J. Steroid Biochem. Molec. Biol. Vol. 46, No. 6, pp. 731–736, 1993
Printed in Great Britain.

0960-0760/93 $6.00 + 0.00

EFFECTS OF ANTIANDROGENS ON TRANSFORMATION AND TRANSCRIPTION ACTIVATION OF WILD-TYPE AND MUTATED (LNCaP) ANDROGEN RECEPTORS

C. A. BERREVOETS, J. VELDSCHOLTE and E. MULDER*
Department of Endocrinology and Reproduction, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

(Received 1 June 1993; accepted 12 August 1993)

Summary—LNCaP cells contain androgen receptors with a mutation in the steroid binding domain (Thr 868 changed to Ala) resulting in a changed hormone specificity. Both the wild-type and mutated androgen receptors were transfected into COS cells. Transcription activation was studied in cells co-transfected with an androgen sensitive reporter (CAT) gene. The wild-type androgen receptor was activated by the agonist R1881, but the antiandrogens did not enhance transcription apart from a partial agonistic effect at high concentrations of cyproterone acetate. The mutated androgen receptor was fully activated by R1881, cyproterone acetate and hydroxyflutamide, but not by ICI 176,334. Receptor transformation to a tight nuclear binding state was studied by preparation of detergent washed nuclei and Western blotting with a specific antibody against the androgen receptor. Nuclei of COS cells transfected with wild-type receptor retained the receptor when the cells had been treated with the agonist R1881, partially retained receptors when treated with antiandrogen cyproterone acetate, but did not retain receptor when treated with hydroxyflutamide or ICI 176,334. The cells transfected with the mutated receptor additionally retained nuclear receptors after treatment with hydroxyflutamide. We conclude that each one of the three antiandrogens tested displayed different characteristics with respect to its effect on transformation and transcription activation.

INTRODUCTION

Effects of androgens in target cells are mediated by binding to the androgen receptor. After binding of the hormone the receptor is transformed to a DNA-binding form with a high affinity for hormone response elements of target genes. Subsequently, the transcription of these genes is modulated by binding of the transformed steroid–receptor complex and interaction with other transcription factors. The specificity of hormonal action is accomplished both by the specific recognition of the target genes and by the specificity of the hormone–receptor interaction, determined by the steroid binding part of the receptor [1, 2].

Antiandrogens act by inhibition of the binding of androgens to the receptor [3–5], but their precise molecular mechanisms of action are at present not known. Several mechanisms have been proposed for the mechanism of action of steroid–receptor antagonists, ranging from induction of an abnormal conformation of the receptor, impaired translocation of the receptor to the nucleus, impaired dissociation of the heteromeric receptor complex, impaired receptor dimerization and binding of the receptor to DNA, to impaired interaction of the DNA-bound receptor with transcription factors [6–12].

In the present study we have compared the androgen receptor from LNCaP tumor cells and the normal, wild-type receptor with respect to antiandrogen mechanism of action. LNCaP cells are derived from a metastatic lesion of a human prostatic carcinoma, contain androgen receptors and respond to androgens with growth in cell culture [13]. In these cells increase in growth rate is also observed in the presence of low doses of estrogens and progestagens, despite the absence of estrogen or progestagen receptors, shown previously with specific antibodies against these receptor proteins [14]. In addition, several, but not all, antiandrogens stimulate growth of these cells. These effects are

*To whom correspondence should be addressed.

due to a mutation in the ligand binding domain of the androgen receptor of LNCaP cells: amino acid 868 (Thr) is replaced by Ala [15].

In previous studies with LNCaP cells we could correlate transcription activation with heat-shock protein dissociation and nuclear retention of receptors [16]. To study nuclear retention and transcription activation under identical conditions for both the mutated LNCaP and the wild-type receptor, these receptors were transfected into the same type of cells. We thus obtained COS cells containing either type of receptor and could eliminate cell-type specific effects. The results show receptor-type specific effects of the different antiandrogens, both with respect to tight nuclear binding and transcription activation.

