid receptor (GR) appear to recognise almost identical HRPs (Chang et al. 1995, Green and Chambon 1988), the mechanism of specific expression of AR-regulated genes is not completely clear.

Hormone antagonists may act on different levels of this reaction cascade, e.g. by disability to induce the complete displacement of the HSPs (Distelhorst and Howard 1990), by blocking dimerisation (Fawell et al. 1990) or by actively driving their cognate receptors into different structural conformations and disturbing the agonist conformation (McDonnell et al. 1994). However different conformations are sometimes compatible with specific, high-affinity desoxyribonucleic acid (DNA) binding (Allan et al. 1992).

Rapid, non-genomic steroid actions are described nowadays. Here effects on second messenger pathways (Lieberherr and Grosse 1994, Machelon et al. 1998) and actions mediated by sex hormone binding globulin (SHBG) were discussed. Receptors for the SHBG and for the androgen binding protein (ABP) are found in plasma membranes of different tissues (Porto et al. 1995, Krupenko et al. 1994). These receptors (R_{SHGB}, R_{ABP}) are connected with G-proteins and influence the cyclic adenosine monophosphate (cAMP) concentration. The peculiarity of this system is:

the interaction R_{SHGB} and SHBG is only possible in the absence of SHBG ligands. The physiological effect takes place after steroid attachment to the R_{SHGB}-SHBG binding (Nakhla and Rosner 1996).

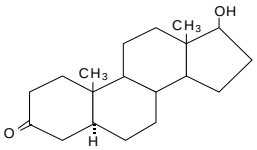
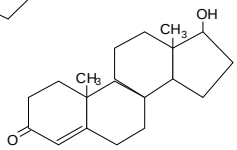
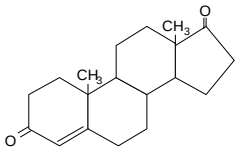
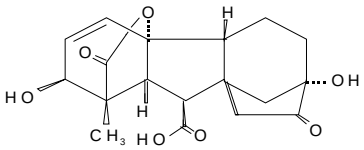
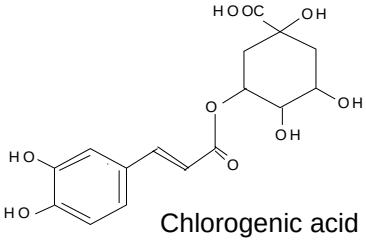
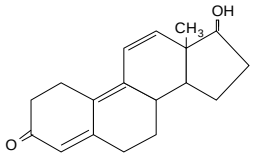
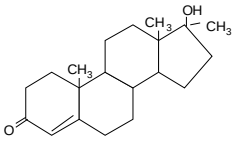
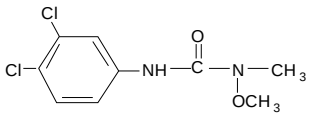
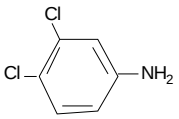
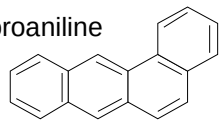
1.3. Structures of endocrine disrupters

The very complex ES of vertebrates may be affected by chemicals or other substances in many different ways. For licensing of chemicals, tests on reproductive toxicology have hitherto been necessary (StMLU 1996), but hormonal activities have not been completely evaluated. Yet the main interest must be detection of disrupting substances, exact assessment of disrupting effects on animals and humans and quantification of these substances in the environment. Substances with androgenic or antiandrogenic effects are divided into the groups shown in table 1.

Two groups of ligands occur *naturally* in the environment. One group comprises the steroids originating from humans and animals. They were at the centre of interest during the seventies, but the low concentrations found in the environment did not occasion any call for action (Gies 1995). They are expected to be degraded very fast by bacteria and tisappear in waste water processing or manure storage (Rurainski et al. 1977, Tabak et al. 1981, Stumpf et al. 1996). The second group includes phytohormones; here gibberellic acid is the only known phytohormone with androgenic action (Gawienowski et al. 1977, Anderson et al. 1982). Other phytohormones showed antiandrogenic action in a breast cancer cell line, e.g. β-carotene, chlorogenic acid and chlorophylline were identified (Rosenberg et al. 1998). They may derive from vegetables, fruit, alcoholic beverages, tea or wood extracts. All examples of this group display only weak androgenic or antiandrogenic effects.

S*ynthetic* androgens include medically used substances for hormone replacement therapy, growth promoters used in farm animals (Danhaive and Rousseau 1988, Conway et al. 2000) and illegally used synthetic androgens for human or animal doping. Synthetic antiandrogens are very valuable in cancer therapy and also for contraception (Foster and Wilde 1998, Kubota et al. 1999).

Table 1: Different AR ligands

Natural substances:	
Natural androgens	Phyto(anti)androgens
 5α-DHT  Testosterone  Androstenedione	 Gibberillic acid  Chlorogenic acid
Synthetic substances:	
Synthetic androgens	Xeno(anti)androgens
 17β-TbOH  Methyltestosterone	 Linuron  3,4-Dichloroaniline  Benz[a]anthracene

The last, and hitherto unexpected, group of androgenic/antiandrogenic substances consists of chemicals or metabolites of chemicals displaying hormonal activtiy as an undesirable side-effect. Only a few such chemicals are known in comparison with the estrogenic or antiestrogenic substances. A list of these androgenic/antiandrogenic substances is shown in table 2.

Table 2: Known androgenic/antiandrogenic substances

Substance	Assay		Reference
Atrazin	*in vitro*	rat AR assay	Danzo 1997
Bisphenol A	*in vitro*	yeast cell reporter assay	Sohoni and Sumpter 1998
Butylbenzylphthalate Dibuthylphtalate (DBP)	*In vivo*	rat	Sohoni and Sumpter 1998 Shultz et al. 2001
	in vitro	yeast cell reporter assay	
Dichloraniline	*in vitro*	rat AR assay	Cook et al.1993
Dieldrin	*in vitro*	rat AR assay/ transactivation assay	Danzo 1997/ Raun Andersen et al 2002
Endosulfan	*In vitro*	transactivation assay	Raun Andersen et al. 2002
Fenitrothion	*In vivo*	rat	Tamura et al. 2001
	In vitro	reporter gene assay	
Fenarimol	*In vitro*	transactivation assay	Raun Andersen et al. 2002
Flavones: chrysin, α-naphtholflavone	*in vitro*	aromatase-yeast AR cell reporter assay	Mak et al. 1999
Gibberillic acid	*in vivo*	chicken comb assay	Gawienowski et al. 1977
Ketotetrahydro-phenanthrene	*in vitro*	yeast cell reporter assay	Ashby et al. 2000
Lindan	*in vitro*	rat AR	Danzo 1997
Linuron	*in vivo*	rat	Cook et al. 1993
	in vitro	rat AR assay	
Methiocarp	*In vitro*	transactivation assay	Raun Andersen et al. 2002
Methoxychlor and metabolites	*in vivo*	rat	Gray et al. 1989
Nonylphenol	*in vivo*	rat	Hossaini et al. 2001
	in vitro	yeast cell reporter assay	Sohoni et al. 1998
o,p'-DDT	*in vitro*	yeast cell reporter assay	Sohoni et al. 1998
Palodesangrens	*in vitro*	rat AR assay	Shirota et al. 1997
Pentachlorphenol	*in virto*	rat AR assay	Danzo 1997
Phytochemicals: e.g. β-carotene, chlorogenic acid...	*in vitro*	breast cancer cell proliferation	Rosenberg et al. 1998
Polychlorinated biphenyls (PCBs)	*in vivo*	cockerel	Platonow and Funnell 1971 / Gray et al. 1999
	In vitro	CHO cell proliferation	Bonefeld-Jorgensen et al. 2001
Polycyclic aromatic hydrocarbons	*in vitro*	reporter gene assay in CHO cells	Vingaard et al. 2000
Prochloraz	*In vitro*	transactivation assay	Raun Andersen et al. 2002
p,p'-DDE	*in vitro*	rat AR assay	Kelce 1995
Pyrethroids	*in vivo*	human	Brody et al. 1983 Sattin 1984
	in vitro	fibroblast AR assay	Eil and Nisula 1990
	in vivo	rat	Mylchreest et al. 1999
Tributyltin	*in vivo*	daphnia magna	Oberdorster et al. 1998 / Tillmann et al. 2001
	in vitro	reporter gene assay in h prostate cancer cells	Yamabe et al. 2000
Triphenyltin	*in vitro*	reporter gene assay in h prostate cancer cells	Yamabe et al. 2000
Vinclozolin and metabolites	*in vivo*	rat	Gray 1994
	in vitro	yeast cell reporter assay / transactivation assay	Sohoni and Sumpter 1998/ Raun Andersen et al. 2002

Almost all substances display antiandrogenic effects, except nonylphenol, tributyltin, ketotetra-hydrophenanthrene and gibberillic acid. For all substances the affinity to the AR was proved, as listed in the table, exept for the flavones, gibberillic acid and PCBs suspected as aromatase inhibitors. The AR binding affinity for DBP is a subject of controversy.

1.4. Strategies for monitoring androgen active substances

As endocrine disruption may occur at different levels, such as synthesis, transport, action, function, metabolism and excretion of hormones, different strategies may be used for monitoring. According to the androgen definition, toxic effects on the different glands of the gonadotropic axis (hypothalamic gland, pituitary and gonads) must be regarded separately because they may also influence hormonal levels.

Efforts have been made by Waller et al. (1996) to predict the ligand affinities to the AR with a theory based on structural requirements by means of a three-dimensional quantitative structure activity relation model. Given that the structure of the substance is known, this approach is useful and thus affords a complementary technique for developing new pharmaceuticals or calculating the affinities of selected chemicals.

Though known androgenic or antiandrogenic substances can be evaluated in concentrations in the ppt range by means of very sensitive methods such as GC-MS, LC-MS and enzyme immuno assay (Meyer et al. 1992, Daxenberger et al. 1999, Le Bizec et al. 2000, Hageleit et al. 2001), no statement on hormonal activity can be made.

Analytical systems for androgens or antiandrogens have to record the potential effect of a chemical, because hormones in general are defined on this basis. For both human and environmental predictions, the effects on either entire organisms (*in vivo*) (Davis and Bortone 1992, Sumpter and Jobling 1995) or cells (Pancrazio et al. 1999) or a cellular structure can be evaluated (*in vitro*). For example, this can be the binding to membrane proteins (Cheun et al. 1998, Schnabl 1999), structure proteins (Danzo and Joseph 1994), enzymes (Schmid 1999, Scheller 1999), ion channels (Cornell et al. 1997) and receptors (Sauerwein and Meyer 1997).

1.4.1. *In vivo* methods

By *in vivo* methods effects on all tissues and functions can be measured and the natural metabolites evaluated. All different levels of endocrine disruption are included. Such tests are therefor of paramount significance.

Testing of androgenic/antiandrogenic substances is mostly done with male rats in different stages of development as listed in table 3.

Table 3: *In vivo* test systems for anti-/androgens with rats

System	Endpoints	Duration	Effectivity
Mature castrated male rat assay (Hershberger et al. 1953)	Weight of ventral prostate, seminal vesicles, levator ani, bulbocavernosus muscle	5-7 d	Androgenic, antiandrogenic
Mature intact rat assay (O'Connor et al. 1999)	Weight of liver, epididymis, prostate and seminal vesicles, hormonal alterations	15 d	Androgenic, antiandrogenic
Peripubertal male rat assay (Ashby 2000)	Weight of testes, epididymides, seminal vesicles, prostate, prepuce separation	14 d	Androgenic, antiandrogenic, estrogenic, thyroid gland, affecting substances
Pregnant rats – pups (Gray et al. 1994)	Development of malformations anogenital distance nipple development	14 d or longer	antiandrogenic

The observed endpoints (measurable effects) include weight changes of for example, accessory sex glands, testes and muscles. These androgen-sensitive tissues show increases in weight due to androgen administration and the opposite effect due to antiandrogen application. As the androgen or antiandrogen concentration influences the secretion of luteinising hormone and of gonadotropin-releasing hormone (Yamada et al. 2000) via feedback mechanisms, another observable endpoints may address changes of hormonal levels. The assay commonly used is that already described by Hershberger in 1953. He used mature castrated rats to test androgenic substances and the same rats with testosterone implants for antiandrogenic substances. The substances were orally administered once per day for 5–7 days.

However, O'Connor and colleagues proved that the sensitivity of this test strongly depends on the breed selection of rats (O'Connor et al. 1999). Other disadvantages of these tests are: the time required, the need for animals, the high biological variability, the species specificity (Ashby 2000) and even the strain specificity. Especially the ethical aspects of these tests exclude them from monitoring experiments.

1.4.2. *In vitro* methods

The developed *in vitro* methods used for screening of xenoandrogens or xenoantiandrogens are based on cellular or subcellular systems. They evaluate the interaction of substances with cellular structures.

At the cellular level either the proliferative activity, depending on androgen action, of the cells themselves or specific proteins are evaluated (reporter gene assays). Here cells provide the transcription apparatus for the production of proteins which are under androgenic control. A negative proliferative effect of androgens can be seen in AR positive breast cancer cells (Poulin et al. 1991, Hackenberg et al. 1991, Bentel et al. 1999) and also in AR transfected breast or prostate cancer cells (Szelei et al. 1997, Yuan et al. 1993). These effects can be abolished by antiandrogen treatment.

The cells of recombinant reporter gene assays contain mostly genetically composed DNA constructs with a natural or genetically facilitated AR expression. The ligand-activated receptors initiate the transcription of a reporter gene (Fuhrmann et al. 1992, Vinggaard et al. 1999, Vinggaard et al. 2000, Térouanne et al. 2000, Blankvoort et al. 2001). A similar approach is used in yeast cells (Mak et al. 1994, Gaido et al. 1997, Sohoni and Sumpter 1998).

These assays are mostly very sensitive: representative detection limits are 3 pg/mL DHT in natural breast cancer cell lines (Hackenberg et al. 1991) and even lower (0.3 pg/mL R1881 in cell reporter gene assays; Vinggaard et al. 1999). Cell systems are also valid for evaluation of non-genomic steroid actions mediated by proteins other than steroid receptors. Assays to evaluate effects on second messenger pathways are described and show changes in cAMP or Ca^{2++} concentrations mediated by membrane proteins (Lieberherr and Grosse 1994, Nakhla and Rosner 1996, Machelon et al. 1998, Falkenstein et al. 2000).

The time required (up to 7d) and the special laboratory equipment needed for cell culturing restrict the applicability of these assays for screening purposes. Moreover, the susceptibility of cells to cytotoxic substances, possibly appearing in environmental samples, or built in side-effects of diverse chemicals themselves, compromise these test systems.

A reporter gene assay for both AR ligands and aromatase inhibitors in yeast cells (Mak et al. 1999) seemed to be more robust against cytotoxic substances. This system also allows predictions about substances interfering with enzymes involved in the androgen metabolism, since these substances simulate androgens in *in vivo*

experiments due to androgen accumulation. These assays are compromised by the specificity of S. cerevisae in terms of membrane permeability and transport of substances, since some cell strains do not react to antiandrogens (Rana et al. 1998). The membrane differences might account for the controversial results obtained by Sohoni and Sumpter (1998) and Gaido et al. (1997) on the androgenic activity of diethylstilbestrol and nonylphenol with their yeast reporter gene assays. In addition, the permeability of the cell walls might limit the sensitivity of these tests, as seen from the different IC_{50} values, 0.3 ng/mL and 1ng/ mL DHT respectively, of the yeast strains used.

The possible differentiation between androgens and antiandrogens with this reporter gene and proliferation assays may lead to false negative results in extreme cases. When androgenic and antiandrogenic substances exist in parallel in the samples, they may reciprocatively obliterate the effects. For androgens this must be taken into consideration, since most of the EDs are expected to be antiandrogenic and might be encompassed by naturally excreted androgens from mammals, e.g. in waste water.

At the subcellular level competitive receptor assays have long been in use. The main objectives were I) receptor quantification in terms of physiological response of tissues to androgens (Sauerwein and Meyer 1989, Shirota et al. 1997), II) receptor qualification such as receptor research on isoforms, truncated forms or structure (Rennie et al. 1983, Macaulay et al. 1988) and III) ligand qualification for medical research (Furr et al. 1987). The concept is based on the competition between the labelled ligand and the ligand to be tested for receptor binding. Research with AR assays on ED has rarely been reported. Mostly animal tissues were used as receptor sources (Kelce et al. 1994); in some cases cultivated human skin fibroblasts were used (Eil and Edelson 1984, Eil and Nisula 1990). These competition assays were also applicable in the entirely cultivated fibroblast cells to obtain information about substance transport into the cells (Wakimoto et al. 1980, Mowszowicz et al. 1981, Breiner et al. 1986).

Whatever the source of AR is, species specifity of AR recognition have to be considered even within mammalia since differences in ligand affinities are known at least for the progestin receptor (PR) (Jewgenow and Meyer 1998). Moreover, several reports indicate that sex hormone receptors from species living in aquatic environments differ from those of man in terms of affinity and specificity (Fitzpatrick

et al. 1994, Le Drean et al. 1995, Sperry and Thomas 1999).

For the development of bioresponse-linked instrumental analysis the target species of interest have to be clearly defined and the assay should be constructed accordingly. Certainly man is not the species most endangered by chemicals in surface waters but lack of knowledge is greatest here.

In 1998 the US Environmental Protection Agency stated the endpoints of ED in the field of androgens or antiandrogens as follows:

1. Receptor binding and function
2. Steroid synthesis inhibition
3. Plasma transport and rate of metabolisation and clearance.

The objective of this work was to focus on the AR binding.

