

# Environmental xenoestrogens, antiandrogens and disorders of male sexual differentiation

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## Abstract

Over the past 20 years, the documented increase in the disorders of male sexual differentiation, such as hypospadias, cryptorchidism, and micropenis, has led to the suspicion that environmental chemicals are detrimental to normal male genital development in utero. Male sexual differentiation is critically dependent on the normal action of androgens, and unbalanced androgen/estrogen ratios can disturb it. Environmental xenoestrogens (such as herbicides, pesticides, PCBs, plasticizers, and polystyrenes) that mimic estrogens or environmental antiandrogens (such as polyaromatic hydrocarbons, linuron, vinclozolin, and pp'DDE) that disturb endocrine balance, cause demasculinizing effects in the male foetus. These environmental chemicals are often referred to as endocrine disruptors: they are thought to mimic endogenous estrogens by entering the cell, binding to the receptor and activating transcription, they may also antagonize normal androgen action. We have established numerous cell lines to assess the estrogenicity and antiandrogenicity of compounds found in the environment and to identify new products present in wastewater effluents that are able to disrupt endocrine functions. Several cell lines responding to estrogens have been obtained in our group, including cells with different enzymatic equipment and cells expressing chimeric receptor or natural estrogen receptors  $\alpha$  and  $\beta$ . These cell lines have proved to be useful for assessing the biological activity of pesticides, fungicides, and chemicals found in plastic or discarded in the environment. In order to generate a powerful tool for the investigation of androgen action and the rapid screening of potential antagonists, we developed a new stable prostatic cell line. The PALM cell line is an original cellular model to characterize the response of hAR, and it provides an easy and rapid bioluminescent test to identify new antagonists. We also developed a model based on a fusion protein between the androgen receptor (AR) and the green fluorescent protein (GFP) to study the intracellular dynamics of AR. The GFP-AR model was applied to define the ability of several xenoestrogens and antiandrogens to inhibit the nuclear transfer of AR. The ubiquitous presence of endocrine disruptors in the environment and the increased incidence of neonatal genital malformation support the hypothesis that disturbed male sexual differentiation may in some cases be caused by increased exposure to environmental xenoestrogens and/or antiandrogens. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Male sexual differentiation; Xenoestrogens; Antiandrogens

## 1. Introduction

There is great concern that the incidence of congenital disorders of male sexual differentiation is increasing. Several reports indicate an increase in the prevalence rates of cryptorchidism, hypospadias, and micropenis (Toppari and Skakkebaek, 1998). It has been hypothe-

sized that the adverse trends in male sexual differentiation are related to environmental xenoestrogens and/or antiandrogens, which may disrupt normal sex differentiation during foetal life. In this short review we summarize the secular trends in the incidence of disorders of male sex differentiation, the occurrence of genital abnormalities in the sons of women exposed to diethylstilbestrol during pregnancy and the adverse effects of prenatal estrogen and antiandrogen treatment in experimental animals and in human male foetus. We also report the main environmental chemicals with known