EXPERIMENTAL

Materials

[^{3}H]R1881, (87 Ci/mmol) and unlabelled R1881 (methyltrienolone) were purchased fron NEN (Boston, MA); butyryl-CoA from Sigma (St Louis, MO). Cyproterone acetate was a gift from Schering (Berlin, Germany), hydroxyflutamide from Schering, USA (Bloomfield, NJ), ICI 176,334 (trade mark 'Casodex') from ICI Pharmaceuticals (Macclesfield, Cheshire, England). All other steroids were purchased from Steraloids (Wilton, NH). [^{14}C]chloramphenicol (50–60 mCi/mmol) was obtained from Amersham (UK). The glucocorticoid/progestagen/androgen responsive CAT construct pG29GtkCAT [17] was generously provided by Dr R. Renkawitz.

Transfections

Construction of expression vectors (pAR0 for wild-type, pARL for LNCaP mutant androgen receptor) and culture of COS-1 cells was as described previously [15].

For transcription regulation studies COS-1 cells were transfected by the calcium-phosphate precipitation method; 5×10^5 cells/dish (30 cm^2) were transfected with either 2.5 μg pAR0 or 2.5 μg pARL and 2.5 μg pG29GtkCAT reporter gene. Carrier DNA (pTZ) was added to a total of 10 μg per dish. After 1 day the cells were exposed to 15% glycerol for 1.5 min and washed twice. Thereafter culture medium with different compounds at the indicated concentrations was added and culture was continued for 1 day.

For nuclear retention studies COS-1 cells were transfected by electroporation (Bio-Rad Gene Pulser). Cells obtained from stock cultures (60×10^6 cells) were resuspended in 3200 μl phosphate buffered saline (PBS) containing either pAR0 or pARL (equal amounts of either plasmid; 1 to 10 μg/ml, depending on the batch of COS cells used) and divided over 4 electroporation-cuvettes (width 0.4 cm). After 10 min at room temperature the cells were exposed to a single voltage pulse (200 V, 960 μF), and allowed to remain in the buffer for 10 min. Finally the cells were pooled and equal amounts plated out in 12 culture flasks at a density of 5×10^6 cells/150 cm^2 and cultured for 2 days.

CAT assays

After culture in experimental media the cells were harvested for CAT (chloramphenicol acetyltransferase) assay, performed essentially as described previously [15, 18]. Reaction mixture (20 μl) was mixed with 7 M urea (5 ml), scintillation fluid (10 ml) added, and ^{14}C activity estimated. For each compound (or combination of compounds) tested, the amount of CAT activity after subtraction of background activity (vehicle only; 0.2% ethanol), was expressed as percentage of the highest level of CAT activity that was found for cells incubated with R1881. Background activity was $<5\%$ of the highest levels of CAT activity (at 10^{-10} to 10^{-9} M R1881). Experiments were performed in triplicate.

Incubation, subcellular fractionation and Western blot analysis

Two days after transfection equal amounts of cells, obtained from one batch of transfected cells, were washed and incubated in serum-free minimum essential medium (MEM) with different compounds for 1 h at 37°C. Subsequently, the cells were washed and scraped in ice cold buffer A [sodium phosphate 10 mM, EDTA 1.5 mM, 1α-thioglycerol 12 mM, dithiothreitol (DTT) 10 mM, PMSF 0.6 mM, leupeptin 0.25 mM, bacitracin 0.5 mM and glycerol 10%, v/v; pH 7.4]. The cells were then lysed by 3 times freeze–thawing (freezing in liquid nitrogen, thawing at 10°C) and centrifuged for 10 min. The crude nuclear (800*g*) pellet was resuspended in buffer A with 0.2% (v/v) Triton X-100. After 5 min the nuclei were pelleted and washed with buffer A. Nuclear extracts were made by incubating the nuclei with extraction buffer (0.5 ml/flask; Tris–HCl 40 mM, EDTA 1.5 mM,

DTT 10 mM, PMSF 0.6 mM, leupeptin 0.25 mM, bacitracin 0.5 mM, NaCl 0.5 M and glycerol 10%, v/v; pH 8.5) for 1 h at 4°C. After centrifugation for 30 min, 105,000*g* at 4°C, the supernatant was used for receptor immunoprecipitation and Western blot analysis as described previously [19]. In brief, the monoclonal antibody F39.4.1 directed against amino acids 301–320 in the N-terminal domain of the androgen receptor [20] was chemically cross-linked directly to protein-A-Sepharose and used for immuno-purification of receptors. SDS–PAGE was carried out and after electrophoresis, the slab gel was subjected to Western blotting. Blots were incubated with antibody F39.4.1 and the antibodies were visualized with a chemiluminescence detection procedure. Densitometric scans of the blots were performed (Bio-Rad Video Densitometer).