1.5. Objective of the studies

Lack of information about the androgen-disruptive activity of licensed chemicals used in the environment calls for development of practicable assay systems. Sources of functional AR have therefore to be made available. The assay systems developed here were used to investigate general questions about the sources of androgen-active substances in the environment: Where do these substances come from and might they arise during metabolisation of chemical substances? The systems were used to define benchmarks for comparing the receptor binding affinities (RBA) of synthetic and natural androgens and EDs. The androgen RBA was also compared with binding affinities of these substances to other steroid hormone receptors (e.g. PR). As some of the already known androgen-active chemicals are in the herbicide category, the androgen RBA of the 29 mostly recommended herbicides of the Federal State of Hessen are evaluated in Section 3.2.1 (Bauer et al. 2002). Section 3.2.2 deals with the question whether AR binding substances may arise during natural metabolisation of chemicals in the environment (Bauer et al. 1998), and Section 3.2.3 evaluates the question if known androgen-active substances used in animal husbandry may entail a risk to the environment after excretion (Bauer et al. 2000 (I)).

2. Materials and Methods

2.1. Preparation of receptors

Two different sources of AR were used for the assays. Bovine AR (bAR) was extracted from the uteri of prepubertal calves. The tissue was obtained from the local slaughterhouse. Homogenisation and ultra-centrifugation were used to obtain bAR-containing cytosol. The maximum concentration of the soluble AR in the supernatant was 8 fmol/mg of protein.

The source of human AR (hAR) was insect cells infected with recombinantly modified baculoviruses. After virus infection, the insect cells produced the recombinant hAR (rhAR) in cytosolic form. This cytosol was obtained by centrifuging the disrupted cells. The concentration of the rhAR was up to 150 fmol/mg protein.

The bovine PR (bPR) was obtained in the same way as the bAR, but the tissue used was from the uteri of cyclic preovulatory heifers.

2.2. Assay systems

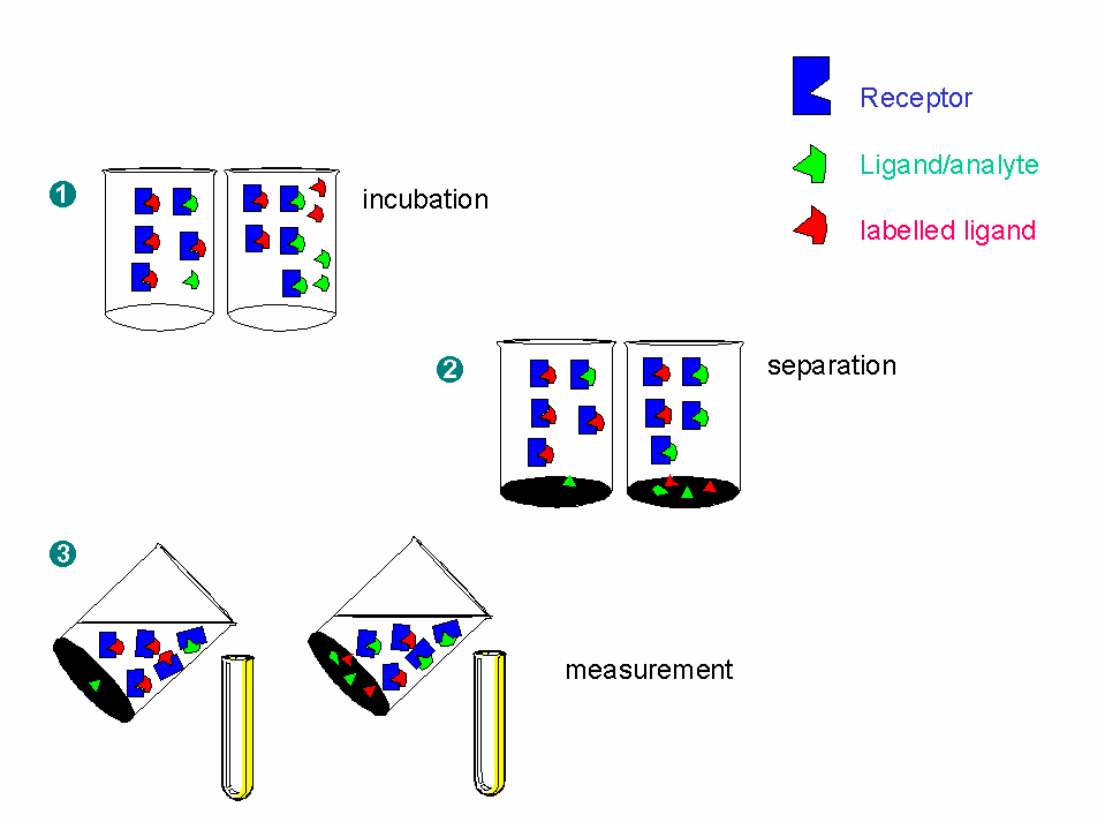
AR and PR were used to develop different assay systems. The underlying principle of evaluating the affinity of a substance to a receptor was the competition of this substance in binding to the receptor in comparison with a labelled high-affinity ligand. For the AR assays tritiated dihydrotestosterone (^{3}H-DHT) was used as labelled ligand. Two forms of AR assays were developed:

2.2.1. In solution AR assay

The cytosol was incubated with increasing concentrations of the analyte and constant concentrations of ^{3}H-DHT. After separating the bound from the free substances by treatment with dextran-coated charcoal in buffered saline and centrifugation, bound ^{3}H-DHT was quantified by counting the radioactive decay. The system is illustrated in Figure 2. To mask the bPR, also present in the cytosol, synthetic progestin, ORG 2058, was used.

By incubation of the receptors with increasing DHT concentrations, the dissociation constants (K_D) were evaluated as described by Scatchard et al. (1949).

Figure 1: In-solution hAR assay

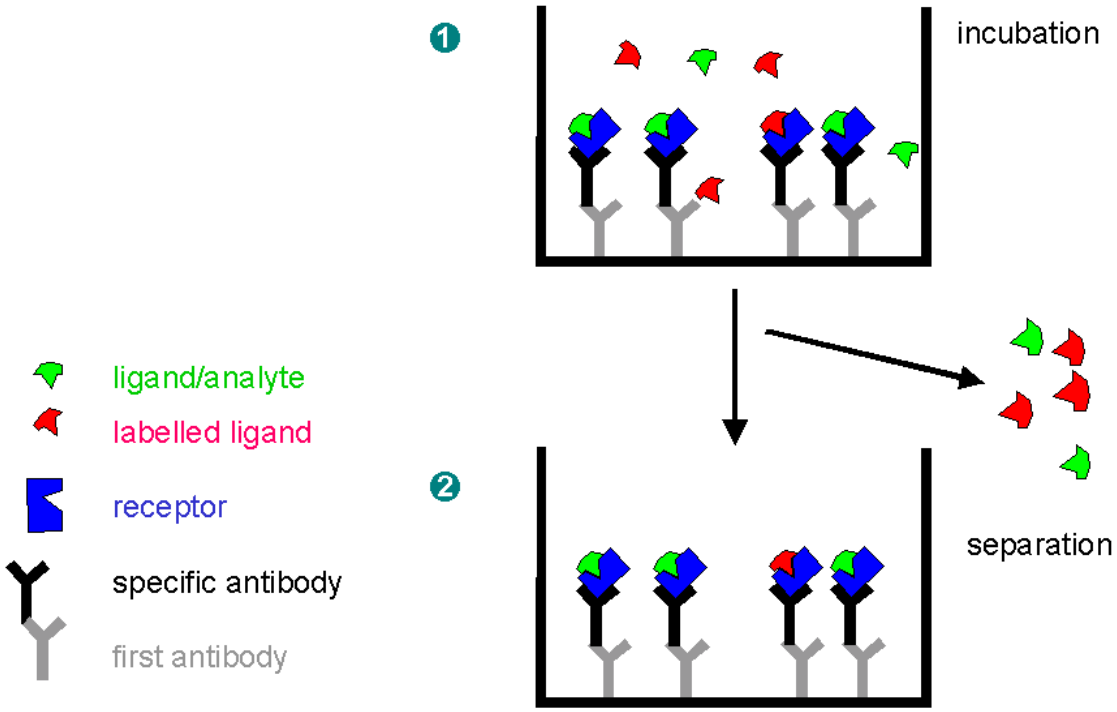


The reversibility of the binding was evaluated by a first incubation of the AR with the analytes and a second incubation with ^{3}H-DHT. The more bound ^{3}H-DHT was, the less receptors were disturbed by the analytes and the higher was the reversibility of binding. Additivity experiments were conducted by incubation of the receptors with mixtures of analytes in different concentrations.

2.2.2. Immuno-immobilised receptor assay (IRA)

The system was modified to improve performance and the sample number. The rhAR were fixed via a double antibody technique on 96-well microtitre plates. To separate the bound from the free ligands, the plates were washed. The receptor-bound ^{3}H-DHT was counted in each well. Figure 3 gives a schematic drawing of the procedure.

Figure 2: Assay principle of the IRA



2.2.3. PR and SHBG assays

The bPR preparation was used with tritiated ORG 2058 (^{3}H-ORG) as stable, labelled ligand for the PR assay. The ligand used for the SHBG assay was ^{3}H-DHT. The recombinantly produced human SHBG was from Calbiochem (Bad Soden, Germany). The principle for both assays was the same as for the in-solution bAR assay.

2.2.4. Data evaluation

The displacement curves were sigmoid with a plateau at the maximum and a baseline, where the compound was not able to completely inhibit binding of the labelled ligand. To calculate the binding data a non-linear, logistic regression with 4 parameters (Sigma plot®) was used:

$$y = y_0 + A/[1 + (x/x_0)^B] \quad , \qquad (1)$$

where y_0 denotes the baseline. A is the plateau value and shows the maximum binding of the receptor, whereas B represents the slope and x_0 gives the point of inflection of the displacement curve. With y set equal to 50, this equation, was used to calculate the exact concentration of the unlabelled compound which gives a 50% displacement of the labelled compound (IC_{50}). To compare the different compounds

tested, the inhibition constants (K_i) were calculated according to the following equation (2):

$$K_i = IC_{50} / (1 + S/K_D) , \qquad (2) \qquad \text{(Cheng \& Prusoff 1973)}$$

Where S represents the concentration of the labelled ligand and K_D is its dissociation constant; the value of K_D was determined by Scatchard analysis for DHT binding to AR (Scatchard 1949). For each compound the RBA in comparison with DHT was calculated according to equation (3):

$$RBA_{(x)} = Ki_{(DHT)} * 100 / Ki_{(x)} \quad (3)$$

2.3. Analytes

The different chemicals and hormones were all of p.a. qualitiy, with the exception of the metabolites of MGA. The MGA metabolites were prepared by Prof. Metzler (University of Karlsruhe) by incubation of liver microsomes with MGA and separation of the products by HPLC. All analytes were dissolved in ethanol or methanol and diluted in buffer until the alcohol concentration was below 10 %. The final concentration of alcohol in the assays was below 2 %.

3. Results and Discussion

3.1. Development of new assay systems

3.1.1. BAR assay

As a starting point for developing further assay systems, an AR assay with AR from animal tissues was developed to establish a screening system for suspected androgenic or antiandrogenic chemicals (Bauer et al. 1998). The first criterion was the availability of suitable tissues for AR extraction. Between mammalians a very high degree of conservation of the DNA and ligand-binding part of the receptor was described (He et al. 1990, Thornton and Kelley 1998), and therefore AR of non-human origin could be used for this assay. The homology of the DNA at the ligand-binding site between human AR and bovine AR is higher than 90% and even higher at the protein level. Juvenile or castrated animals were preferable because of their low endogenous hormone levels. The nuclear location of the receptors after androgen binding makes them difficult to extract and mostly useless for receptor tests. An initial screening of different tissues showed that the highest content of AR can be found in the uterus of juvenile cattle with 8 fmol/mg protein. The low concentration of proteolytic enzymes is a further benefit of uterus tissues (van Loon et al. 1988). The second criterion was the selection of a suitabl labelled ligand. The ligand has to be specific to AR, since the presence of other different hormone receptors in the preparation can not be circumvented. Moreover, the ligand has to be stable and bind only slightly to plasma proteins (unspecific binding). [^{3}H]-DHT was selected since it is a ligand specific to the AR. The affinity to the ER and GR are below 0.06% in comparison with 100% to AR and 1.1% to the PR (Rapp 1996). To avoid interference with PRs, they were saturated with the specific synthetic ligand ORG 2058 (Formento et al. 1987). The stability of [^{3}H]-DHT was shown for the assay conditions (4° C) and room termperature for 16 hours, during which no decrease of the binding could be detected. In addition, low unspecific binding was obtained and the linear Scatchard plot indicated a single specific binding side. With this assay system the RBA of different synthetic or natural ligands was appreciable. The detection limit of this assay was 1.4 ng/mL of DHT as calculated by three times standard deviation of the blank value. The inflection point of the assay calibration curve was at 4.9 ng/mL. For testosterone the detection limit was 6.4ng/mL.

3.1.2. Cloning of the human AR and production of functional protein

The hAR is needed to build up an assay system to get information about potential EDs in man. As human tissue or tissue cells are not commercially available, recombinant protein expression appeared to be an appropriate method of obtaining AR free of any other steroid receptors in a stable and constant amount.

Different expression systems are described to produce recombinant proteins. For the hAR, expression systems with E. coli (Young et al. 1990, Roehrborn et al. 1992), yeast (Purvis et al. 1991, Mak et al. 1994), insect cells (Xie et al. 1992, Chang et al. 1992) and human cell lines (Quarmby et al. 1990, Hall et al. 1992) were reportedly successful. In this work the AR expression was carried out with: 1) E.coli, 2) S. pombe, 3) insect cells. The cDNA produced by reverse transcription polymerase chain reaction technique from human prostate mRNA was used by the author to insert different parts of the hAR DNA, including the ligand-binding domain, into expression plasmides for E. coli or S. pombe. The receptor expression experiments resulted in mostly insoluble AR protein not applicable to assay development. In contrast, the insect cells were useful for producing functional rhAR.

160 fmol/mg of protein of active receptor was obtained from the baculovirus infected insect cells. Western blot analysis confirmed the expected size of 98.5 kDa. The equilibrium K_D of DHT from the androgen receptor system was 2.5×10^{-9} M. The order of RBAs to the natural steroids were DHT > 19-nortestosterone (19-NT) > testosterone > estradiol > progesterone.

The different binding affinities of synthetic and natural steroids were examined to prove the functionality of the receptor and to have a benchmark for comparison. rhAR showed high specific binding to DHT of the same order of magnitude as described for the AR from human skin fibroblasts (Keenan et al. 1975, Breiner et al. 1986) and for rat androgen receptor (Carroll et al. 1984). Others reported AR K_D values one rank below these data, but this might be attributable to differences in the test systems used. For the RBA of testosterone in comparison with DHT, a range between 38% for hAR from intact MCF7 cells and 19% for hAR from human genital skin fibroblasts has been described (Bergink et al. 1983, Breiner et al. 1986). In the present system an RBA value of 31.3% was found. The affinity of 19-NT (75.2%) to the hAR is also in the range described for bovine and rat AR (Meyer and Rapp 1985, Lemus et al. 1997). Various doping agents and therapeutically used progestogens were also evaluated (Bauer et al. 2000 (I)). The doping agents, boldenone and

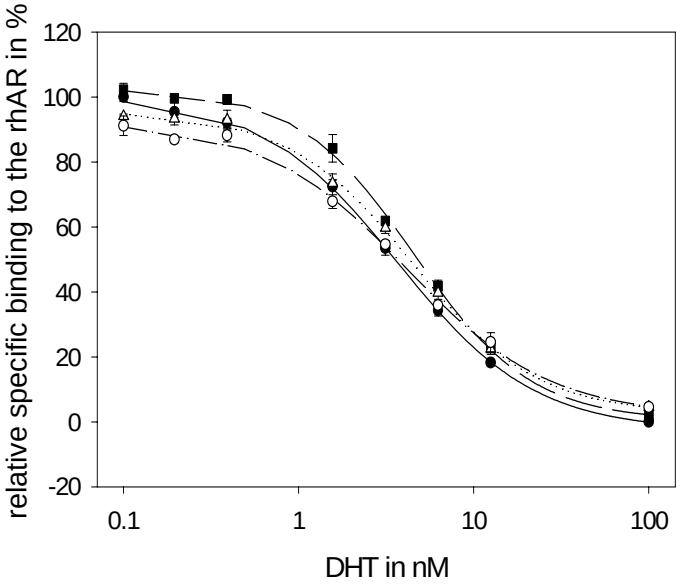
methyltestosterone, showed a high affinity to the rhAR between DHT and testosterone. The therapeutically used substances, allyltrienolone, medroxy-progesterone actate (MPA) and chlormadinoneacetate (CMA), also showed a high affinity to the hAR between 75% and 15%. MPA is a subject of controversy as AR agonist and antagonist (Gräf et al. 1974, Bentel et al. 1999, Kemppainien et al. 1999), and CMA is discussed as androgen antagonist (Labrie et al. 1987, Poulin et al 1991). These earlier findings are in line with the values observed. The RBA of progesterone and estradiol, which was below 1/20 of that of DHT, documents that these steroids are also bound in preparations containing exclusively hAR.

3.1.3. Development of a screening assay on micro titre plates (IRA)

It was possible to fix the rhAR in active form to microtitre plates by the double antibody technique. The functionality could be verified by incubation with [^{3}H]-DHT and increasing DHT concentrations. After separating the wells and transfer into scintillation vials the bound radioactivity was measurable. In this format also a competitive assay could be performed with the detection limit of 1ng/mL of DHT and a point of inflection in the calibration curve at 3.3 ng/mL of DHT. A highly significant difference ($p < 0.001$) could be calculated between the blank value and the sample already at a concentration of 0.3 ng/mL of DHT with the students t-test (Sigma Stat ®).

With this assay system direct evaluation of different environmental water samples was possible (figure 3) (Bauer et al. 2000 (II)). No decrease in binding could be measured by evaluating DHT-spiked tap water, river water or sewage plant effluent.