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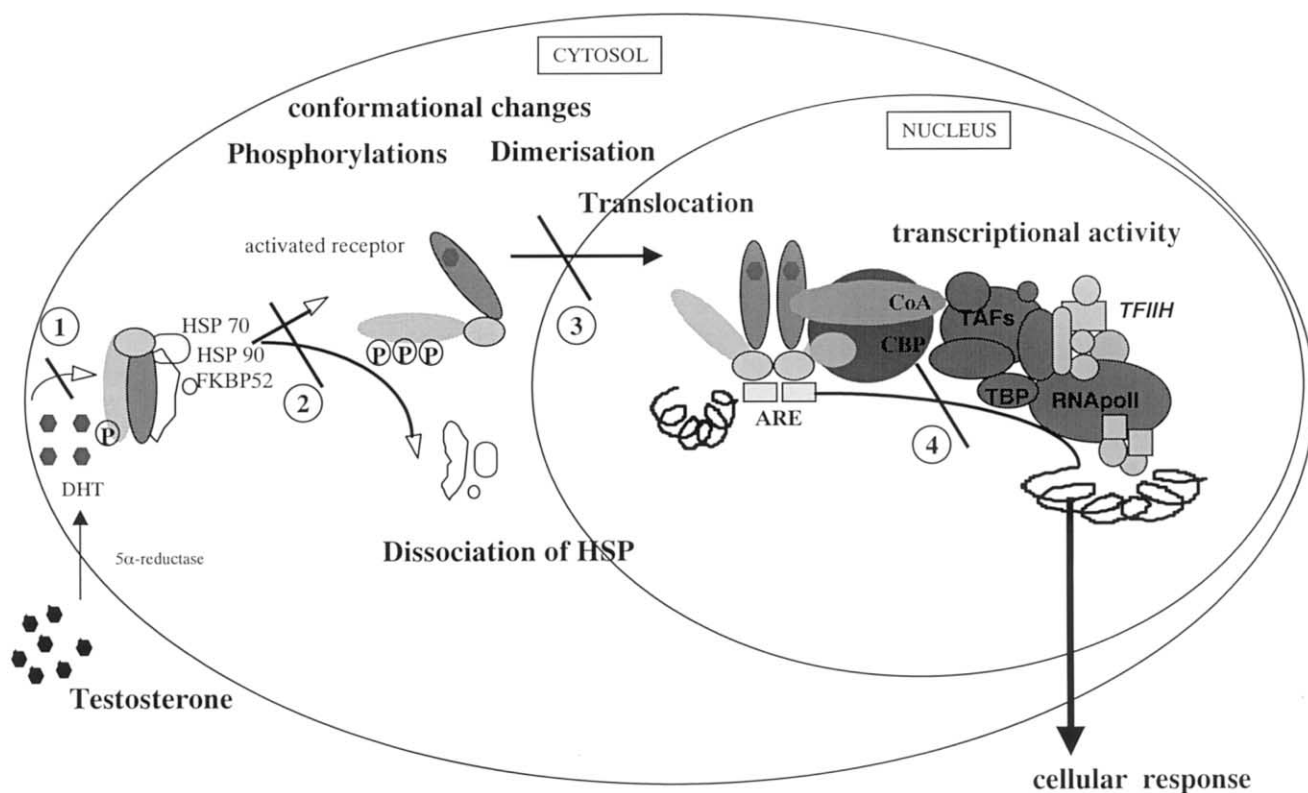


Fig. 1. Potential action of endocrine disruptors in an androgen target cell: (1) Competition for the LBD; (2) conformation change of AR; (3) nuclear transfer; and (4) DNA binding and transcriptional activation.

estrogenic and/or antiandrogenic effects. Special attention is given to the testing strategies for evaluation of estrogenic-like or antiandrogenic activity of potential environmental disruptors (Sohoni and Sumpter, 1998).

## 2. Epidemiologic studies

International data taken from registries indicate an increase in the prevalence of neonatal cryptorchidism and hypospadias. For example, in England, the prevalence rate of cryptorchidism has doubled within the last 25 years (1952: 1.4; 1977: 2.9%). During this same period, the incidence of hypospadias has significantly increased in Europe (England, Hungary, and France), as well as in the United States. This increasing trend in abnormalities of male sex differentiation raises the question of whether they are caused by environmental endocrine disruptors during pregnancy (Sultan et al., 2000).

## 3. Effects of diethylstilbestrol

The effects of diethylstilbestrol (DES) provide an unfortunate model of how a potent estrogenic chemical prescribed during gestation can alter foetal sex differen-

tiation in human. The occurrence of genital abnormalities in the sons of women exposed to DES during pregnancy is noteworthy: 20.8% of the males exposed to DES in utero had epididymal cysts (vs 4.9% in controls), 4.4% had hypospadias (vs 1.1% in controls), 11.4% presented with cryptorchidism and hypoplastic testes (vs 2.1% in controls), and 1.5% had micropenis (vs 0% in controls) (Sonnenschein and Soto, 1998).