RESULTS

Reporter gene activation

The expression vector for either the wild-type or the mutated androgen receptor from LNCaP cells was co-transfected together with an androgen responsive reporter gene into COS-1 cells and the differences in effects on transcription of antiandrogens were investigated. The relative binding affinities of antiandrogens for the androgen receptor are only a few percent of those for androgens, therefore higher amounts of the antiandrogens had to be added than of the androgen R1881 [4, 15, 16]. The results in Fig. 1 show that R1881 could induce CAT activity in cells with the wild-type receptor. Of the antiandrogens only cyproterone acetate induced CAT activity at elevated concentrations. R1881 and cyproterone acetate also bind to progesterone receptors. The reporter gene contains a GRE (pG29G-tk-CAT, [17]) and would be responsive to the progestagenic activities of R1881 and cyproterone acetate in addition to their (anti)androgenic activities, if progesterone receptors were present. However, the COS cells did not contain progesterone receptors and no CAT activity was induced by progestagens in the absence of the androgen receptor vectors.

When the mutant receptors were expressed in COS cells, CAT activity was fully induced by the antiandrogens hydroxyflutamide and cyproterone acetate, but not by ICI 176,334 (Fig. 1). Induction of CAT activity with cyproterone acetate was even higher than with R1881. In competition studies (Fig. 2) hydroxyflutamide and ICI 176,334 could suppress R1881 effects in cells with wild-type receptor, whereas cyproterone acetate suppressed activity to approx. 50%. Only ICI 176,334 suppressed the effect of R1881 on CAT activity in the cells with mutant receptor, indicating that this compound remained antagonistic in the presence of the mutated receptor.

Androgen receptor in nuclear extracts

Receptors were isolated from nuclei of COS cells after transfection either with the wild-type receptor or mutated androgen receptor and incubation with R1881 or antiandrogens. The presence of receptors in nuclear extracts is indicative for the transformation process of the steroid–receptor complex to a tight nuclear binding form in a complex with DNA [1, 2]. Figure 3 shows that extracts from cells with wild-type receptor contained appreciable amounts of receptors after incubation with R1881, a still detectable amount after incubation with cyproterone acetate, but hardly any receptor after incubation with the other two antiandrogens. Cells with the mutated receptor additionally retained receptors after incubation with hydroxyflutamide. In both cells with wild-type or mutated receptor incubation with ICI 176,334 did not result in the presence of nuclear receptors, in addition this compound could prevent the uptake of receptors when added together with R1881 (data not shown).

DISCUSSION

Transcription activation by steroid receptors has been shown to be cell type and promoter context specific. Variable activation of a steroid independent transcription activation function, present in the N-terminal region of steroid receptors is probably involved in cell specific partial agonistic effects of some antagonists of steroid hormones [5, 7, 19]. The partial agonist activity of the antiprogestagen RU 38.486 in some cells has been explained by such a mechanism [10, 22]. It could be envisaged that cell specific protein factors influence this effect. In LNCaP cells several antiandrogens show agonistic properties. To exclude any cell specific property apart from an effect of the mutated androgen receptor, we transfected both wild-type and mutated receptor into COS cells and studied transformation and transcription activation under comparable conditions.