Figure 3: Displacement of ^{3}H-DHT binding by unlabelled DHT spiked into different water samples



IC_{50} values of DHT in different water qualities (nM):	
○ pure water	3.37
■ tap water	4.29
▲ river water	4.17
Δ sewage effluent	3.83

This demonstrated that the assay system developed is useful for screening environmental samples from their androgenic content.

3.2. Application

A lot of different sources of EDs are maintainable, as pointed out in section 1.3. One critical point is the use and distribution of chemicals in the environment since these substances are new and unknown components. Unknown are items such as distribution, metabolisation, clearance and accumulation. For natural substances, these items are probably also not clear, but the survival of the living being shows no critical interference with the natural substances. A special danger for humans seems to arise when these chemicals are used in the food production chain. As a consequence of efforts made to increase agricultural productivity pesticides are often used, but these substances are rarely tested for their endocrine disruptive activity. With the IRA it was possible to screen 29 of the most recommended pesticides of the Federal State of Hessen, Germany.

3.2.1. Binding affinities of pesticides

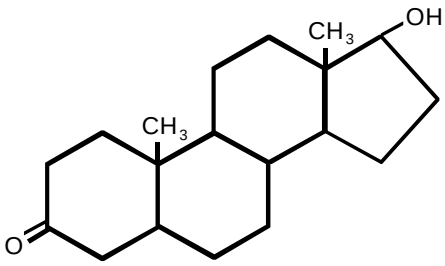
28 of the pesticides tested had an RBA to the rhAR that was below 0.011% as compared with DHT (100%) (Bauer et al. 2002). This agrees with the binding

properties reported for different pyrethroids to human skin fibroblast AR, e.g. the RBA of fenvalerate and permethrin was 0.000027% and 0.000015% as compared with methyltrienolone (Eil and Nisula, 1990). The affinity of methyltrienolone was demonstrated to be comparable to DHT in human fibroblast AR (Eil and Edelson, 1984).

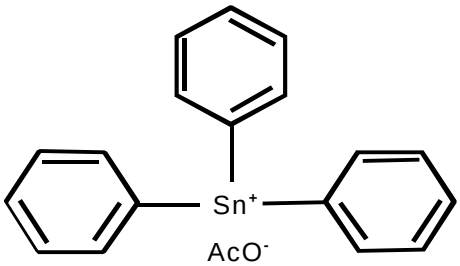
Among the herbicides evaluated, only fentinacetate (triphenyltinacetate, structure: figure 18) had a significantly higher RBA to the rhAR of about 1.42% (K_i = 124.5 nM = 51 µg/L). It was possible to show that the binding of fentinacetate to rhAR is reversible. Fentinacetate is used as fungicide on crops, but also as algaecide and molluscicide on boats and ships. It is used on, for example, potatoes, celery, onions, sugar beet, peanuts, beans, wheat and cocoa (Tomlin 1997).

Figure 4: Structure formulas of DHT and fentinacetate.

DHT



Fentinaceatate



Recently triphenyltin (TPT, corresponds to fentin) was found to induce imposex in female ramshorn snails (Schulte-Oehlmann et al. 2000). Fecundity was reduced at a concentration as low as 230 nM TPT in water during incubation for 4 months. Similar effects have already been described for tributyltin (TBT), which is an organic tin compound comparable to fentinacetate. TBT induces superimposition of male organs such as a penis or vas deferens on the female genital system (Jenner 1979, Smith 1981, Oehlmann et al. 1995, 1996, Horiguchi et al. 1998). For these effects alterations of the steroid hormone-metabolising enzymes, in particular of the cytochrome p 450 aromatase, are likely to be responsible (Spooner et al. 1991; Bettin et al. 1996).

TPT showed androgen-like action in an androgen-dependent transcription and cell proliferation assay (Yamabe et al. 2000). Low concentrations of 1 nM TPT showed the same proliferative and transcription activating effects as does 10 nM DHT in a human prostate cancer cell line. In contrast to DHT, the effect of TPT was

not suppressed by simultaneous application of flutamide, an antiandrogen used in medical therapy protocols. Using a transcription assay in human hepatoma cells, Maness et al. (1998) could demonstrate that flutamide and also hydroxyflutamide (OH-flutamide) in concentrations higher than 1 µM are complete transcription agonists. OH-flutamide is the antiandrogenically active metabolite of flutamide metabolized *in vivo* but probably at lower concentrations. As the required concentrations of OH-flutamide to displace the agonistic effect of TPT might be higher in comparison with the concentrations required for DHT, effective concentrations are reached at which OH flutamide also has agonistic effects. Hence, it is probably not possible to show the same effect of flutamide for both substances.

Information about the androgenic or antiandrogenic effects of fentin compounds evaluated by in vivo assays with mammalia is sparse. The acceptable daily intake (ADI) value is stated to be 0.5 µg/kg body weight per day by the WHO because of the reduction of the body weight of the dam in reproductive studies in rats and rabbits (Lu 1995).

3.2.1.1. Additivity

A very disturbing factor in the fields of EDs is synergistic effects. Such effects have described by several authors: Arnold et al. (1997) reported synthetic effects from their assay systems. This paper was withdrawn later on (McLachlan 1997) but again information about synergistic effects of ED was made available in 2000. At the workshop on “Hormones and endocrine disrupters in food and water” in Copenhagen 2000 Gray (2000) reported that procymidone and the endocrine disrupter dibutylphtalate (DBP) react synergistically when administered to rats. Synergistic effects of natural steroids were also reported in turtles (Bergeron et al. 1999) and therefore suspected as endocrine disrupters. These results indicate that the various effects of ED are not yet completely understood.

It could be shown for six different pesticides plus DHT or DHT derivatives to behave additively in this assay system when compared as binary mixtures. When the substances were categorised according to strong, middle and weak affinity to the rhAR, these substances showed additivity in all possible combinations.

3.2.1.2. Reversibility

In this test the binding of a substance to the rhAR was demonstrated by a decrease of the specific binding of [^{3}H]-DHT. This is possible by specific

displacement of [^{3}H]-DHT but may also occur through total disturbance of the receptor. The stability of the receptor during incubation must therefore be demonstrated. For six relevant herbicides we could prove reversibility. After the first incubation with the different herbicides at different concentrations they were again displaced with [^{3}H]-DHT in a second incubation step. Here the recoverable receptor binding was between 72% and 96% of the recoverable receptor content in the same experiment with DHT.

The results showed for all the pesticides, except one, a low binding affinity to the AR. The chemicals themselves were evaluated, but the behaviour in nature in the way of degradation is not clear. Here some danger might arise, as already indicated by Kelce et al. (1994). These evaluations showed that the two main metabolites of vinclozolin had a 12- and 310-fold RBA to the AR, respectively, in comparison with the original substance. This is comparable with a concentration of 0.44 mg/L of vinclozolin. The ADI of vinclozolin was set at 0.07 mg/kg body weight by the WHO (Lu 1995), i.e. showing a safety factor of only 6 to the IC_{50} value. To investigate this question, different phenylurea herbicides and known derivatives were evaluated with the bAR assay.

3.2.2. Evaluation of phenylurea herbicides

The RBA of 3,4-dichloracetanilid (3,4-DCAc), linuron, flutamide, 3,4-dichloroaniline (3,4-DCA), 3,4-dicholorpenylurea (3,4-DCPU) and diuron confirms the ability of these compounds to interact with the AR (Bauer et al. 1998). Similar data for the RBA of flutamide have been reported (Cook et al. 1993, Simard et al. 1986). It was possible to show that the common metabolite 3,4-DCA, which may also originate from sources other than herbicides, had double affinity to the AR after further metabolisation to 3,4-DCAc. This further metabolisation is found after incorporation of 3,4-DCA into fish. The RBA of 3,4-DCAc is higher than the RBA of linuron, for which an antiandrogenic effect on rats was demonstrated with the same order of AR affinity (Cook et al. 1993). The affinities to the AR measured for the substances were very low, and, vice versa, the concentrations of linuron in the animal experiment had to be high to show antiandrogenic effects. The increasing affinity of substances after metabolisation is of concern. To be able to judge whether the affinities of the various substances to the androgen receptor are of biological relevance, one has to consider the concentrations found in the aquatic systems. For 3,4-DCAc, the IC_{50} value is 10

mg/L, but unfortunately environmental 3,4-DCAc concentrations have hitherto not been reported. If they are in the same range as the 3,4-DCA concentrations, e.g. 140 ng/L (Gülden et al. 1997), the safety factor would be high enough. However, other factors such as permanent exposure or potential accumulation have to be considered to be able to rule out the risk of reproductive functions, in particular for aquatic organisms.

Most of the industrial chemicals discriminated as "EDs" display only weak hormonal activity representing unintentional side-effects. In contrast to these substances, there is a wide variety of other substances being developed with the aim of significantly influencing the endocrine system. These synthetic hormones are used for medical purposes (e.g. contraception, hormone replacement therapy) or as growth promoters in farm animals. In the USA and in Canada, the synthetic steroids, melengestrol acetate (MGA) and trenbolone acetate (TBA), are licensed in cattle fattening besides the natural compounds estradiol, progesterone and testosterone. For TBA and MGA neither the complete spectrum of biological activities nor the potential endocrine-disrupting activity of their excreted metabolites in the environment is fully understood, and so their affinity to the rhAR, the bPR and the rhSHBG was evaluated from the original substances and from the main metabolites.

3.2.3. Studies of binding affinities of growth promotors

TBA is hydrolysed after administration to 17β-trenbolone (17β-TbOH), the active androgenic compound. About 75.6% of the original substance is excreted as 17α-trenbolone (17α-TbOH) (Pottier et al. 1981). The evaluations (Bauer et al. 2000 (I)) are summarised as follows: the high affinity of 17β-TbOH to the rhAR and to the bPR was reduced after metabolisation into 17α-TbOH and triendione (TbO) to less than 1/24 of the original substance. In comparison with DHT, 17β-TbOH showed a lower affinity to rhSHBG and the other metabolites had negligible affinity to rhSHBG. According to the RBA of 17α-TbOH and 17β-TbOH, the binding activity of excreted 17α-TbOH to the rhAR is comparable to excretion of 3.4% of 17β-TbOH. In addition, 2% of non-metabolised 17β-TbOH is excreted. In consequence, residues with significant binding capacity and potentially endocrine-disrupting activity are excreted after TBA treatment.

The growth promoter, MGA, exhibited a very high affinity to the bPR, but only marginal affinities to the rhAR and to the rhSHBG. For the three major metabolites

built *in vitro*, a residual affinity to the bPR was determined in the range between 85% and 28% of the affinity of progesterone.

For medically used megestrol acetate, which has a very similar structure to that of MGA, androgen-like activity has been reported for human breast cancer cells (Poulin et al. 1991) as well as an affinity to the hAR from MCF7 cells which is of the same magnitude as for testosterone (Bergink et al. 1985). Considering the divergent action properties of these two structurally closely related compounds, it is obvious that safety evaluations for MGA cannot be done by extrapolation from related substances.

3.3. Ligands of the AR

The test systems described were developed to record not a single substance but the class of androgens or antiandrogens. The values reported are seen always in relation to the strongest natural ligand of the AR, DHT. The RBA in comparison with DHT is reported as a percentage figure when DHT is set to 100%. The evaluated RBA with the AR assays showed a broad diversity. The highest affinity was displayed by the natural and synthetic steroids used for medical purposes, in animal husbandry and in human doping. Some of the synthetic gestagens, often used in contraceptive pills, also exhibit a high affinity to the AR, to antagonise androgen action. A lower, but still remarkable affinity was demonstrated for progesterone and estradiol, being of the same order of magnitude as 17α-TbOH, the main metabolite of TBA. Among the chemicals and herbicides evaluated, fentinacetate showed by far the highest affinity to the AR, being comparable to progesterone and estradiol. The results agree with results reported from other assay systems. It is noteworthy that in receptor assays applied to detect potential estrogenic activities of chemicals, the RBA's of BPA or nonylphenols are 30-170-fold lower than the RBA of fentinacetate reported herein (Kuiper et al. 1998, Blair et al. 2000).

3.3.1. The IRA in comparison with other test systems

To evaluate a complex item such as EDs, different assay systems are needed. The applicability of an assay is correlated with the detection limit of the substances evaluated. Since the detection limit directly depends on the K_D of the AR to the natural specific ligands, there is a great difference between the IRA and cell assays

where the time of incubation increases the sensitivity since the ligands may create multiple effects due to repeated receptor binding and transcription. In view of the naturally effective concentrations of androgens in men and woman ranging between 1-5,6 ng/mL (Wuttke 1990, Voss 1973 III) the developed assay is able to detect critical contaminations. However, in prepubertal children, much lower endogenous concentrations of androgens are reported: 0.3-1.5 ng/mL for males (Bidlingmaier et al. 1980, Goji and Tanikaze, 1993) and for females. Children might therefore be very sensitive to steroidal hormones. These concentrations are also a matter of interest since some of the EDs are known to accumulate in the organism and might be set free in particular phases (breast milk) (Greizerstein et al. 1999). To evaluate lower substance concentrations, a sample extraction can easily be performed.

Compared with cell-based assay systems, the presence of agonistic or antangonistic binding compounds within the same sample does not affect reliability since binding by itself is the only criterion evaluated. In spite of the fact that androgenic or antiandrogenic activity of substances in a mixture cannot be differentiated, the interference with the receptor reliably implies the presence of substances that interfere within the androgenic system. The proof of additivity for compound mixtures at the binding activity level provided herein demonstrates that false negatives can be precluded. In conclusion, the criterion of the ability of a given substance or a mixture of various substance to bind to the AR is sufficient for the screening system aimed at; further questions concerning stimulatory or inhibitory effects are secondary at this point.

This assay system is realised on microtitre plates and thus facilitated the handling of large sample numbers. For the entire assay a time of 3 hours is needed compared with an incubation time overnight. Compared with cell-based systems, no restrictions are imposed by the cell wall permeability or toxic substances. Direct screening of water samples is possible. Due to the required radioactivity it has hitherto been a method for licensed laboratories.

3.3.2. Prospects

To have an assay to screen for androgens and antiandrogens, it would be preferable to change the radioactive-labelled ligand into other types of labels. This may be successful with europium-labelled ligands or, with the right requisites, also with fluorescence-labelled ligands. Here sensitive readers and readily excitable fluorophors are needed. Ligands labelled with enzymes of low molecular weights, e.g. microperoxidase, might be useful alternatives.

The recombinant receptor produced herein will also be useful to develop receptor affinity columns for chromatography. With this approach, all receptor ligands are extracted from environmental samples and can then be analysed and quantified by GC-MS. These columns have already been described for the estrogen receptor (Scippo 2000). By combining different receptors on one column, the extraction of all hormonally active substances might be achieved.

4. Abstract

Several investigations have shown that a wide variety of chemicals present in the environment are capable of interacting with the endocrine system. To analyse critical substances for their endocrine disruptive potency, various assay systems have been developed during the last few years. These bioresponse-linked analytical methods evaluate the effects of substances by their interaction with cellular structures. One level of interaction of chemicals with the endocrine system is hormone-receptor binding. Chemicals can be evaluated for their hormonal effectsby using the corresponding hormone receptors. To date a large number of estrogenic or antiestrogenic compounds are known, in contrast to very few known androgenic or antiandrogenic compounds. This also indicates the lower number of assays available for androgenic effects.

The lack of information about the androgenic disruptive activity of licensed chemicals used in the environment makes the development of practical assay systems necessary.

This work comprises the development of new androgen receptor (AR) assays and their application. It was possible to make different sources of AR available. Functional bovine AR (bAR) was extracted with a concentration of up to 8 fmol/mg protein from the uterus, and recombinant human AR (rhAR) was produced with baculovirus and insect cells in concentrations of up to 150 fmol/mg protein.

Two different AR assays were developed. The bAR extracts, with the lower AR concentration, were useful for developing a liquid AR assay. With the rhAR preparations, a new assay formate was developed. The rhAR was immobilised in active form on 96-well microtitre plates using receptor specific antibodies. The so-called IRA (Immuno-immobilised Receptor Assay) was entirely validated, even for application with real water samples. The 96-well assay formate guaranteed convenient handling and high sample throughput.

In the following, different classes of man-made chemicals were evaluated for their AR binding. For chemicals used in the food production chain it seems very important to evaluate possible endocrine disruptive activities, because the incorporation of these compounds must be taken into consideration. Here, 31 licensed pesticides, several pesticide metabolites and synthetic hormones including some metabolites were evaluated. The synthetic hormones, trenboloneacetate (TBA)

and melengestrolacetate (MGA), which are licensed in the US and Canada for cattle fattening, were of special interest. These synthetic hormones and their metabolites were additionally analysed for their progestin receptor and their human sex hormone binding affinity in order to have a benchmark for comparison.

The values reported are given in relation to the strongest natural ligand of the AR, dihydrotestosterone (DHT). The relative binding affinities (RBA) in comparison with DHT are reported as a percentage figure, with DHT set as 100%. The RBAs evaluated with the AR assays were very divergent for the different chemicals tested. The highest affinities were exhibited by the natural and synthetic steroids used for medical purposes, in animal husbandry and in human doping. These substances were: DHT, 17β-trenbolone (active compound of TBA), allyltrienolone, 19-nortestosterone, boldenone, medroxyprogesteroneacetate, methyltestosterone and testosterone. Some of the synthetic gestagens, often used in contraceptive pills, also had a high affinity to the AR. A lower, but still appreciable affinity was shown for progesterone and estradiol, of the same order of magnitude as 17α-TbOH, the main metabolite of TBA. Among the chemicals and herbicides evaluated, fentinacetate showed by far the highest affinity to the AR, being comparable to progesterone and estradiol. For a further 13 of the pesticides evaluated the affinity to the AR could also be demonstrated.

The assay system developed herein is useful as a convenient high throughput screening tool to analyse not only chemicals but also surface waters or drugs. Even the evaluation of illegal doping products or residue monitoring is practicable.