This set of data emphasizes the sensitivity of foetal external genitalia to excess synthetic estrogen exposure.

Table 1  
Chemical products tested for estrogenic and antiandrogenic activities by stable cell lines

Insecticides	DDT, DDE
Pesticides	Lindane Arochlor
Herbicides	Atrazine
Fungicides	Vinclozolin (M1–M2)
Industrial chemicals	Polychlorinated biphenyls (PCB)
Detergents	Nonyphenols
Plasticizers	Benzylbutylphthalate Diphenylphthalate Bisphenol A
Phytoestrogens	Genistein Daidsein

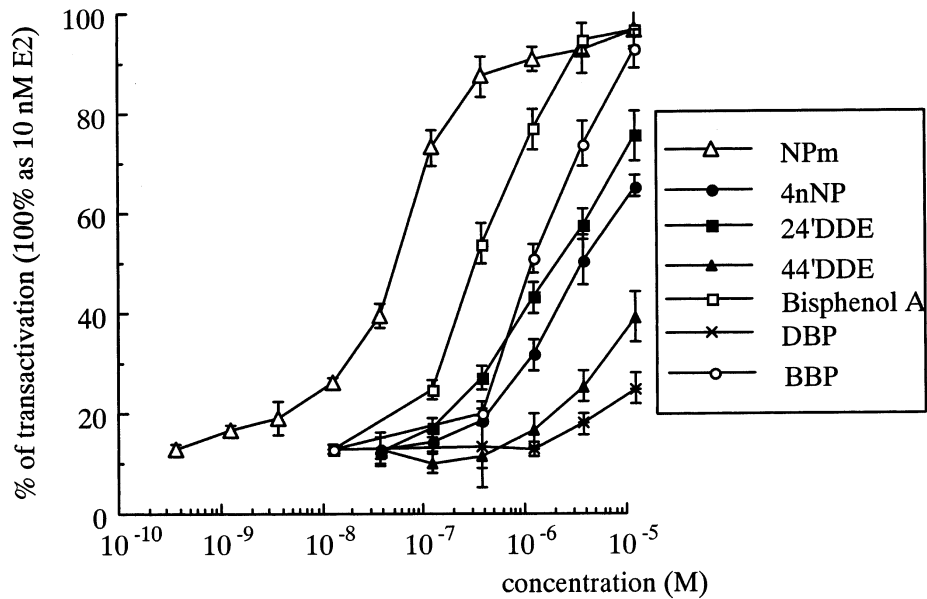


Fig. 2. Induction of luciferase activity by xenoestrogens in the HeLa cell line stably transfected with the reporter plasmid ERE–Luciferase and the expression vector ER $\alpha$ . Results are expressed as a percentage of luciferase activity measured per well. The 100% value represents the value obtained in presence of E $_2$ 10 $^{-8}$  M.