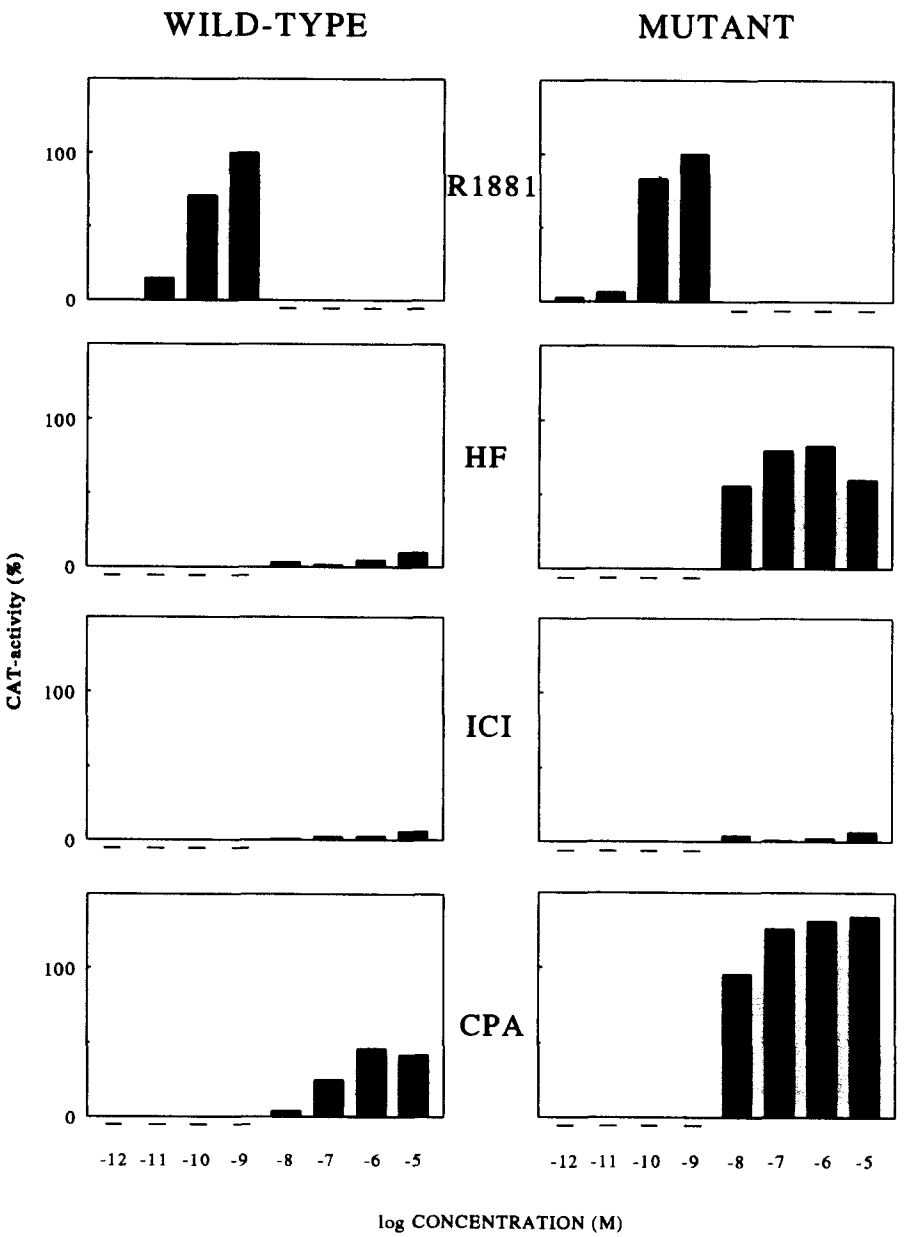

Fig. 1. Transcription activation. Induction of CAT activity in COS cells transfected with plasmids containing either the wild-type androgen receptor or the androgen receptor from LNCaP cells and co-transfected with an androgen sensitive reporter gene. Cells were incubated with different compounds: R1881, hydroxyflutamide (HF), ICI 176,334 (ICI) and cyproterone acetate (CPA). Activity is expressed in % of the activity found for 1 nM R1881 (set at 100%). — : not determined.

The single mutation in the steroid binding domain of the mutated androgen receptor profoundly affected nuclear retention and transcription activation in COS cells for several, but not all antiandrogens. The antagonistic effect of ICI 176,334 was not influenced by the mutation; this compound was shown to inhibit transformation to a tight nuclear bound state and to inhibit gene transcription induced by androgens. In a previous study [16] we could show that in the intact LNCaP cells ICI 176,334 also prevented the heat-shock protein dissociation step in the process of androgen receptor activation preceding tight nuclear binding. Studies on heat-shock protein interaction could not be included in the present study: due to high intercellular variation in the levels of expression of the androgen receptors after transient transfection, several side-effects were observed (e.g. presence of non-ligand binding forms of receptors). At a lower mean expression level reliable results could be obtained, but the limited sensi-