Compared with cell-based systems, the presence of agonistic or anti-agonistic binding compounds within one sample does not affect reliability since binding it self is the only criterion evaluated.

Zusammenfassung

Unterschiedliche Untersuchungen haben gezeigt, dass eine große Anzahl von Chemikalien, die in der Umwelt vorkommen, das endokrine System beeinflussten. Um Substanzen auf diese Wirkung hin zu untersuchen, wurden in den letzten Jahren einige Testsysteme entwickelt. Diese Analysemethoden untersuchen die biologischen Wirkungen von Substanzen auf zelluläre Strukturen. Eine Ebene der Wechselwirkungen von Chemikalien mit dem endokrinen System ist die Hormon-Rezeptor Bindung. Um Substanzen auf ihre hormonelle Wirkung zu untersuchen, können die entsprechenden Rezeptoren verwendet werden. Zur Zeit ist bereits eine große Anzahl an östrogen oder antiöstrogen wirkenden Verbindungen bekannt, im Gegensatz zu einer geringeren Zahl an androgen oder antiandrogen wirkenden Verbindungen. Dieses Verhältnis spiegelt auch die geringe Anzahl an verfügbaren Testsystemen für androgene Wirkungen wieder. Auf Grund der fehlenden Informationen bei lizensierten Chemikalien über deren adverse Wirkung im Bezug auf Androgen Wirkung sind solche Testsysteme aber erforderlich.

Diese Arbeit beinhaltet die Entwicklung von neuen Androgenrezeptor (AR) Testsystemen und deren Anwendung. Es war möglich AR aus verschiedenen Quellen zu gewinnen. Funktionelle bovine AR (bAR) konnten bis zu einer Konzentration von 8 fmol/mg Protein aus Gewebe extrahiert werden. Bei der rekombinanten Herstellung von humanen AR (hAR) mit Baculoviren und Insektenzellen konnten bis zu 150 fmol AR pro mg Protein gewonnen werden.

Es wurden zwei verschiedene AR Testsysteme entwickelt. Die bAR Extrakte mit der niedrigeren AR Konzentration konnten zur Entwicklung eines AR Testsystems in Lösung verwendet werden. Mit den rekombinanten hAR (rhAR) Präparationen wurde ein neues Testformat entwickelt. Die rhAR konnten mit spezifischen Antikörpern in aktiver Form an 96-Well Mikrotitrationsplatten fixiert werden. Der so genannte IRA (Immuno-immobilisierter Rezeptor Assay) wurde sogar für die Anwendung mit realen Wasserproben validiert. Das 96-Well Format steht für eine bequeme Handhabung und einen hohen Probendurchsatz.

Darauffolgend wurden verschiedene Klassen von Chemikalien auf ihre Fähigkeit an den AR zu binden untersucht. Bei Chemikalien, die bei der Produktion von Lebensmitteln verwendet werden, erscheint es besonders wichtig, eine mögliche das endokrine System störende Wirkung zu untersuchen, da man bei diesen Substanzen nicht ausschließen kann, dass sie mit der Nahrung aufgenommen werden. In dieser

Arbeit wurden 31 zugelassene Pestizide, einige Pestizidmetaboliten und synthetische Hormone und ihre Metaboliten untersucht. Von besonderem Interesse waren die synthetischen Hormone Trenbolonacetat (TBA) und Melengestrolacetat (MGA), die in den USA und in Canada bei der Rindermast zugelassen sind. Diese synthetischen Hormone und ihre Metaboliten wurden außerdem auf die Affinität zum Gestagenrezeptor und zum humanen Sex-Hormone-Binding-Globulin untersucht, um die Affinitäten untereinander zu vergleichen.

Die angegebenen Werte sind immer Vergleichswerte zu dem stärksten natürlichen Liganden des AR, Dihydrotestosteron (DHT). Die relative Bindungsaffinität (RBA) ist als Prozentsatz im Vergleich zu DHT mit 100% wiedergegeben. Die gefundenen RBA-Werte sind sehr unterschiedlich. Die höchste Affinität hatten die natürlichen und synthetischen Steroide, die in der Medizin, in der Tiermast und zum Doping verwendet werden. Diese Substanzen beinhalten: DHT, 17β-Trenbolon (die aktive Komponente von TBA), Allyltrienolon, 19-Nortestosteron, Boldenon, Medroxyprogesteronacetat, Methyltestosteron und Testosteron. Aber auch einige der synthetischen Gestagene zeigten eine hohe Affinität zum AR. Diese werden zum Teil in Medikamenten verwendet, um der Androgenwirkung gegen zu steuern. Eine niedrigere aber immer noch bemerkenswerte Affinität zeigten Progesteron und Östradiol in der selben Größenordnung wie 17α-Trenbolon, dem Hauptmetaboliten von TBA. Unter den untersuchten Herbiziden und Chemikalien zeigte Fentinacetat bei weitem die höchste Affinität zum AR, vergleichbar mit Progesteron und Östradiol. Bei weiteren 13 der untersuchten Pestizide konnte eine Affinität zum AR nachgewiesen werden.

Das entwickelte Testsystem ist ein gut zu handhabendes Werkzeug zur Untersuchung nicht nur von Chemikalien, sondern auch von Wasserproben oder Arzneimitteln. Sogar die Untersuchung von illegalen Dopingprodukten oder Rückstandsanalytik ist möglich. Verglichen mit Systemen, die mit ganzen Zellen arbeiten, beeinträchtigt das Vorhandensein von angonistischen und antagonistischen Substanzen die Aussagekraft des Ergebnisses nicht, da die Rezeptorbindung das einzige Bewertungskriterium ist.

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Cancer Research 1993; 53: 1304-1311

6. Curriculum vitae

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School:

1978-1982	primary school Grafrath
1983-1991	Viscardi-Gymnasium, Fürstenfeldbruck
1991	qualification for university entrance

Vocational training:

1991	study of pharmaceutics
1992-1996	study of food analysis at the Ludwig Maximilian University in München
1996	graduated with diploma (1 Staatsexamen)

Practical work:

1992	Paracelsus Apotheke (pharmacy)
1996	Barnhouse Naturprodukte (food producer)
1997-2000	employee at the Institute of Physiology TU München-Weihenstephan
Since 2001	employee at Cefak KG, Kempten (pharmaceutical industry)

7. List of publications

Publications by the author:

Refereed:

- Bauer ERS, Meyer HHD, Stahlschmidt-Allner P, Sauerwein H.
  Application of an androgen receptor assay for the characterisation of the androgenic or antiandrogenic activity of various phenylurea herbicides and their derivatives.
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- Bauer ERS, Bitsch N, Brunn H, Sauerwein H, Meyer HHD
  Development of an immuno-immobilized androgen receptor assay (IRA) and its application for the characterization of the receptor binding affinity of different pesticides
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Lectures:

- "Analytik und Vorkommen von androgen wirksamen Substanzen in der Umwelt." Kolloquium über ausgewählte Kapitel der Physiologie, physiologische Chemie und Tierernährung, Tierärztliche Fakultät der Ludwig-Maximilian-Universität München. 25.01.1999.

- "Efficient expression of recombinant human androgen (hAR) receptor from baculovirus infected insect cells and development of a microtiterplate androgen receptor assay for AR-binding chemicals."
  At the Euroresidue IV conference on “Residues of Veterinary Drugs in Food" , 8-10.05.2000 Veldhoven, The Netherlands

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- Bauer E, Meyer HHD, Allner B, Sauerwein H
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- Bauer ERS, Daxenberger A, Petri T, Sauerwein H, Meyer HHD
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  27-30.05.2000 Rigshospitalet Copenhagen University Hospital.

- Obst U, Brenner-Weiß G, Seifert M, Sauerwein H, Bauer E, Meyer HHD, Hock B
  Receptors as analytical tools for bioresponse-linked instrumental analysis.
  In: Bioresponse-Linked Instrumental Analysis, Editor: B. Hock, Teubner-Reihe Umwelt 2001, 79-103.

By the author of this dissertation the following work was done:

- Collection of the material form the slaughterhouse and preparing the receptors.
- Development of the liquid assay with bAR and evaluation of the substances.
- Microbiological and cloning work with E. coli and S. pombe for protein production.
- Insect cell culturing and production of hAR. Westernplots and protein quantification.
- Development of IRA and evaluation of all substances.
- Data evaluation, preparation of the data for publication (figures and tables) and writing the whole manuscripts of the three published papers.
- Besides, the cloning work of hAR into baculo virus was done by Dr. Petri (Schering AG) with the pSG5-HAOa plasmide of Prof. Cato (University Karlssruhe).

8. Appendix

8.1. Development of an immuno-immobilized androgen receptor assay (IRA) and its application for the characterization of the receptor binding affinity of different pesticides

Bauer ERS, Bitsch N, Brunn H, Sauerwein H, Meyer HHD
Chemosphere 2002, 46: 1107-1115.

8.1.1. Abstract

Pesticides are synthetic chemicals used not only for improving food and feed production but also for the protection of materials and of human health and well-being. Some of these substances are suspected for adverse effects attributable to an interaction with the endocrine system of vertebrates by mimicking or inhibiting endogenous hormones. One of the biological targets important in this relation is the androgen receptor. To be able to screen environmental samples for the presence of compounds which might interfere with androgen action, we aimed to develop a receptor assay based on recombinant human AR (rhAR). We herein describe an rhAR assay in which the receptor is immobilized in microtiter plates via a specific antibody. The assay can be used for high throughput screening of chemicals spread into the environment. 29 of the most recommended pesticides of the Federal Country Hessen, Germany, were tested for their ability to displace [^{3}H]-DHT bound to the rhAR. This evaluation included the major part of the most common herbicides, insecticides and fungicides and covered three potential groups of endocrine disrupting chemicals. For 28 of the substances evaluated, the relative binding affinity to the rhAR was below 0.1% when compared to DHT (100%), only fentinacetate exhibited an affinity of 1.42%. An exchange assay indicated that the binding inhibition was reversible. In consequence, fentinacetate seems to be a hormonally active substance which may be present in vegetables or fish, but also on clothing. We conclude that further investigations on this compound and it's metabolites are necessary.

Key words: endocrine disruptors, Fentinacetate, Additivity, Microtiter plates, Xenobiotics

8.1.2. Introduction

As a consequence of modern manufacturing processes and efforts expended to increase agricultural productivity, a wide variety of man made chemicals are found in the environment. More than 30 years ago, it was demonstrated that one of these substances, a metabolite of DDT released into the environment for insect control, interacts with the endocrine system (Bitman et al., 1968) and binds to the estrogen receptor (Nelson, 1974). During the past 10 years it became obvious that other substances might also interfere with the endocrine system of vertebrates. It is proposed that these substances cause sexual malformation, infertility and cancer, in particular in aquatic species via interfering in the hormonal systems regulating sexual development and behavior (Toppari et al., 1996). In view of the plethora of different chemicals in the environment, only a small amount of substances are tested for potential disrupting effects within the hormone system. The effects of steroid hormones are principally mediated by binding to their specific receptor and the subsequent modification of gene transcription within the nucleus. Therefore, the affinity to a steroid hormone receptor can be used to evaluate the capacity of various substances suspected for endocrine disrupting to effect steroid hormone actions. In contrast to the estrogen receptor for which several practicable test systems have been developed for binding chemicals (e.g. Arnold et al., 1996; Seifert et al., 1998), test systems for human androgen receptor (hAR) binding chemicals are scarce. Androgens are the most important hormones in the sexual development of males and any disruption by substances from the environment, attributable to either agonistic or antagonistic androgen receptor binding might have important consequences. At present, different *in-vitro* assays based on cellular systems are available (Poulin et al., 1990; Fuhrmann et al., 1992, Bentel et al., 1999; Vinggaard et al., 1999, 2000). At the subcellular level, mainly competitive radioreceptor assays based on rat or bovine AR are applied (Simard et al. 1986, Sauerwein & Meyer 1997, Bauer et al., 1998). In these cytosolic receptor preparations, other steroid receptors besides AR are present and may exhibit cross reactivities with the labeled ligands or the substances analyzed, e.g. for several synthetic androgens significant binding to the progestin receptor has been documented (Schilling & Liao, 1984, Bauer et al. 2000). In addition, species specificity of AR recognition has to be considered, since differences in ligand affinities have been shown at least for the progestin receptor even within mammalia (Jewgenow & Meyer, 1998). Until now, no cell free recombinant hAR

(rhAR) assay is described to screen environmental samples for AR binding activity. Herein we report the development and application of a microtiter plate receptor assay with rhAR for the evaluation of the receptor binding affinity (RBA) of the 29 herbicides recommended in the Federal Country Hessen in Germany.

8.1.3. Materials and Methods

Materials

5α-Dihydro[1,2,4,5,6,7-^{3}H]testosterone ([^{3}H]-DHT, 5α-androstan-17β-ol-3-one) (4.70 TBq/mmol) was obtained from Amersham (Buckinghamshire, UK). DHT was from Sigma-Aldrich (Steinheim, Germany). Charcoal (Norit A), bovine serum albumin (BSA, Fraction V, MG 67000) and dextran (research grade, MG 65000-73000) were purchased from Serva (Heidelberg, Germany). The complete cloning kit, Insect cells, linearized virus DNA and transfer plasmid (pAcSG-His NT-C) were bought from of PharMingen (San Diego, California USA). Sodium phosphate, sodium carbonate, sodium chloride, tween 20 and glycerol (all p.a.) were purchased from Merck (Darmstadt, Germany). Protease inhibitor mix was obtained from Boehringer (Mannheim, Germany). Scintillation Cocktail Xylofluor was bought from Baker (Deventer, Holland) and strip well breakable high adsorption 96-well microtiter plates were obtained from Corning (New York, USA). The monoclonal mouse anti-human AR antibody (441) sc-7305 was from Santa Cruz (Delaware, California, USA). The source of supply of the pesticides used is given in table 1. All pesticides were dissolved in Ethanol (p.a.), except of carbendazim for which Methanol (p.a.) was used. The concentration of the stock solution depended on the solubility of each pesticide and ranged between 7×10^{-1} mol/L (Fenpropimorph) and 1.45×10^{-3} mol/L (Carbendazim).

Methods

Recombinant hAR expression

The recombinant hAR (rhAR) was prepared as described previously (Bauer et al., 2000). In brief, the cDNA of the hAR was inserted into the transfer plasmid pAcSG-His NT-C. The recombinant Baculovirus was obtained by transfection of insect cells (Sf9) with linearised Baculo gold virus DNA and the recombinant plasmid. For protein production, the exponentially growing insect cells were infected with the virus. After 40h the cells were collected by centrifugation and suspended in 2

volumes of disruption buffer (50 mM Na_2HPO_4, 150 mM NaCl, 10% v/v glycerol pH 7.2, containing 0.16% m/v protease inhibitor mix). By three freezing and thawing cycles the cells were disrupted and the supernatant was collected after a 100,000g centrifugation for 15 min at 4°C.

Immuno immobilised AR assay (IRA)

Breakable microtiter plates were coated with 1µg goat anti mouse IgG /well in 100µL carbonate buffer (50 mM $NaHCO_3$ pH 9.6) overnight and blocked for 30 minutes with assay buffer (40 mM Na_2HPO_4, 145 mM NaCl pH 7.2, 0.1% BSA). The receptor preparation was diluted 1:60 in assay buffer (including 0.16% m/v protease inhibitor mix) and a 100 µL aliquot per well was incubated 16 h at 4°C with 0.44 nM ^{3}H-DHT in the presence or absence of increasing concentrations of the pesticides or DHT. The receptor was fixed on the microtiter plate with 50µL of the 1:500 diluted specific hAR antibody. After washing the plates two times with washing buffer (8 mM Na_2HPO_4, 0.05% Tween 20, pH 7.2), the wells were separated by breaking and transferred into scintillation vials and counted in 3mL scintillation fluid. Specific binding represents the difference of total binding and non specific binding in presence of a 200-fold surplus of unlabelled DHT.

Exchange assay

The rhAR was diluted 1:60 in assay buffer and aliquots of 0.5 mL were incubated with different concentrations of DHT, difenoconazole, pyridate, fentinacetate, tebuconazole, flusilazole and fenarimol for 8h at 0-4°C. Free ligands were separated by adding 100µL of dextran coated charcoal (DCC, 4% charcoal, 0.4% dextran in assay buffer) for 5 min and centrifugation for 15 min at 3000g. 0.4 mL of the supernatant were incubated with 0.5 nM of ^{3}H-DHT for 16h at 0-4°C. The free ligands were again separated by adsorption to 100µL DCC and centrifugation. 0.4 mL of the supernatant was transferred into scintillation vials, mixed with 3 mL of scintillation fluid and counted.