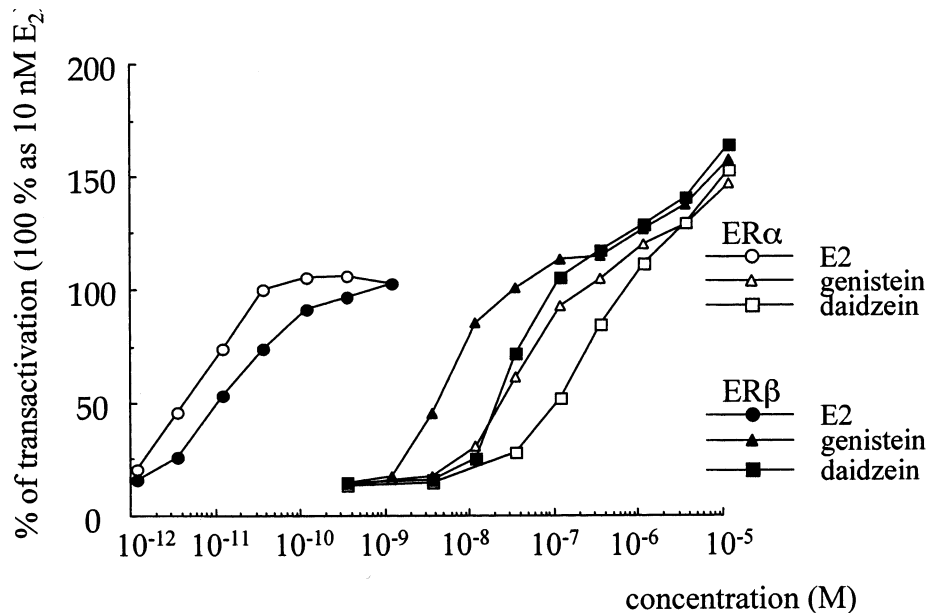


Fig. 3. Induction of luciferase activity by phytoestrogens in the HeLa cells stably transfected with the reporter plasmid ERE–luciferase and the expression vector ER $\alpha$  or ER $\beta$ . HE $\alpha$ LN and HE $\beta$ LN cells were treated for 24 h with estradiol, genistein, and daidzein. Results are expressed as a percentage of luciferase activity measured per well. The 100% value represents the value obtained in presence of E $_2$ 10 $^{-8}$  M.

The adverse effects of DES have been extensively studied in experimental animals (Gray, 1998). After DES exposure in pregnant mice, male offspring exhibited micropenis, hypospadias, and cryptorchidism along with underdevelopment of the vas deferens, epididymis, and seminal vesicles. These defects are similar to those of DES-exposed human male foetus (Cheek and McLachlan, 1998).

#### 4. Effects of antiandrogens

Accidental exposure of male foetus to antiandrogen treatment similarly results in an undervirilized or female external phenotype.

It has been clearly demonstrated that these deleterious effects of antiandrogens depend on the dose and the chemical structure of the substance — but mainly

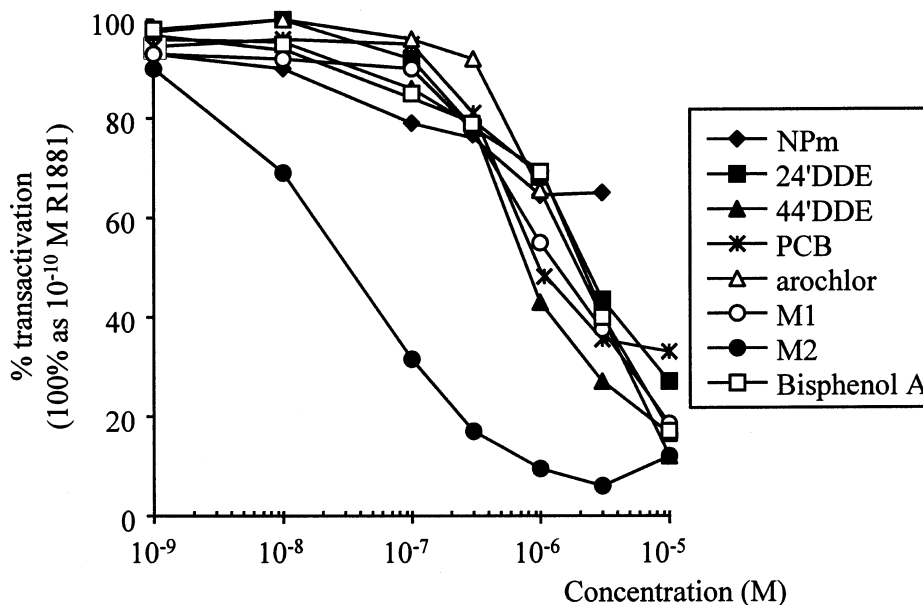


Fig. 4. Detection of antiandrogenic activities of xenoestrogens with the PALM cell line. Cells were treated with various concentrations of chemicals (1 nM to 10  $\mu$ M) with 0.1 nM R1881. Luciferase activities were expressed relative to AR activities with 0.1 nM R1881 which was set at 100%.

on the timing of exposure: the first trimester of gestation is the most sensitive period in terms of foetal sex differentiation.