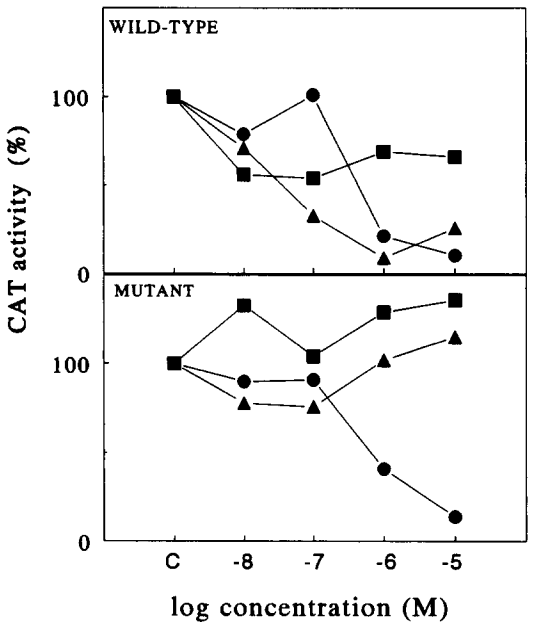

Fig. 2. Effects of antagonists on androgen induced transcription. COS cells were transfected with different plasmids as described in the legend of Fig. 1 and incubated both with 0.1 nM R1881 and a series of concentrations of different antagonists. Activity is expressed in % of the activity found for 0.1 nM R1881 (C; control incubation, without antagonist). (▲), HF, hydroxyflutamide; (●), ICI, ICI 176,334; (■), CPA, cyproterone acetate.

tivity of the available antibodies did not permit estimation of heat-shock protein dissociation in COS cells.

Progestagen and estrogen antagonists have been tentatively divided into two types depending on their level of action. One type would interfere with dimerization and subsequent binding to DNA. The other type of antihormones would induce DNA-binding of the receptor, but act by blocking the transcription activation function localized in the C-terminal steroid binding domain of the receptor [10, 21–23]. In the present study each one of the three antiandrogens tested displayed different characteristics with respect to its effect on transformation and transcription activation (summarized in Table 1). Both hydroxyflutamide and ICI 176,334 prevented formation in COS cells of a tight nuclear binding form of the wild-type receptor. However, a subtle change induced by a point mutation in the ligand binding domain of the LNCaP receptor did convert hydroxyflutamide, but not ICI 176,334 to a full agonist. Cyproterone acetate could convert some wild-type receptors to the tight nuclear bound form and showed partial agonism. Agonistic effects of cyproterone acetate were also previously observed in studies on androgen receptors transfected into CV-1 cells [24], the cell-type of which COS cells were derived. This partial agonistic effect is a cell specific effect, in HeLa cells transfected with the wild-type receptor hardly any agonistic effect of cyproterone acetate was observed [15]. Cyproterone acetate was a full agonist for the mutated receptor, both in HeLa and in COS cells.

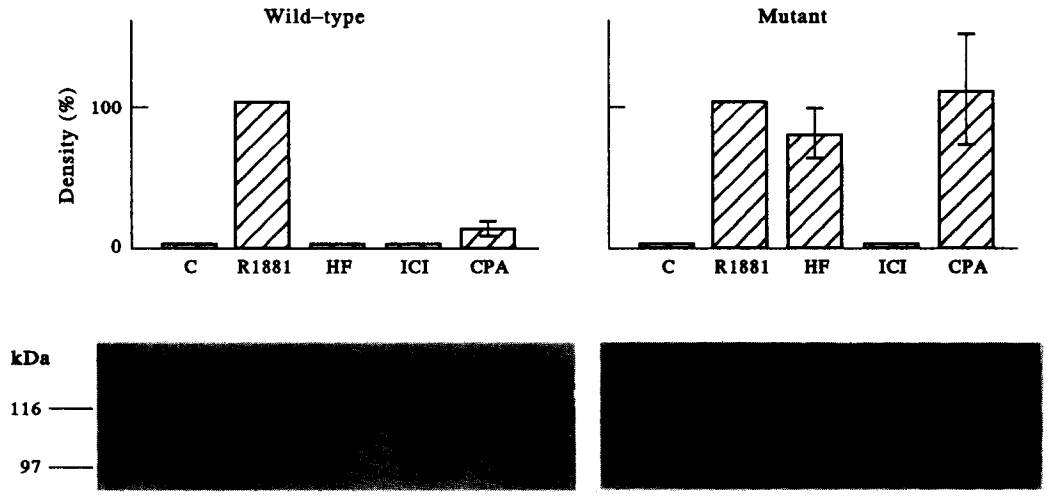

Fig. 3. Immunochemical detection of androgen receptors in nuclear extracts. COS-cells obtained from one batch of transfected cells, were incubated with different compounds and androgen receptors were isolated from nuclei. Either 1 nM R1881 or 5 μM of the antagonists hydroxyflutamide (HF); ICI 176,334 (ICI) or cyproterone acetate (CPA) were added. (C; control incubation without added compounds). The figure shows both an example of the immunoblot and results of densitometric scans (intensity of R1881 set at 100%). Error bars indicate SD of 3 experiments.