Table 1: All pesticides evaluated in the assays ordered by the substance class

*	Substance	Substance Class	CAS No	Substance Name	Purity
H	dichlorprop-P [a]	Aryloxyalkanoic acid	15165-67-0	(R)-2-(2,4-dichlorophenoxy)propionic acid	99.0
H	mecoprop-P [a]	Aryloxyalkanoic acid	16484-77-8	(R)-2-(4-chloro-o-tolyloxy)propionic acide	99.0
F	difenoconazole [b]	Azole	119446-68-3	cis,trans-3-chloro-4-[4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether	97.0
F	flusilazole [a]	Azole	85509-19-9	bis(4-fluorophenyl)(methyl)(1H-1,2,4-triazol-1-ylmethyl)silane	99.0
F	propiconazole [c]	Azole	60207-90-1	(±)-1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4-triazole	96.8
F	tebuconazole [b]	Azole	107534-96-3	(RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol	98.5
F	carbendazim [b]	Benzimidazole	10605-21-7	methyl benzimidazol-2-ylcarbamate	99.9
H	ethofumesate [a]	Benzofuranyl alkanesulfonate	26225-79-6	(±)-2-ethoxy-2,3-dihydro-3,3-dimethylbenzofuran-5-ly methanesulfonate	99.0
H	metazachlor [a]	Chloroacetanilide	67129-08-2	2-chloro-N-(pyrazol-1-ylmethyl)acet-2',6'-xylidide	99.0
H	bifenox [a]	Diphenyl ether	42576-02-3	methyl 5-(2,4-dichlorophenoxy)-2-nitrobenzoate	99.0
P	ethephon [a]	Ethylene generator	16672-87-0	2-chloroethylphosphonic acid	98.0
H	bromoxynil-octanoate [a]	Hydroxybenzonitrile	1689-99-2	2,6-dibromo-4-cyanophenyl octanoate	99.2
F	fenpropimorph [b]	Morpholine	67564-91-4	(±)-cis-4-[3-(4-tert-butylphenyl)-2-methylpropyl]-2,6-dimethylmorpholine	95.0
F	fenpropidin [a]	Morpholine analogue	67306-00-7	(RS)-1-[3-(4-tert-butylphenyl)-2-methylpropyl]piperidine	97.5
I	dimethoate [c]	Organophosphorus	60-51-5	O,O-dimethyl S-methylcarbamoylmethyl phosphorodithioate	99.1
I	ethyl-parathion [c]	Organophosphorus	56-38-2	O,O-diethyl O-4-nitrophenyl phosphorothionate	99.5
F	fentinacetate [a]	Organotin	900-95-8	triphenyltin(IV) acetate	98.0
F	metalaxyl [c]	Phenylamide	57-837-19-1	methyl N-(methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate	99.3
I	cypermethrin [c]	Pyrethroid	52315-07-8	(RS)-α-cyano-3-phenoxybenzyl (1RS,3RS;1RS,3RS)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate	91.0 [d]
I	deltamethrin [c]	Pyrethroid	52918-63-5	(S)-α-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate	99.0
I	fenvalerate [c]	Pyrethroid	51630-58-1	(RS)-α–cyano-3-phenoxybenzyl (RS)-2-(4-chlorophenyl)-3-methylbutyrate	99.0
I	permethrin [b]	Pyrethroid	52645-53-1	3-phenoxybenzyl (1RS,3RS;1RS,3RS)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate	97.5
I	tetramethrin [b]	Pyrethroid	7696-12-0	cyclohex-1-ene-1,2-dicarboximidomethyl (1RS,3RS;RS,3RS)-2,2-dimethyl-3-(2-methylprop-1-enyl) cyclopropanecarboxylate	99.2
F	fenarimol [b]	Pyrimidinyl carbinol	60168-88-9	(±)-2,4'-dichloro-α-(pyrimidin-5-yl) benzhydryl alcohol	99.2
P	chlormequat-chloride [a]	Quaternary ammonium	999-81-5	2-chloroethyltrimethylammonium	99.5
H	terbuthylazine [a]	1,3,5-Triazine	5915-41-3	N^2-tert-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine	99.0
H	metribuzin [a]	1,2,4-Triazinone	21087-64-9	4-amino-6-tert-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one	99.7
H	bentazone [a]	-	25057-89-0	3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide	99.9
H	pyridate [a]	-	55512-33-9	6-chloro-3-phenylpyridazin-4-yl S-octyl thiocarbonate	99.0

*: I = Insecticide, H = Herbicide, F = Fungicide, P = Plant growth regulator
Substances obtained from: [a] Riedel de Haen (Seelze, Germany), [b] Dr. Ehrensdorfer (Augsburg, Germany), [c] Promochem (Wesel, Germany), [d] technical

Data Evaluation

The displacement curves were sigmoid with a plateau at the maximum or a baseline, because not all compounds were able to completely inhibit binding of the labeled ligand. To calculate the binding data we selected a non-linear, logistic regression with 4 parameters (Sigma Plot®). The equation (1) used was

$$y = y_0 + A/[1 + (x/x_0)^B] \quad (1)$$

where y_0 gives the baseline of this curve. A is the plateau value of the curve and shows the maximum binding of the receptor, whereas B represents the slope and x_0 gives the point of inflection of the displacement curve. Using this equation, the exact concentration of the unlabelled compound which gives a 50% displacement of the labelled compound (IC_{50}) was calculated by setting y = 50. To compare the different compounds tested, the inhibition constants K_i were calculated according to the following equation (2).

$$K_i = IC_{50} / (1 + S/K_D) \quad (2) \qquad \text{(Cheng \& Prusoff 1973)}$$

S represents the concentration of the labelled ligand and K_D is its dissociation constant. The K_D-value was determined by Scatchard analysis for DHT binding to AR (Scatchard 1946).

For each compound the relative binding affinity (RBA) in comparison to DHT was calculated according to the equation (3):

$$RBA_{(x)} = Ki_{(DHT)} * 100 / Ki_{(x)} \quad (3)$$

To evaluate whether the various compounds tested act truly additive when applied in combination, their binding was initially compared to DHT binding and thus a DHT equivalent concentration could be defined. Binding affinity (BA) of DHT is defined as 1 (= RBA / 100%).

The DHT equivalent concentration (c_e) is given by:

$$c_e = c_s * BA \quad (4)$$

with: c_s = concentration of the measured substance

BA = binding affinity of the measured substance.

Additivity of two substances is given if both substances A and B are assayed separately at an optional calibration point within the measuring range, and the calculated sum of $c_{S(A)}$ plus $c_{S(B)}$ equals the $c_{e(A+B)}$ of the two substances measured in mixture:

$$c_{e(A+B)} = c_{e(A)} + c_{e(B)} \quad (5)$$

$$= c_{S(A)} \times BA_{(A)} + c_{S(B)} + BA_{(B)} \quad (6)$$

Table 2: Comparison of the different K_i values and the relative binding affinities (RBA), calculated from each examined ligand according to the equation $RBA_{(x)} = K_{i(DHT)} * 100 / K_{i(x)}$, to the recombinant human androgen.
Values are means ± SD (n≥3) or ± differences (n=2).

Substance	K_i values in nM	RBA in % (number of replicates)
DHT	1.7654 ±0.4608	100.00 ±26.1 (18)
Fentinacetate	124.45 ±21.032	1.42 ±0.24 (7)
Difenoconazole	15715 ±942.90	0.011 ±0.0007 (3)
Tetramethrin	20534 ±3922.1	0.0089 ±0.0017 (2)
Flusilazole	22509 ±3889.6	0.0081 ±0.0014 (2)
Bromoxynil-octanoate	23164 ±2965.1	0.0078 ±0.0010 (2)
Pyridate	24711 ±2471.2	0.0070 ±0.0007 (4)
Bifenox	27797 ±4703.3	0.0065 ±0.0011 (3)
Parathion IC55 [a]	32789 ±12492.	0.0063 ±0.0024 (2)
Tebuconazole	29109 ±494.86	0.0060 ±0.0001 (2)
Fenarimol	39501 ±3278.6	0.0036 ±0.0003 (2)
Propiconazole	95929 ±5372.0	0.0018 ±0.0001 (2)
Fenpropimorph	1510720	0.0001
Carbendazim	n.d.	< 0.07
Cypermethrin	n.d.	< 0.01
Deltamethrin	n.d.	< 0.01
Permethrin	n.d.	< 0.01
Terbutylazin	n.d.	< 0.01
Bentazone	n.d.	< 0.001
Chlormequat-chloride	n.d.	< 0.001
Dichlorprop	n.d.	< 0.001
Dimethoate	n.d.	< 0.001
Ethephon	n.d.	< 0.001
Ethofumesate	n.d.	< 0.001
Fenpropidin	n.d.	< 0.001
Fenpropimorph	n.d.	< 0.001
Fenvalerate	n.d.	< 0.001
Mecoprop-P	n.d.	< 0.001
Metalaxyl	n.d.	< 0.001
Metazachlor	n.d.	< 0.001
Metribuzin	n.d.	< 0.001

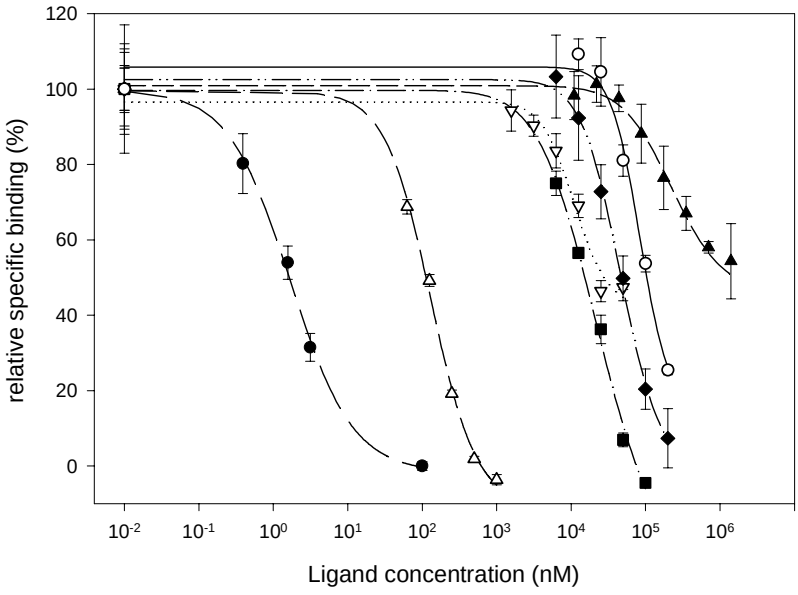
[a] For the calculation of the K_i and the RBA of parathion, the inhibition concentration of 55% (IC 55) was used, as parathion was not able to displace more than 50% of the bound ^{3}H-DHT.
n.d. not determinable. No displacement of ^{3}H-DHT was detectable by the evaluation of even the maximal soluble concentration of those pesticides. Here only excluded values are listened which must be higher than the real RBA reconfirmed from the evaluated negative values.

8.1.4. Results

Assay criteria :

The evaluated K_D of DHT of the rhAR was 2.5 x 10^{-9} M (data not shown). The inter assay variation of this test was 26.5% (n=18) and the intra assay variation was 5.7% (n=3). The range of concentrations used for testing the different pesticides depended on the maximal solubility of each individual analyte. 18 out of the 29 pesticides investigated were unable to displace ^{3}H-DHT even when applied at their maximal soluble concentration. Due to this, no Ki and, in consequence, no exact RBAvalue could be determined for these substances. Instead, the substances were classified as shown in table 2, i.e. for those substances for which no ^{3}H-DHT displacement was observed even at the maximally soluble concentration, an RBA < 0.001% was assigned. For cypermethrin, deltamethrin, permethrin and terbutylazin RBAs <0.01% and For carbendazim <0.07% were assigned. The other herbicides showed significant displacement of ^{3}H-DHT binding within the concentrations evaluated. The RBAs calculated are listed in table 2. In figure 1, the competition curves of DHT, fentinacetate, difenoconazole, fenpropimorph, bromoxynil-octanoate, fenarimol and propiconazole are shown. Binding of fentinacetate, difenoconazole, pyridate, flusilazole, tebuconazole and fenarimol to the hAR was reversible as demonstrated byexchange assays in which 72% to 95% of the rhAR binding activity could be recovered (table 3).

Figure 1:
Displacement curves showing the relative abilities of (●)DHT, (△) fentinacetate, (■) difenoconazole, (▽) bromoxynil-ocatanoate, (◆) fenarimol, (○) propiconazole and (▲) fenpropimorph to compete with [^{3}H]-DHT for binding to the rhAR. Data points are means ±SD; the curves depicted were calculated according to the four parameter logistic function.



Additive responses of different pesticides in the IRA:

Representative IRA binding studies comparing the hAR binding activity of two compounds alone or in combination are shown in Figure 2a for DHT, fentinacetate and difenoconazole. By combining DHT with fentinacetate and fentinacetate plus difenoconazole, each pair in two different ratios, information about mixtures containing a strong and an average AR binding substance and vice versa could be obtained. The concentrations of these substances at each calibration point (c_s) within the mixture was multiplied with the known binding affinity (BA) of the individual substances (Table 2: DHT 1, fentinacetate 0.014 and difenoconazole 0.00011) to obtain the expected DHT equivalent concentration, according to equation (6). If $c_{e(A+B)}$ matches $c_{s(DHT)}$ additivity is assumed for the mixture (Figure 2b).

Substance	Tested concentrations (nM)	Equivalent DHT conc. according to RBA	Recoverable active AR in comparison to the DHT experiment (%)
DHT	1	1	100.00 ±17.13
	6.25	6.25	
	$5.0*10^2$	500	
Fentinacetate	$5.0*10^2$	1.50	72.06 ±4.1
	$2.0*10^3$	6.	
	$5.0*10^4$	149.75	
Difenoconazole	$1.6*10^4$	0.59	83.33 ±2.45
	$1.3*10^5$	4.75	
	$5.0*10^5$	19	
Pyridate	$1.6*10^4$	0.63	73.26 ±11.81
	$1.3*10^5$	5	
	$5.0*10^5$	20	
Flusilazole	$1.6*10^4$	1.03	95.75 ±5.97
	$1.3*10^5$	8.25	
	$5.0*10^5$	33	
Tebuconazole	$1.6*10^4$	0.63	83.58 ±8.15
	$1.3*10^5$	5	
	$1.0*10^6$	40	
Fenarimol	$3.1*10^4$	1	83.61 ±5.41
	$2.5*10^5$	8	
	$1.0*10^6$	32	

Table 3:

Percentage of recoverable active AR, calculated from the bound ^{3}H-DHT values, after a preincubation with listed herbicides at different listed concentrations in comparison to the recoverable active AR after preincubation with DHT with three different concentrations. Values are means ± SEM (n=3).

If the curves are significantly different, the binding affinity of at least one

substance has changed in the mixture and nonadditive effects must be assumed. The Ki values of the calculated displacement curves are listened in table 4 and did not differ much from the Ki values of the evaluated DHT displacement curves. Similar experiments were carried out for the following binary mixtures containing pairs of two weak (flusilazole, tebuconazole) and a combination of a weak and a strong AR binding chemical (bromoxynil-octanoate, DHT). Calculated Ki values were listened in table 4. Additivity was confirmed in all cases.

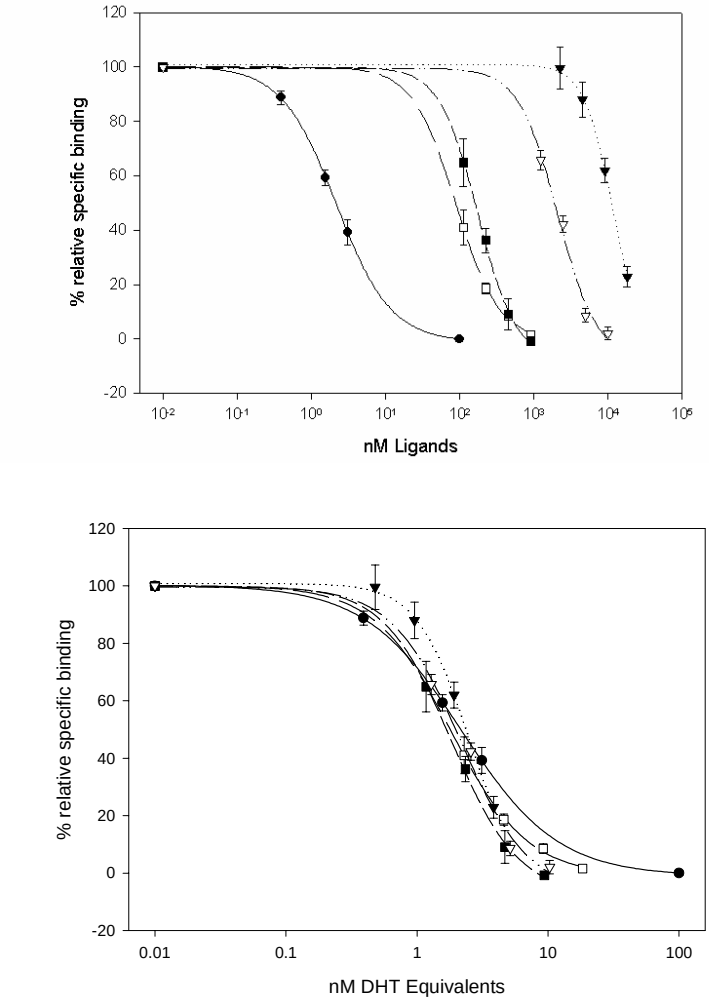


Figure 2:

Determination of additivity. **a:** Mixtures of DHT and fentinacetate (1:90 [□] and 1:900 [■]) and fentinacetate and difenoconazole (1:9 [▽]and 1:90 [▼] were measured. Fentinacetate and difenoconazole was already measured separately to define the binding affinities (Fig 1). The theoretical DHT equivalents of the mixture ($c_{e(A+B)}$) were calculated using these binding affinities. Relative specific binding was depicted against the calculated DHT equivalents. This results in almost identical curves (**b**) and additivity can be assumed (n=3).

Table 4: Determination of additivity. Mixtures of different pesticides in different mixture ratios were measured in triplicates. The Ki values were calculated according to equation (3). With the already evaluated binding affinities (table 2) the theoretical DHT equivalent Ki value was calculated of the mixture according to equation (6). The quite similar Ki values in comparison to pure DHT (1.77 ±0.46 nM) indicate additivity of the pesticides in this test.