Both xenoestrogenic and antiandrogenic substances can disrupt the synthesis, transport, and metabolism of androgen. Most environmental antiandrogenic agents antagonize androgen action within the target cell by competing with the androgen receptor (AR) and inducing a conformational change of the AR or by reducing transcriptional activation of target genes at the crucial period. Whereas chemical exposure may be transient, some of the effects are irreversible (Kelce and Gray, 1999).

The mechanisms of action of endocrine disruptors within an androgen target cell are presented in Fig. 1.

In conclusion, three sets of evidence: secular trends in the incidence of disorders of male sexual differentiation, the occurrence of genital abnormalities in the sons of women exposed to DES during pregnancy, and the adverse effects of prenatal estrogen/antiandrogen treatment in experimental animals, have pushed several authors to advance the hypothesis that foetal exposure to xenoestrogens and/or antiandrogens may account for the reported chronological changes in the incidence of disorders of male sexual differentiation.

## 5. Effects of xenoestrogens

Based on their interactions with the ER-binding sites, environmental xenoestrogens are a diverse group of chemicals (Table 1).

For those which preferentially bind to the ER $\beta$  re-

ceptor, one may speculate that subsequent down-regulation of the AR is involved in the development of urogenital malformation during foetal life (J.-A. Gustafsson, personal communication). It should be mentioned that several environmental estrogens are antiandrogens — metabolites of Vinclozolin and 44'-DDE, for example — and inhibit AR-mediated gene activation (Kelce et al., 1997).

The Eurogen program, supported by the European Community, has been implemented recently: the overall study objective is to determine whether there is an association between environmental factors in the pre-

Table 2

Summarized results of estrogenic and antiandrogenic activities of different chemicals on stable cell lines expressing ER $\alpha$ , ER $\beta$  and AR, respectively.  $\nearrow$  = agonist activity  $\searrow$  = antagonist activity

Chemicals	ER $\alpha$	ER $\beta$	PALM
4nNP	$\nearrow$	$\nearrow$	0
DBP	$\nearrow$	$\nearrow$	0
BBP	$\nearrow/\nearrow$	$\nearrow/\nearrow$	0
Genistein	$\nearrow/\nearrow$	$\nearrow/\nearrow/\nearrow$	
Daidsein	$\nearrow$	$\nearrow/\nearrow$	
NPm	$\nearrow/\nearrow$	$\nearrow/\nearrow$	$\searrow$
24'DDE	$\nearrow$	$\nearrow$	$\searrow$
44'DDE	$\nearrow$	$\nearrow$	$\searrow$
Bisphenol A	$\nearrow$	$\nearrow$	$\searrow$
PCB	0	0	$\searrow$
Arochlor	0	0	$\searrow$
M1	0	0	$\searrow$
M2	0	0	$\searrow/\searrow$
Lindane	0	0	0
Atrazine	0	0	0

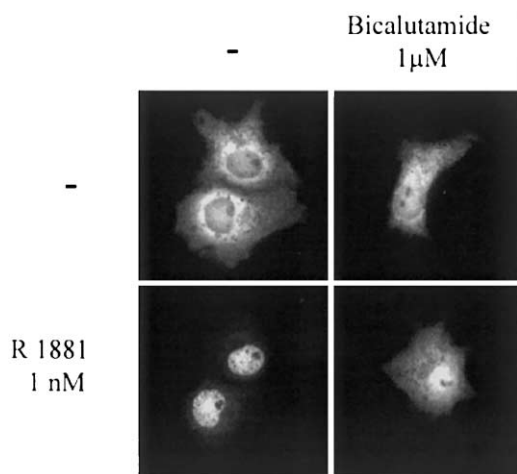


Fig. 5. COS-7 cells were transiently transfected with the plasmid coding the fusion protein between the green fluorescent protein and the androgen receptor (GFP-AR). After 48 h, cells were incubated for 3 h in presence of R1881 (1 nM), bicalutamide (1  $\mu$ M) alone or in competition with R1881. Cells were observed and recorded directly with an epifluorescence microscopy coupled to a CCD camera.