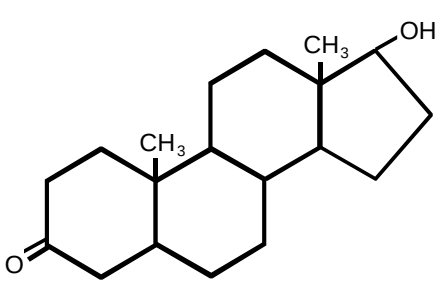
Substance 1	Substance 2	Mixture ratio	K_i (nM) of the mixture	K_i (nM) calculated as DHT equivalent
DHT	Fentinacetate	1 : 90	77.00	1.9275
DHT	Fentinacetate	1 : 900	143.20	2.1902
Fentinacetate	Difenoconazole	1 : 9	1719.22	2.6115
Fentinacetate	Difenoconazole	1 : 90	9293.79	2.4613
Tebuconazole	Flusilazole	1 : 1	19273.97	1.3588
Tebuconazole	Flusilazole	1 : 4	18846.93	1.2100
DHT	Bromoxynil-octanoate	1 : 90	158.39	1.7721
DHT	Bromoxynil-octanoate	1 : 900	1919.56	1.2800

8.1.5. Discussion

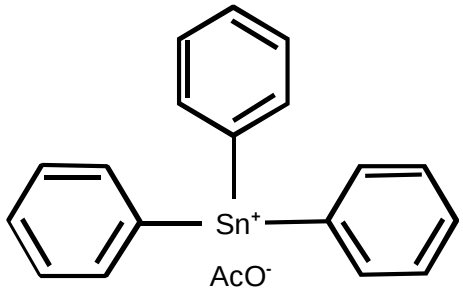
Despite the known affinity of individual xenobiotics to the AR, there is a vast number of chemicals which isnot characterized in this regard. It is difficult to predict androgenicity exclusively on the basis of the chemical structure (Waller et al., 1996), and therefore bioresponse-linked l analysis is needed. Animal models as proposed by Hershberger et al. (1953) seem to be very effective tools, but high costs, time consumption and the requirement of animals are disadvantages. During the last years few *in-vitro* test systems to study the androgenicity of substances have been developed which are mainly based on cellular systems. In addition to systems using cultivated breast cancer cells, in which the hormonal activity is evaluated via recordings of proliferative activity (Poulin et al., 1990; Bentel et al., 1999), cultivated

mammalian cells are used as the basis for reporter gene assays (Fuhrmann et al., 1992, Vinggaard et al., 1999, 2000; Térouanne et al., 2000, Yamabe et al., 2000). Hereby the ligand-activated receptors are initiating the transcription of a reporter gene. A similar approach is used with reporter gene assays in yeast cells.

Figure 3: Structure formulars of DHT and fentinacetate.



DHT



Fentinacetate

To be able to work with native hAR, human fibroblasts were cultivated and used for androgen binding assays as entire cells (Wakimoto et al., 1980; Mowszowicz et al., 1981; Breiner et al., 1986). Although these assays are very sensitive, their performance might by impaired by the occurrence of cytotoxic substances in environmental samples. Besides, if different substances which display either agonistic or antagonistic activities are present in parallel in reporter gene based assays, false negative results might be obtained which can not be entirely excluded by the use of adequate controls. In contrast to the approaches described so far, cell free assay systems do not have these disadvantages. We therefore aimed to develop a cell free assay for the human AR. Using baculoviruses and insect cells we were able to obtain a rhAR preparation devoid of any other steroid hormone receptor. Moreover, the recombinant receptor expression provided a stable source of functional hAR.

We have described earlier that this particular recombinant receptor preparation exhibits the same binding characteristics as does native hAR (Bauer et al., 2000); in conclusion, it is suitable for the development of a receptor assay. In order to be able to use the assay as an effective screening tool for the detection of AR-binding chemicals, it was designed in a microtiter plate format. Compared to cell-based assay systems, the presence of agonistic or antangonistic binding compounds within the same sample does not affect the reliability since binding by itself is the only

criterion evaluated. Although it can not differentiate between androgenic or antiandrogenic activity of substances in a mixture, the interference with the receptor reliably implies the presence of substances that interfere within the androgenic system. The proof of additivity for compound mixtures at the binding activity level provided herein demonstrates that false negatives can be precluded. In conclusion, the criterion of the ability of a given substance or a mixture of various substance to bind to the AR is sufficient for the screening system we were aiming at; further questions for stimulatory or inhibitory effects are secondary at this point.

Our evaluation included the substantial part of the used herbicides, fungicides and pesticides and covered three of the most suspicious groups of endocrine disrupting chemicals used in plant protection. 28 of the pesticides tested, had a RBA to the rhAR that was lower than 0.011% when compared to DHT (100%). This agrees with the binding properties reported for different pyrethroids to human skin fibroblast AR, e.g. the RBA of fenvalerate and permethrin was 0.000027% and 0.000015% when compared to methyltrienolone (Eil & Nisula, 1990). The affinity of methyltrienolone was demonstarted to be comparable to DHT in human fibroblast AR (Eil & Edelson, 1984).

Amongst the herbicides evaluated, only fentinacetate (triphenyltinacetate, structure: figure 3) had a significantly higher RBA to the rhAR of about 1.42% (K_i = 124.5 nM = 51 µg/L). The binding of fentinacetate to rhAR was reversible. Fentinacetate is used as fungicide on crops, but also as algaecide and molluscicide on boats and ships. It is used e.g. on potatoes, celery, onions, sugar beet, peanuts, beans, wheat and cacao (Tomlin, 1997).

Recently Triphenyltin (TPT correspond to fentin) was found to induce imposex in female ramshorn snails (Schulte-Oehlmann et al., 2000). Fecundity was reduced at a concentration as low as 230 nM TPT in water during incubation for 4 months. Similar effects have been described earlierfor tributyltin (TBT) which has is an organic tin compound comparable to fentinacetate. TBT induces the superimposition of male organs such as a penis or vas deferens onto the female genital system (Jenner, 1979, Smith, 1981, Oehlmann et al., 1996, Horiguchi et al., 1998). For these effects alterations of thesteroid hormone metabolizing enzymes, in particular of the cytochrome p 450 aromatase, are likely to be resonsible (Spooner et al., 1991; (Bettin et al., 1996).

TPT showed androgen like action in an androgen-dependent transcription and

cell proliferation assay (Yamabe et al. 2000). Low concentrations of 1 nM TPT showed the same proliferative and transcription activating effects as does 10 nM DHT in a human prostate cancer cell line. In contrast to DHT, the effect of TPT was not suppressed by simultaneous application of flutamide, an antiandrogen used in medical therapy protocols. Using a transcription assay in human hepatoma cells, Maness et al. (1998) could demonstrate that flutamide and also hydroxyflutamide (OH-flutamide) in concentrations higher than 1µM is a complete transcription agonist. OH-flutamide is the antiandrogenically active metabolite of flutamide metabolized *in vivo* but probably at lower concentrations. As the required concentrations of OH-flutamide to displace the agonistic effect of TPT might be higher in comparison to the concentrations required for DHT, effective concentrations are reached at which OH-flutamide has also agonistic effects. Hence, it is perhaps not possible to show the same effect of flutamide on both substances.

The information about the androgenic or antiandrogenic effects of fentin compounds evaluated by in vivo assays with mammalia is quit rare. But the Acceptable Daily Intake (ADI) value is stated to 0.5 µg/kg body weight per day by the WHO because of the reduction of the body weight of the dam in reproductive studies in rats and rabbits (Lu, 1995).

Among the chemicals evaluated by steroid hormone assays, fentinacetate showed by far the highest affinity to the AR. The results agree with results found by other assay systems. It is noteworthy that in receptor assays applied for the detection of potential estrogenic activities of chemicals the RBA's of Bisphenol A or Nonylphenols are 30-170 fold lower than the RBA of fentinacetate reported herein. (Kuiper et al., 1998, Blair et al. 2000).

However, to be able to judge potential interferences of individual substances with regard to their interference within the androgenic system, alterations of steroid metabolizing enzymes might also be important. Further investigations are necessary to evaluate especially the endocrine disruptive activity of fentinacetate in *in vivo* experiments. Moreover, generation studies should be performed because endocrine disrupters can be expected to display their effect sometimes not before the second generation (Gill et al., 1979).

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Analyst 1998, 123: 2485-2487.

8.2.1. Abstract

The potency of different substances for ^{3}H-dihydrotestosterone (^{3}H-DHT) displacement from the bovine androgen receptor was tested. The phenylurea herbicide Linuron and its derivative 3,4-dichloroaniline (3,4-DCA), which are found in sediments and surface waters, are known to displace bound testosterone from the rat androgen receptor. Because 3,4-DCA is rapidly taken up by fish and metabolised into 3,4-dichloroacetanilide (3,4-DCAc), it was investigated whether the displacement effects are attributable to 3,4-DCA or to 3,4-DCAc. The potency of 3,4-DCAc androgen receptor binding was compared with several phenylurea compounds. In a radioreceptor assay with calf uterus cytosol as androgen receptor preparation, the specific binding of ^{3}H-DHT, the endogenous ligand, was completely displaceable by increasing concentrations of 3,4-DCAc. The relative binding affinities (RBA) of the various compounds were about $1/10^{4}$ to $1/10^{5}$ of the RBA of DHT. 3,4-DCAc had the relative highest affinity (1.31×10^{-4} followed by Linuron, Flutamide, 3,4-dichlorophenylurea and Diuron with the lowest RBA of 2.4×10^{-5}). Thus, the metabolism of xenobiotic compounds has to be considered to estimate potential ecotoxiocological effects. This test can be used to screen for androgen- and antiandrogen- like substances not only in environmentally relevant samples such as surface waters, but might also be applied for drug testing and for residue controls.

8.2.2. Introduction

Recently several investigations have shown that a wide range of chemicals , present in the environment, are capable to interact with the endocrine system. In particular, abnormal development of secondary sex characteristics and reduced fertility have been observed in species living in the aquatic environment[1,2]. The observed abnormalities have been traced back to the presence of endocrinally

active substances, which act as endocrine disrupters. These substances might influence the endocrine system at any level and therefore extensive in vivo tests would be necessary to detect potential endocrine disrupting effects. A relatively fast way to screen for such substances is to check their receptor binding affinity. For the oestrogen receptor, significant affinities have been demonstrated for several structurally heterogeneous substances[2]. Chemicals which interact with the androgen receptor have scarcely been investigated. Androgens are male reproductive hormones, they are synthesised mainly in testes and have important functions in regulating growth and development of the external sexual organs and of secondary sex characteristics. Testosterone is the main endogenous androgenic compound; it is metabolised to the biologically active form, dihydrotestosterone (DHT). Androgens act through specific intracellular receptors. Binding of the ligand induces the activation of the receptor molecule which then binds to specific response elements in the DNA and leads to alterations in the transcription rate of specific genes. Chemicals which act like androgens or which block the androgen receptor therefore interfere with physiological androgen functions and lead to impairments in sexual development and in reproduction.

There are some data available demonstrating that phenylurea herbicides like Linuron are able to displace testosterone bound to the androgen receptor[3]. Linuron is currently marketed as a selective herbicide for pre- and/or post emergence control of weeds in crops. Besides, Diuron, a structurally similar phenylurea herbicide, is used to keep weeds from growing on track systems and other areas (e.g. sporting grounds). There it is used in concentrations up to 3 g/m^2 [2]. This agrochemical is degraded in surface waters or in sediments to 3,4-dichloroanilide (3,4-DCA)[4]. Above that, 3,4-DCA is an important intermediate in the chemical production of different agricultural chemicals[5] and is detectable in sewage plant effluents of dye factories. There it is thought to represent a metabolite of several chemicals which occur as waste products in the course of dye production[6]. In addition, a 1.7-fold lower androgen receptor binding affinity has been reported for 3,4 DCA than for Linuron[3]. For Diuron there have no data been available until now. Recent investigations have shown, that 3,4-DCA is rapidly taken up by fish and metabolised to 3,4-dichloroacetanilide[7]. In comparison to 3,4 DCA, this metabolite is structurally closer to the therapeutically used antiandrogen Flutamide for which a significant affinity to the androgen

receptor has been demonstrated[8]. It is thus possible that this metabolite might have a higher relative binding affinity than 3,4-DCA itself. We therefore established an androgen receptor assay using receptor preparations from calf uterus and ^{3}H-dihydrotestosterone (^{3}H-DHT) as labelled ligand to compare for the receptor binding affinity of various phenylurea herbicides, 3,4-DCA and its metabolite.

8.2.3. Experimental

Material:

5α-Dihydro[1,2,4,5,6,7-^{3}H]testosterone (5α-androstan-17β-ol-3-one) (4.70 TBq/mmol) and ORG 2058 (16α-ethyl-21-hydroxy-19-norpregn-4-en-3,20-dione) were obtained from Amersham (Buckinghamshire, UK) and 3,4-dichloroaniline (98%) and 5α-dihydrotestosterone from Sigma-Aldrich (Steinheim, Germany). Linuron and Diurone (certified standards) were from Promochem (Wesel, Germany) and 1-(3,4-dichlorophenyl)urea (98%) from Dr. Ehrensdorfer (Augsburg, Germany). 3,4-dichloroacetanilide has been from Merck Schuchardt (Hohenbrunn, Germany). Magnesium chloride hexahydrate, Tris, EDTA and glycerol (all p.a.) were purchased from Merck (Darmstadt, Germany). Charcoal (Norit A), dextran (research grade , MG 65000-73000) have been from Serva (Heidelberg, Germany) and protease inhibitor mix was obtained from Boehringer (Mannheim, Germany). Scintillation Cocktail Xylofluor was purchased from Baker (Deventer, Holland) and the Ultraturrax equipment from Jahnke & Kunkel (Staufen, Germany)

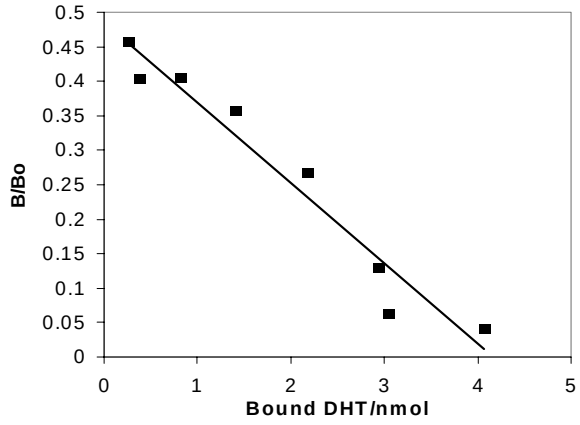


Figure 1: Scatchard plot of the competitive binding of ^{3}H-DHT and DHT. $K_D = 8.6 * 10^{-9}$M, $R^2 = 0.9478$

Preparation of uterine cytosols:

Immediately after slaughter uteri from prepuberal calves were collected and transported on ice to the laboratory. The cytosolic fraction was prepared as described by Sauerwein & Meyer (1989)[9]. Briefly, the tissue was cut into small pieces and mixed with 4 volumes of homogenisation buffer (6mM $MgCl_2$, 5mM Tris, 1mM EDTA, 10% v/v glycerol, pH 7.4 containing 0.16% w/v protease inhibitor mix, as recommended by the manufacturer). After homogenisation with an Ultraturrax equipment and centrifugation for 1 h at 285,000 x g and 4°C, the resulting supernatant was aliquoted and either stored at – 60°C or used immediately.

Radioreceptor assay:

Aliquots of 0.5 mL cytosol were incubated at 0-4°C for 16 h with a constant amount of 0.4nM ^{3}H-DHT and 2,5µM ORG 2058 in the presence or absence of increasing concentrations of DHT, 3,4-dichloroacetanilide (3,4-DCAc), Linuron, 3,4-dichlorophenylurea (3,4-DCPU), Flutamide, 3,4-dichloroaniline (3,4-DCA) or Diuron. ORG 2058 was used to block gestagen receptors. To separate bound and free ligand, the cytosol was incubated with 100µL dextran-coated charcoal (4% charcoal and 0.4% dextran in homogenisation buffer) for 5 min before centrifugation at 2000 x g for 15 min at 4°C. 0.4 mL of the supernatant were transferred to scintillation vials, mixed with 3 mL Xylofluor and counted.

The displacement curves were sigmoid with a plateau at the maximum and a baseline, because some compounds were not able to inhibit ^{3}H-DHT binding completely. We therefore chose a non-linear, logarithmic regression with 4 parameters (Sigma Plot®) to calculate the displacement curves from the measured binding data. In the used equation:

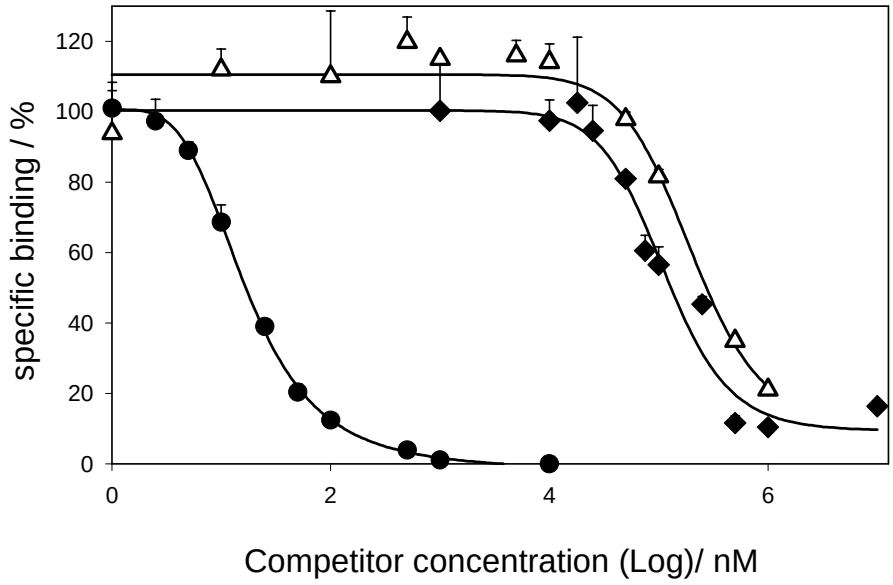
$$Y = Yo + A/(1+[X/Xo]^B)$$

where Xo gives the point of inflection of the displacement curve, A is the plateau of the curve which shows the maximum binding of the receptor, B represents the slope and Yo gives the baseline. This regression is a model for the displacement data. Although the function may not represent the data in all areas exactly, the R^2 values (table 1) as well as the comparison of the doses needed to displace 50% of ^{3}H-DHT binding as calculated by the regression versus graphical deduction without

the use of the regression lines, demonstrate that the regression used is indeed adequate to describe the displacement curves.

To compare the different compounds tested, the inhibition constants K_i were calculated according to the equation $K_i = IC_{50}/(1+S/K_D)$[10]. IC_{50} is the concentration of the unlabeled compound which gives a 50% displacement of ^{3}H-DHT binding, which is different from Xo and must be calculated separately. S represents the concentration of ^{3}H-DHT and K_D is the dissociation constant of DHT, as determined by Scatchard analysis (Fig.1).

Figure 2: Displacement curves showing the relative abilities of DHT (●) , 3,4-DCAc (◆)and 3,4-DCA (△) to compete with ^{3}H-DHT for binding in calf uterus cytosol. Data points are means + SD from 4 to 8 replicates assayed in 2 to 4 tests; the curves depicted were calculated according to the four parameter logistic function.



8.2.4. Results

In Figure 2 the potency of some of the compounds tested in displacing ^{3}H-DHT from the androgen receptor is shown; the RBA values of all substances tested are given together with the individual R values of the regression curves in Table 1. The dissociation constant (K_D) of DHT in the androgen receptor system used herein amounted to 8.6 nM.

Compared to the endogenous ligand DHT, the relative binding affinities (RBA) of the different compounds investigated were much lower, i.e. 7.6 x 10^3 to 4.3 x 10^4 -

fold higher concentrations were necessary to achieve a half maximal displacement of ^{3}H-DHT binding. 3,4-DCAc showed the highest relative binding affinity of the 6 substances tested, followed by Linuron, 3,4-DCPU, Flutamide, 3,4-DCA and finally Diuron. With DCAc the maximal displacement of ^{3}H-DHT binding was comparable to that of DHT, whereas the other five compounds tested could not displace more than 50 to 80 % of the bound ^{3}H-DHT even at maximal concentrations close to the limit of solubility.

Table 1: From the Ki values, the relative binding affinities (RBA) in comparison to DHT from each examined ligand were calculated according to the equation: $RBA_{(x)} = Ki_{(DHT)}/Ki_{(X)}$.

Ligand	RBA	R^2 of the regression	Number of assays	Number of replicates per test
DHT	1	0.99	2	2
DCAc	0.000131	0.94	4	2
Linuron	0.000100	0.97	2	2
DCPU	0.000075	0.97	2	2
Flutamide	0.000065	0.87	3	2
DCA	0.000062	0.92	1	3
Diuron	0.000024	0.93	2	2

8.2.5. Discussion

The androgen receptor assay described herein provides a useful tool to characterise potential endocrine-like substances. The level of detectable interference in the endocrine system is hereby limited to androgen receptor binding and a differentiation between androgenic or antiandrogenic effects is not possible. However, the assay system is an effective in vitro approach which allows for the screening of a broad spectrum of either individual compounds or mixtures with regard to their androgen receptor interaction. This is of particular relevance for the risk evaluation of surface water contaminants but may also be used to test suspect drug

preparations from the black market being used as anabolics in meat production and/or in sport doping. In a first application of the radioreceptor assay to clarify the anabolic potential of two preparations designated as “doping products” we were able to exclude androgenic activity being the active component because no displacement of ^{3}H-DHT was observed. Besides its relevance in environmental toxicology and testing of drug preparations, there is also potential to use the receptor assay for residue controls in various body fluids or in tissues. To exclude the interference of endogenous androgens present in these samples, combinations with immunological methods might be used, e.g. immunoaffinity chromatography with antibodies against endogenous ligands prior to the receptor assay.

The present study about the relative binding affinities of 3,4-DCAc, Linuron, Flutamide, 3,4-DCA, 3,4-DCPU and Diuron confirms the ability of these compounds to interact with the androgen receptor. Similar data for the RBA of Flutamide have been reported in the literature[3+8] : in rat prostate androgen receptor preparations 3,4-DCPU did not displace bound testosterone and in the same report a four times lower binding affinity of Linuron was described[3] in comparison to our results. These differences might be attributable to the different species used for androgen receptor preparations in the literature: recently it has been documented that the gestagen receptor from various mammals has species-specific RBA values for dihydroprogesterone[11]. There is no evidence for differences of the RBA values of steroid hormone receptors obtained from different tissues within a given species. The androgen receptor binding potency of the 3,4-DCA metabolite, 3,4-DCAc and for the phenylurea herbicide Diuron was investigated for the first time herein. The receptor binding affinity of Diuron was only 25% of the RBA of Linuron: the two-fold higher affinity of DCAc in comparison to DCA and Flutamide indicates that metabolism might imply potentiation of androgen receptor binding affinity. To be able to judge as to whether the affinities of the various substances to the androgen receptor are of biological relevance, the concentrations found in the aquatic systems have to be considered. For Diuron, the most commonly used phenylurea pesticide, the ground water concentrations reported for Germany range between 8.6×10^{-14} and 2.4×10^{-8} M [12]. In effluents from purification plants, the maximal concentration documented for Diuron and Linuron were 4.9×10^{-8}M and 2×10^{-9} M, respectively[2]. These concentrations are far below the ones needed to displace the endogenous ligand of terrestrial animals as reported herein. However, other factors such as permanent

exposition or potential accumulation have to be considered to be able to rule out a risk for reproductive functions in particular for aquatic organisms.

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8.3. Characterisation of the affinity of different anabolics and synthetic hormones to the human androgen receptor, human sex hormone binding globulin and to the bovine progestin receptor.

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8.3.1. Abstract

For the steroidal growth promoters trenbolone acetate (TBA) and melengestrol acetate (MGA) neither the complete spectrum of biological activities nor the potential endocrine disrupting activity of their excreted metabolites in the environment is fully understood. The potency of these substances in [^{3}H]-dihydrotestosterone ([^{3}H]-DHT) displacement from the recombinant human androgen receptor (rhAR) and from human sex-hormone binding globulin (hSHBG) was evaluated. In addition, the potency for [^{3}H]-ORG2058 displacement from the bovine uterine progestin receptor (bPR) was tested. For comparison, different anabolics and synthetic hormones were also tested on their binding affinities. For 17β-trenbolone (17β-TbOH), the active compound after TBA administration, an affinity the rhAR similar to dihydrotestosterone (DHT) and a slightly higher affinity to the bPR than progesterone were demonstrated. The affinity of the two major metabolites, 17α-trenbolone and triendione, was reduced to less than 5% of the 17β-TbOH-value. The affinity of these three compounds and of MGA to the hSHBG was much lower compared with DHT. MGA showed the 5.3-fold higher affinity than progesterone to the bPR but only a weak affinity to the rhAR. The major MGA metabolites have an affinity to the bPR between 85% and 28% of the affinity of progesterone. In consequence, MGA and TBA metabolites may be hormonally active substances which will be present in edible tissues and in manure. We conclude that detailed investigations on biodegradation, distribution and bio-efficacy of these substances are necessary.

Keywords:

Tenbolone acetate, melengestrol acetate, synthetic hormones, receptor affinity, endocrine disruptors

8.3.2. Introduction

Presently there is a wide-spread discussion that man made chemicals found in the environment may interact with endocrine systems. In particular, abnormal development of secondary sex characteristics and reduced fertility observed in species living in the aquatic environment were attributed to hormonal effects of pollutants (1,2). Most of the industrial chemicals discriminated as “endocrine disruptors” display only weak hormonal activities (e.g. dichloroaniline) representing unintended side effects. In contrast to these substances, there is a wide variety of other substances developed with the intention used to significantly influence the endocrine system. These synthetic hormones are used for medical reasons (e.g. contraception or hormone replacement therapy) or as growth promoters in farm animals. In the USA and in Canada, the synthetic steroids melengestrol acetate (MGA, 17α-acetoxy-6-methyl-16-methylene-pregna-4,6,16-triene-3,20-dione) and trenbolone acetate (TBA, 17β–hydroxy-estra-4,9,11-trien-3-one-17-acetate) are licensed in cattle fattening besides the natural compounds estradiol, progesterone and testosterone. The main indications for MGA used are oestrus induction and/or synchronisation; above that, MGA is used as growth promoter in heifers. It exerts its biological activity primarily as a progestagen. Its activity is 125 times higher than that of progesterone when administrated parenterally (3). It is suspected to exert its anabolic effects via stimulating the ovarian synthesis of endogenous anabolic steroids such as oestradiol (4), but androgenic side effects are also discussed and controversally assessed (3,5). The metabolism of MGA by heifers had not been investigated in detail until now, but it must be expected that hydroxylated metabolites and their glucuronides or sulfates are found in the excretions as demonstrated after application to humans (6). TBA establishes its anabolic action via androgenic and anti-glucocorticoidal effects (7). Immediately after adsorption, TBA is hydrolysed to 17β-Trenbolone (17β-TbOH, 17β–hydroxy-estra-4,9,11-trien-3-one) (8). It is known to bind to the AR with similar affinity as dihydrotestosterone (DHT), and its affinity to the PR was presumed to be in the same magnitude as progesterone (9). The main metabolites of TBA which are excreted from cattle are 17α-trenbolone (17α-TbOH, 17α–hydroxy-estra-4,9,11-trien-3-one) and, at lower concentrations, triendione (TbO, estra-4,9,11-trien-3,17-dione) plus the most active substance 17β-TbOH (8).

Since the mechanisms of action of MGA and TBA are not yet exactly clear, we evaluated the receptor binding affinity (RBA) of these substances and their metabolites to the androgen receptor (AR) and to the progestin receptor (PR). While the effects of steroidal substances are principally displayed by binding to the adequate receptor and subsequent initiation of gene transcription in the nucleus by the receptors concerned, receptor binding studies are a cheap and rapid tool for an initial evaluation of potential endocrine disrupting action of unknown substances.

The natural steroids in human and animal excrements are expected to be degraded by bacteria very fast and will disappear at the waste water processing or manure storage. The synthetic steroids are synthesized especially for a longer physiological half-live, and migh therefore be more resistant against microbial degradation. E.g. diethylstilboestrol (DES) is reportedly present in not aerated manure after 55 days at more than 20% of the initial concentrations as original substance (10). 17ß-TbOH was detectable in soil after fertilisation with manure originating from TBA treated heifers (11). Ethinylestradiol and mestranol were found to be metabolised to a lesser extent than natural steroids in effluents from sewage plants (12). Taken together, complete degradation of excreted hormones can not be considered as granted, and therefore their potential endocrine disrupting potencies have to be evaluated. Besides the interaction of these potential endocrine disruptors with the respective receptors, they might also influence the endocrine system by binding to steroid transport proteins, e.g. sex-hormone binding globulin (SHBG). To investigate these items we evaluated the affinity of different natural and synthetic steroids to the recombinant human AR (rhAR), to bovine uterine PR (bPR) and to human SHBG (hSHBG).

8.3.3. Materials and Methods

Materials:

5α-Dihydro[1,2,4,5,6,7-^{3}H]testosterone ([^{3}H]-DHT, 5α-androstan-17β-ol-3-one) (4.70 TBq/mmol) and [^{3}H]ORG 2058 (16α-ethyl-21-hydroxy-19-nor[6,7-^{3}H]pregn-4-ene-3,20-dione) (1.48 TBq/mmol) were obtained from Amersham (Buckinghamshire, UK). Dihydrotestosterone (DHT, 5α-androstan-17β-ol-3-one), boldenone (1,4-androstadien-17β-ol-3-one), chlormadinone acetate (CMA, 17α-acetoxy-6-chloro-4,6-pregnadiene-3,20-dione), medroxyprogesterone acetate (MPA, 17α-acetoxy-6α-

methyl-4-pregnen-3,20-dione) and second antibody (mouse IgG, peroxidase labelled, A 2304) were from Sigma-Aldrich (Steinheim, Germany). Testosterone (4-androsten-17β-ol-3-one), methyltestosterone (4-androsten-17α-methyl-17β-ol-3-one), 19-nortestosterone (19-NT, 4-estren-17β-ol-3-one), estradiol (1,3,5(10)-estratrien-3,17β-diol), progesterone (4-pregnen-3,20-dione), Charcoal (Norit A), bovine serum albumin (Fraction V, MG 67000) and dextran (research grade, MG 65000-73000) were purchased from Serva (Heidelberg, Germany). 17β-Trenbolone (17β-TbOH, 17β-hydroxy-estra-4,9,11-trien-3-one), 17α-trenbolone (17α-TbOH, 17β-hydroxy-estra-4,9,11-trien-3-one), trendione (TbO, estra-4,9,11-trien-3,17-dione), allyltrienolone (17β-hydroxy-17-allylestra-4,9,11-trien-3-one) and epitestosterone (4-androsten-17α-ol-3-one) were a kind gift from Roussel-Uclaf (Romainville, France). Melengestrol acetate (MGA, 17α-acetoxy-6-methyl-16-methylenpregna-4,6-dien-3,20-dione) was obtained from Upjohn (Kalamazoo, Michigan, USA), and MGA-metabolites 6, 7 and 10 were a kind gift from Prof. M. Metzler (Karlsruhe University, Germany). The human sex hormone binding globulin was obtained from Calbiochem (Bad Soden, Germany). The complete cloning kit, insect cells, baculovirus Gold DNA and transfer plasmid (pAcSG-His NT-C) were bought from of PharMingen (San Diego, California, USA). Insect cell culture medium Sf900II, Graces insect cell medium and insect cell approved fetal bovine serum were obtained from Life Technologies (Frederick, Maryland, USA). Magnesium chloride, sodium phosphate, Tris, EDTA, sodium chloride and glycerol (all p.a.) were purchased from Merck (Darmstadt, Germany), and protease inhibitor mix was obtained from Boehringer (Mannheim, Germany). Scintillation Cocktail Xylofluor was bought from Baker (Deventer, Holland) and the Ultraturrax equipment from Jahnke & Kunkel (Staufen, Germany). The specific anti AR antibody (AR 441) was from Santa Cruz (Delaware, California, USA).

Cloning of the human androgen receptor and protein production:

The baculovirus expression system was used for the production of the recombinant human androgen receptor, essentially as described (13,14). The starting material for the construction of the recombinant baculovirus used in this investigation was the pSG5-HAOa plasmid containing the complete androgen receptor DNA. This plasmid had been provided as a kind gift by Prof. A. Cato, Forschungszentrum Karlsruhe, Germany. pSG5-HAOa was cut with SmaI within the N-terminal coding

sequence of the hAR cDNA and with the BamHI restriction enzyme in the multiple cloning side downstream of the receptor DNA. The resulting cDNA fragment coding for an 880 aminoacid fragment of the androgen receptor from amino acid 38 to the end (15) was inserted in frame into the StuI / BgI II cut Baculovirus transfer vector pAcSG-His NT-C. Transfection of Sf 9 insect cells with this transfer vector together with baculovirus Gold DNA, the isolation and cloning of recombinant baculovirus as well as stock virus preparation and titration were performed as described (16). The resulting recombinant protein begins now with an histidine tag, followed by a protein kinase A domain and a thrombin cleavage site. It is 918 AS in length and has a molecular weight of 98.5 kDa. Its binding characteristics towards a wide variety of androgens were not affected by the small truncation/modification at the N-terminus in comparison to the unmodified recombinant human androgen receptor produced also by the Baculovirus expression system (data not shown). For protein production a suspension insect cell culture growing in serum free Sf900II medium in the logarithmic phase was infected with a multiplicity of three viruses per cell and incubated for 40 hours at 27°C shaking at 100 rpm. Subsequently the cells were spun down at 80 x g for 10 minutes and suspended in two pellet volumes of disruption buffer (50 mM Na_2HPO_4, 150 mM NaCl, 10% v/v glycerol pH 7.2, containing 0.16% m/v protease inhibitor mix). By three freezing and thawing cycles on liquid nitrogen and on wet ice the cells were disrupted. After centrifugation for 15 minutes at 15.000 x g at 4°C the supernatant containing the soluble active recombinant human androgen receptor was aliquoted and immediately frozen in liquid nitrogen.

Preparation of uterine cytosols:

Uteri from cyclic preovulatory heifers were collected immediately after slaughter and frozen in liquid nitrogen. The cytosolic fraction was prepared as described previously (17). The frozen tissue was cut into small pieces and homogenised with an Ultraturrax equipment in 4 volumes of homogenisation buffer (6mM $MgCl_2$, 5mM Tris, 1mM EDTA, 10% v/v glycerol, pH 7.4, containing 0.16% w/v protease inhibitor mix). After centrifugation at 285,000g for one hour at 4°C the supernatant was aliquoted and immediately frozen in liquid nitrogen:

Preparation of MGA-Metabolites:

The MGA-Metabolites M6, M7 and M10 had been produced *in vitro* by incubation of bovine liver microsomes with MGA and separation by HPLC. GC-MS spectra indicated that the three main metabolites were all monohydroxylated MGA-products. M10 was not completely stable during storage and had lost about 50% of its original concentration at the point of investigation.

Evaluation of the protein concentration:

The protein concentration was evaluated with the BCA technique (18).

Preparation of Western blots:

To prepare Western blots the standard protocol of Santa Cruz (Delaware, California, USA) was used.

Radio receptor/SHBG assays:

AR assay:

The AR assay was carried out as described previously (19) with the following variations. The rhAR preparation was diluted 1:50 in assay buffer (5 mM Na_2HPO_4, 150 mM NaCl, pH 7.2, containing 0.16% w/v protease inhibitor mix and 0.1% bovine serum albumin). Aliquots of 0.5 mL were incubated at 0-4°C for 16 hours with an constant amount of 0.4 nM ^{3}H-DHT and in the presence or absence of increasing concentrations of analytes to be tested. DHT was used in all experiments as standard. To separate bound and free ligand, the receptor preparation was incubated with 100µL of dextran-coated charcoal (4% charcoal and 0.4% dextran in assay buffer) for 5 min before centrifugation at 2000 x g for 15 min at 4°C. 0.4 mL of the supernatant was transferred into scintillation vials, mixed with 3 mL Xylofluor and counted. Specific binding represents the relative difference of total binding and non specific binding observed in the presence of a 250 fold surplus of unlabelled DHT. All measurements were carried out in triplicates.