natal period and the development of disorders of male sex differentiation. Prospective epidemiological studies of genital malformation will be conducted in birth cohorts from Europe (Denmark, Finland, Spain, England, and France), Japan and the United States, as these countries are expected to have significant differences in the prevalence of urogenital malformations. In a simultaneous case-control study, diet, drug usage, exposure to chemicals during pregnancy, and lifestyle parameters will be evaluated as possible causal factors in neonatal genital malformations.

## 6. Recombinant receptor–reporter gene assay

Recombinant receptor–reporter gene bioassays to evaluate the estrogenic/antiestrogenic, androgenic/antiandrogenic activities of environmental chemicals and to identify new products present in food and water are also important.

Simple cell models that express a gene under the control of defined promoters responding to specific drugs and produce a signal easy to quantitate are of great interest for rapid screening of the biological effects of artificial or natural compounds. In integrated systems, these cell models are very useful to study the synergy or antagonism of different substances and, in the field of environmental research, they are excellent tools to identify compounds capable of disrupting endocrine functions.

We have established numerous cell lines using a technology based on the bioluminescent gene reporter assay. Analysis and selection of stable transfectants were simplified using a low-light imaging system. We have

used these systems to evaluate the biological activity of compounds found in the environment and to identify new products present in wastewater effluents.

### 6.1. Stable bioluminescent cells responding to estrogens

Several cell lines responding to estrogens have been obtained, including cells with different enzymatic equipment and cells expressing chimeric receptors or natural estrogen receptor  $\alpha$  or  $\beta$  (Balaguer et al., 1999, 2000). These cell lines could not only be used by pharmaceutical companies, but they would also be helpful for monitoring the biological activity of pesticides and chemicals found in plastic or discarded in the environment.

The detection limit of these bioassays is lower than  $10^{-12}$  M estradiol, and high-throughput screening could be performed using a 96-well microplate format.

The estrogenic activity of detergents (nonylphenols), plasticizers (bisphenol A, phthalates), and pesticides (DDE products) was characterized with our reporter cell lines. Using HELN ER $\alpha$  cell line, derived from Hela cell line, we observed a luciferase activity induced by these chemicals tested at concentrations above  $10^{-8}$  M (Fig. 2). Similar results were obtained on the ER $\beta$  cell line (results not shown).

On the contrary, phytoestrogens which exhibited a biphasic activation were more potent at low concentrations (10–100 nM) on the ER $\beta$  than in the ER $\alpha$  cell line (Fig. 3). At high concentrations (1–10  $\mu$ M) the estrogenic potency was similar on the ER $\alpha$  and the ER $\beta$  cell lines but was greater than that induced by estradiol.

### 6.2. Cells expressing the androgen receptor and a bioluminescent reporter gene

In order to generate a powerful tool for the investigation of androgen action and the rapid screening of novel agonists and antagonists, we developed a new stable prostatic cell line (Terouanne et al., 2000). A line of androgen receptor (AR)-deficient PC-3 cells was stably transfected with a human AR (hAR) expression vector and the reporter gene MMTV-luciferase. It was characterized by its response to androgens and antiandrogens, as reflected by the expression of measured luciferase.

The PC-3 cells were transfected with pSG5-puro-hAR and pMMTV-neo-Luc. Twenty-five days after the initiation of double selection, clones that expressed luciferase were identified by monitoring the chemiluminescence emanating from inducible colonies in the presence of androgen.

Numerous neomycin-resistant and puromycin-resistant clones were selected as luminescent. A number of these expressed functional hAR as shown by DHT induction. One highly inducible clone was selected and named PALM, for PC-3-androgen receptor–luciferase–MMTV.

The androgen concentrations required to induce half-maximal luciferase gene expression were  $3 \times 10^{-11}$  M for R1881,  $2 \times 10^{-10}$  M for DHT and  $3 \times 10^{-9}$  M for testosterone. The three agonists had the same maximal activity at  $10^{-6}$  M and the fold induction was equal to 20. These results were better than those obtained with the transiently transfected PC-3 cell line.

The PALM cell line is a new and original cellular model to characterize the response of hAR, and it provides an easy and rapid bioluminescent test to characterize new agonists or antagonists. Moreover, no cellular damage occurs with the use of a simple luminescence buffer and the androgen effect can thus be quantified at different times within the same cells.

Different xenoestrogens, pesticides, herbicides, and fungicides, were tested alone or in presence of 0.1 nM or 0.1  $\mu$ M R1881. None of them presented androgenic activities. Maximum values obtained with 0.1  $\mu$ M R1881 were not inhibited with higher concentrations of tested chemicals. Their antiandrogenic activities are reported in Fig. 4. A summary of estrogen-like activity as well as the antiandrogenic activity of the tested environmental disruptors is presented in Table 2.

## 7. Cells expressing GFP-AR

The analysis of the subcellular localization of the steroid receptors has usually been performed by immunocytochemistry. It is generally acknowledged that the estrogen receptor (ER) and the progesterone receptor (PR) are predominantly nuclear, with a continuous shuttle between the nucleus and the cytoplasm. The intracellular localization of the mineralocorticoid receptor (MR), the glucocorticoid receptor (OR), and the AR are more controversial.

Depending on the immunostaining protocol, these receptors have been described as being either in the cytoplasm or in the nucleus in the absence of ligand, and exclusively in the nucleus after incubation with the ligand. These techniques require the fixation and permeabilization of cells, which can lead to artefacts in the pattern of subcellular localization. Moreover, the AR can be in different states, i.e. associated with the heat-shock proteins in an unliganded form or associated with DNA or transcription factors in the liganded form. The accessibility of the epitope to antibodies may vary for these different forms and this could induce artefactual results in the immunostaining. Having considered all the limits of immunocytochemistry we developed a model using a chimera of AR fused to the green fluorescent protein (GFP) (Georget et al., 1997). This fluorescent reporter permitted the visualization of the AR in living transfected cells.

We first verified that the fusion protein (GFP-AR) conserved the functional characteristics of AR. We

demonstrated the advantages of this GFP-AR tool vs immunodetection. The intracellular dynamics of AR were evaluated and quantified in living cells, which suggested some applications of the GFP-AR model, such as antiandrogen screening and androgen insensitivity study. An example of inhibition of nuclear trafficking is reported in Fig. 5.

Using this method, it was possible to select new compounds capable of binding to the androgen receptor but unable to trigger the translocation to the nucleus. Unlike classical antiandrogens, these compounds do not exhibit low agonist activity even at high concentration.

Besides the classical *in vivo* tests to identify chemical with endocrine disrupting activity, such as the uterine weight bioassay, the sex accessory gland weight or the induction of developmental malformations in offspring, we plan to implant bioluminescent cell lines in nude mouse in order to evaluate *in vivo* the biological consequences of environmental estrogens and antiestrogens.

## 8. Conclusions

In conclusion, the systematic screening of *environmental* chemicals and the chemicals present in *human foods and water* is needed to identify putative causal agents and to assess their ability to disrupt the endocrine system.

The EDSTAC is considering a screening battery to detect (anti)estrogenic and (anti)androgenic activities using *in vitro* assays (Tier 1).

The battery should detect receptor-mediated effects: chemicals that test positive in Tier 1 should be labelled as *potential endocrine disruptors* and subjected to *in vivo* testing (Tier 2).

There is an urgent need for *prospective* multicenter studies to describe the epidemiological trend in newborn male congenital malformations.

## Acknowledgements

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