PR assay:

The PR assay was performed as described and validated previously (20) with slight variations. The uterine cytosol was diluted 1:30 in homogenisation buffer and incubated with 1.5 nM ^{3}H-ORG in the absence or presence of increasing analyte concentrations. The evaluation curves were carried out in triplicates. The incubation

and separation conditions were the same as described for the androgen receptor assay. Progesterone was used in all measurements for standard.

SHBG-binding assay:

SHBG was diluted in homogenisation buffer to 6.6µg per litre. The globulin solution was incubated with the constant amount of 0.4 nM ^{3}H-DHT and with the 0, 250, 2500 and 25000 fold concentration of analytes compared to ^{3}H-DHT. After 16 h incubation at 0-4°C the separation of bound and free steroids was performed as described for the AR assay. Specific binding represents the relative difference between total binding and non specific binding (1µM DHT added). The non specific binding was always below 7% of total binding. The calibration curves were carried out in triplicates.

Data Evaluation

The displacement curves of the receptor assays were sigmoid with a plateau at the maximum and a baseline always below 5%, indicating that all compounds were able to inhibit binding of the labelled ligand almost completely. To calculate the measured binding data we therefore selected a non-linear, exponential regression with four parameters (Sigma Plot®). The equation used was

$$y = y_0 + A/[1 + \exp(-(x/x_0)/B)]$$

where y_0 gives the baseline of this curve. A is the plateau value of the curve and shows the maximum binding of the receptor while B represents the slope and x_0 gives the point of inflection of the displacement curve. To compare the different compounds tested, the inhibition constants K_i were calculated according to the following equation.

$$K_i = IC_{50} / (1 + S/K_D)$$

IC_{50} is the concentration of the unlabelled compound which gives a 50% displacement of the labelled compound, which is different from x_0 and must be calculated separately. S represents the concentration of the labelled ligand and K_D its dissociation constant. The K_D-values were determinated by Scatchard analysis for DHT binding to AR and for progesterone to PR (Figure 1).

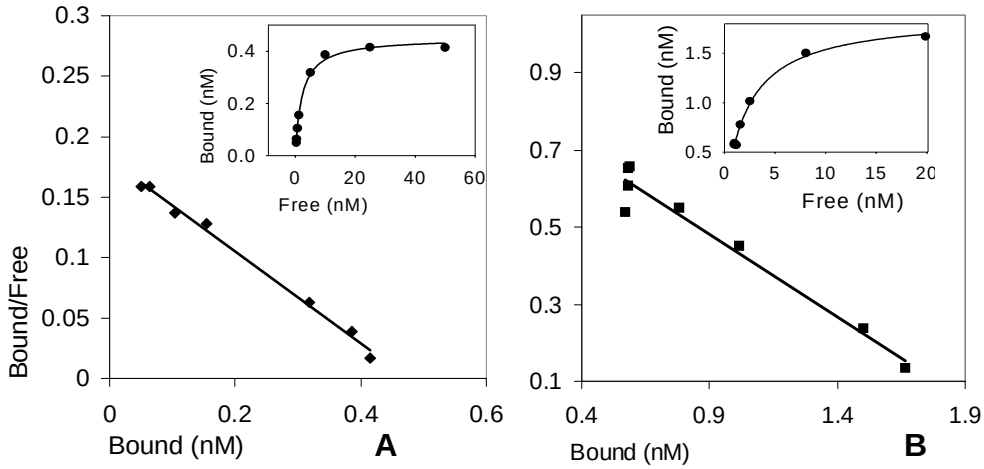

Figure 1. A: Scatchard plot of the competitive binding of ^{3}H-DHT and DHT to the rhAR. K_D = 2.66 x 10^{-9} M, r^2 = 0.9932. Inset: Saturation plot of the same preparation of the rhAR. K_D is herein the concentration of the free hormone at the half maximal binding of the receptor and was 2.24 x 10^{-9} M, r^2 = 0.9950. For the calculation of the Ki-values, the mean of both measurements was used. **B:** Scatchard plot of ^{3}H-ORG 2058 and progesterone to the bPR. K_D = 2.33 x 10^{-9} M, r^2 = 0.9593. Inset: Saturation plot of uterine bPR, 2.26 x 10^{-9} M, r^2 = 0.9942. For the calculation of the Ki-values the mean of both measurements was used. Data depicted were means of triplicates.

8.3.4. Results:

160 fmol/mg protein of active receptor were obtained from the baculovirus infected insect cells. Western blot analysis confirmed the expected size of 98.5 kDa (data not shown). The equilibrium dissociation constant (K_D) of DHT from the androgen receptor system used herein was 2.5 x 10^{-9} M (Figure 1A). The order of relative binding affinities (RBA) to the natural steroids were DHT > 19-nortestosterone > testosterone > oestradiol > progesterone (Table I). In figure 2 the displacement with DHT, 17β-TbOH, 17α-TbOH, TbO and MGA of ^{3}H-DHT from the rhAR is shown.

Table I. Comparison of the different relative binding affinities (RBA), calculated from each examined ligand according to the equation $RBA_{(x)} = K_{i(DHT)} * 100 / K_{i(x)}$, to the recombinant human androgen receptor (rhAR), to the bovine progestin receptor (bPR) and to the human sex-hormone binding globulin (hSHBG).

Substance	RBA (%)		Residual specific ^{3}H-DHT binding (%) to hSHBG in presence of a 2500-fold concentration of the analytes
	rhAR DHT= 100%	bPR Progesterone = 100%	
DHT	*100.00*	1.38	*0.00*
17β-TbOH	108.86	137.40	29.39
Allyltrienolone	75.42	1082.74	19.20
19-NT	75.22	19.51	5.61
Boldenone	48.76	0.24	0.74
MPA	48.61	222.89	81.96
Methyltestosterone	35.93	7.79	1.37
Testosterone	31.31	1.16	0.56
CMA	14.61	1080.53	32.85
Oestradiol	4.88	2.34	n.e.
17α–TbOH	4.49	2.04	94.76
Progesterone	3.83	*100.00*	46.57
Epitestosterone	1.63	0.06	10.69
TbO	0.36	1.03	87.96
MGA	0.31	526.31	25.24
MGA-Metabolit 6	< 1.30	84.75	n.e.
MGA-Metabolit 7	< 1.30	45.53	n.e.
MGA-Metabolit 10	< 1.30	28.26	n.e.

n.e.: data not evaluated

Table I lists the RBAs of all substances tested to the rhAR in comparison to the affinity of DHT and to bPR in comparison to the affinity of progesterone. In addition, the residual specific binding of 0.4 nM ^{3}H-DHT to the human SHBG incubated simultaneously with the 2500-fold concentration of the analyte is listed in this table. A lower percentage of ^{3}H-DHT binding represents a higher affinity of the analyte.

The highest RBA to the rhAR showed 17β-TbOH with 109% in comparison to 100% for the natural ligand DHT and 31% for testosteone. MGA, 17α-TbOH, TbO and the endogenous hormones, estradiol and epitestosterone had only low affinities between 4.9% and 0.31%.The affinities of other natural and synthetic steroids are also shown for comparison (Table1).

Saturation binding and the Scatchard plot for the bPR with progesterone is shown in figure 1b. The Scatchard plot was linear indicating a single population of binding sites. The K_D determined herein was 2.3 x 10^{-9} M. All synthetic progestogens showed a higher RBA to the bPR than progesterone. MGA had a 5.3 fold RBA compared to progesterone, whereas its metabolitesexhibited binding affinities to the receptor between 85% and 28% of that of progesterone. 17β-TbOH had a marginally higher affinity (1.4 fold) to the bPR than progesterone. The affinities of the other natural and synthetic androgens were below 20%. 250 fold concentrations of DHT, boldenone, testosterone and methyltestosterone were able to entirely block the specific binding of ^{3}H-DHT to hSHBG as shown in figure 3. In contrast, MGA and 17β-TbOH a much weaker affinity for hSHBG as a 25000 fold concentration of these two compounds was necessary to entirely block the specific binding of ^{3}H-DHT to hSHBG. The other TBA metabolites, 17α-TbOH and TbO, had virtually no binding to hSHBG. The binding to hSHBG of the other natural and synthetic androgens and progestagens are shown for comparison (Fig.3 and Table 1).

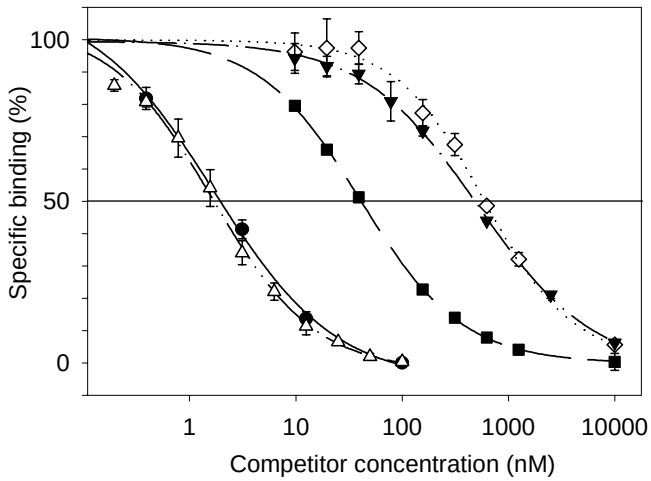

Figure 2. Displacement curves showing the relative abilities of (●) DHT, (Δ) 17β-TbOH, (■) 17α-TbOH, (▼) TbO and (◇) MGA to compete w ith ^{3}H-DHT for binding to the recombinant hAR. Means ±SD from three replicates per test are given. The curves depicted were calculated according to the four parameter sigmoid function with $r^2 > 0.99$.

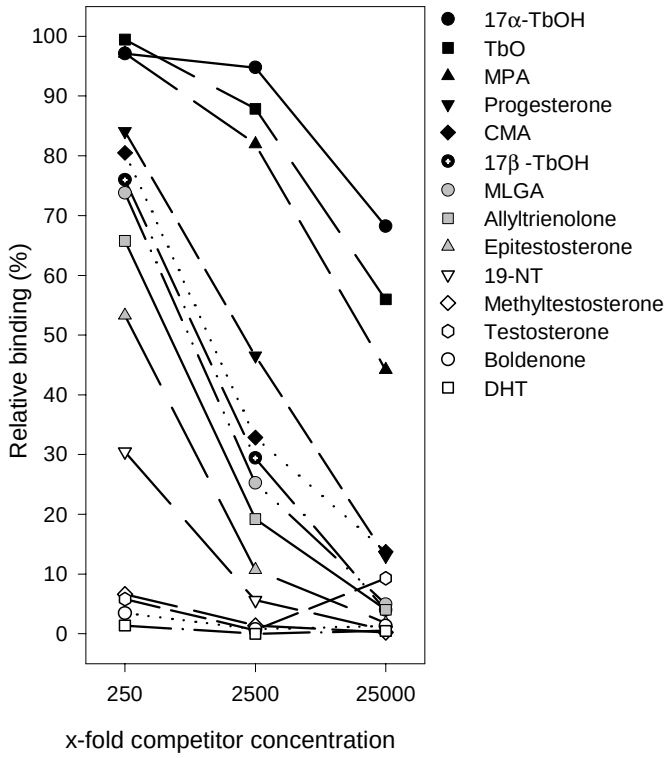

Figure 3. Displacement curves showing the relative abilities of DHT, boldenone, testosterone, methyltestosterone, 19-NT, epitestosterone, allyltrienolone, MGA, 17β-TbOH, CMA, progesterone, MPA, TbO, 17α-TbOH to compete with the ^{3}H-DHT binding to the human Sex-Hormone Binding Globulin. The 250, 2500 and 25000 fold concentrations of the substances in comparison to ^{3}H-DHT were examined in duplicate. Means are given.

8.3.5. Discussion:

With the recombinant baculovirus infected insect cells it was possible to produce human androgen receptor devoid of any other steroid hormone receptors. In contrast, receptor preparations of animal tissues in principle contain all receptors to be found in this tissue. In particular, a selective enrichment of AR is very difficult from natural tissues, because the concentrations of free AR are found to be negligible in non gonadectomized intact animals due to high endogenous androgen levels which then result in occupied receptors retained in the nuclei. Because these receptor forms are not appropriate for our investigations and also to avoid problems due to cross reactivities from other steroid receptors, the recombinant receptor preparation is an adequate solution. RhAR showed a high specific binding to DHT in the same order of magnitude as described for the AR from human skin fibroblasts (21,22) and for rat androgen receptor (23). Others reported AR K_D values one rank below our data, but this might be attributable to differences in the test systems used. For the RBA of testosterone in comparison to DHT, a range between 38% for hAR from intact MCF7

cells and 19% for hAR from human gentile skin fibroblasts has been described (24,21). We found an RBA value of 31.3% in our system. The affinity of 19-NT (75.2%) to the hAR is also in the range described for bovine and rat AR (9,25). The RBA of progesterone and oestradiol which was below 1/20 of that of DHT documents that these steroids are also bound in preparations containing exclusively hAR.

To investigate PR binding activities, animal tissues can successfully be used. The relatively high concentration of PR in preovulatory uteri besides the very low level of other steroid receptors and of endogenous progesterone leads to a practicable receptor concentration in cytosolic preparations. The K_D of progesterone was in the same magnitude as described (26). The order of androgenic substances for displacement of [^{3}H]-ORG 2058 was progesterone >> 19-NT = DHT = testosterone = methyltestosterone; this corresponds to previous reports (9). We also found the same order of competition of bound [^{3}H]-DHT from hSHBG, which was DHT > testosterone > 19-NT > 17β-TbOH > 17α-TbOH as previously reported (27,28).

Besides these substances used as growth promoters, we evaluated the RBA of some doping agents and therapeutically used progestogens to have a benchmark for comparison. The doping agents: boldenone, 19-NT and methyltestosterone had a high affinity to the hAR between 75 and 36 percent of that of DHT. The affinity of these substances to the bPR is lower, but 19-NT might also act via the progestin receptor. Their affinity to hSHBG is of interest since it indicates a different mode of interference within the endocrine system. Originally bound steroids might be displaced and thus the ratio of free and SHBG bound steroids will be altered. Above that, membrane receptors for SHBG have been reported which show steroid depending signalling pathways (29). Thus these substances may not act only via the AR.

The high affinity of 17β-TbOH to rhAR and the bPR was reduced after metabolisation into 17α-TbOH and TbO to less than 1/24 of the original substance. In comparison to DHT 17β-TbOH showed a lower affinity to hSHBG and the other TBA metabolites had negligible affinities to hSHBG. About 75.6% of the original substance is excreted as 17α-TbOH (8). According to the RBA of 17α-TbOH and 17β-TbOH the binding activity of excreted 17α-TbOH is comparable to a excretion of 3.4% of 17β-

TbOH. In addition, 2% of excreted non metabolised 17β-TbOH are excreted. Thus residues with signigicantly binding capacity and potentially endocrine disrupting activity are excreted after TBA treatment.

In an analougous techique with ^{3}H-triamcinolonacetonid we evaluated the affinity of 17β-TbOH to the bovine glucocorticoide receptor (bGR) (data not shown). In comparison to Cortisol the RBA was 9.37%. Together: 17β-TbOH has an affinity to the rhAR, bPR and bGR.

The growth promoter MGA exhibited a very high affinity to the bPR, but only marginal affinities to the rhAR. For the medically used megestrol acetate which has a very similar structure to MGA, androgen like activity has been reported for human breast cancer cells (35) as well as an affinity to the hAR from MCF7 cells which is in the same magnitude as for testosterone (36). Considering the divergent properties of action of these two structurally close related compounds, it is obvious that safety evaluations for MGA can not be done by extrapolations from related substances. For the three major *in vitro* build metabolites of MGA, M6, M7 and M10, a residual affinity to the bPR was determined in the range between 85% and 28% of the affinity of progesterone. The values are lower than the affinities of the evaluated synthetic progestogens but still in the range of natural progesterone. This observation implies that after metabolisation of MGA in cattle, PR active substances might be excreted.

Besides these substances used as growth promoters, we evaluated the RBA of some doping agents and therapeutically aused progestagens to have a benchmark for comparison. The doping agents: boldenone, 19-NT and methyltestosterone had a high affinity to the hAR between 75 and 36 percent of that of DHT. The affinity of these substances to the bPR is lower, but 19-NT might also act via the progestin receptor. Their affinity to the hSHBG is of interest since it indicates a different mode of interference within the endocrine system. Originally bound steroides might be displaced and thus the ratio of free and SHBG bound steroids will be altered. Above that, membrane receptors for SHBG have been reproted wich show steroid depending signalling pathways (29). Thus these substances may not act only via the AR.

The therapeutically used substances allyltrienolone, CMA and MPA showed a higher affinity to the bPR than progesterone but they also exhibit an affinity to the

rhAR, 75 %, 15 % and 49 % respectively. For methyltrienolone a high affinity to the progestin receptor has been reported (30, 31) and thus the high affinity of the structurally related allyltrienolone is not surprising. CMA and MPA are used therapeutically as powerful progestogens with a 330 fold higher effect than progesterone and MPA is discussed as AR agonist and antagonist (32-34). These earlier findings are in line with our RBA values.

In conclusion, receptor and SHBG assays are a useful tool to establish basic data for the assessment of potential environmental risks arising from the use of growth promoters in animal husbandry. For MGA and for TBA evaluations on their biodegradation as well as of the distribution and the bioefficacy of the breakdown products are necessary before considering them as safe growth promoters.

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