Table 1. Summary of the effects of antagonists on the wild-type and mutated receptors

	Growth of LNCaP cells	Transcription activation (COS cells)		Nuclear retention (COS cells)	
		Wild-type	Mutant	Wild-type	Mutant
HF	+	−	+	−	+
ICI	−	−	−	−	−
CPA	+	+/−	+	+/−	+

−: no effect; +/−: limited effect; +: full effect. HF: hydroxyflutamide; ICI: ICI 176,334; CPA: cyproterone acetate.

REFERENCES

1. Beato M.: Gene regulation by steroid hormones. *Cell* **56** (1989) 335–344.
2. Carson-Jurica M. A., Schrader W. T. and O'Malley B. W.: Steroid receptor family: Structure and function. *Endocrine Rev.* **11** (1990) 201–220.
3. Neumann F. and Töpert M.: Pharmacology of antiandrogens. *J. Steroid Biochem.* **25** (1986) 885–895.
4. Raynaud J.-P. and Ojasoo T.: The design and use of sex-steroid antagonists. *J. Steroid Biochem.* **25** (1986) 811–833.
5. Wakeling A. E.: Steroid antagonists as nuclear receptor blockers. *Cancer Surveys* **14** (1992) 71–85.
6. Moudgil V. K., Anter M. J. and Hurd C.: Mammalian progesterone receptor shows differential sequence specificity to sulfhydril group modifying agents, when bound to agonist and antagonist ligands. *J. Biol. Chem.* **264** (1989) 2203–2211.
7. Segnitz B. and Gehring U.: Mechanism of action of a steroidal antiglucocorticoid in lymphoid cells. *J. Biol. Chem.* **265** (1990) 2789–2796.
8. Berry M., Metzger D. and Chambon P.: Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. *EMBO Jl* **9** (1990) 2811–2818.
9. Fawell S. E., White R., Hoare S., Sydenham M., Page M. and Parker M.: Inhibition of estrogen receptor DNA binding by the pure antiestrogen ICI-164,384 appears to be mediated by impaired receptor dimerization. *Proc. Natn. Acad. Sci. U.S.A.* **87** (1990) 6883–6887.
10. Klein-Hitpass L., Cato A. C. B., Henderson D. and Ryffel G. U.: Two types of antiprogestins identified by their differential action in transcriptionally active extracts from T47D cells. *Nucleic Acids Res.* **19** (1991) 1227–1234.
11. Guiochon-Mantel A., Loosfeld H., Ragot T., Bailly A., Atger M., Misrahi M., Perricaudet M. and Milgrom E.: Receptors bound to antiprogestin form abortive complexes with hormone responsive elements. *Nature* **336** (1988) 6995–698.
12. Sabbah M., Gouilleux F., Sola B., Redeuilh G. and Baulieu E. E.: Structural differences between the hormone and antihormone estrogen receptor complexes bound to the hormone response element. Proc. Natn. Acad. Sci. U.S.A. **88** (1991) 390–394.
13. Horoszewicz J. S., Leong S. S., Kawinski E., Karr J. P., Rosenthal H., Ming Chu T., Mirand E. A. and Murphy G. P.: LNCaP model of human prostatic carcinoma. *Cancer Res.* **43** (1983) 1809–1818.
14. Schuurmans A. L. G., Bolt J., Voorhorst M. M., Blankenstein R. A. and Mulder E.: Regulation of growth and epidermal growth factor receptor levels of prostate tumor cells by different steroids. *Int. J. Cancer* **42** (1988) 917–922.
15. Veldscholte J., Ris-Stalpers C., Kuiper G. G. J. M., Jenster G., Berrevoets C., Claassen E., Van Rooij H. C. J., Trapman J., Brinkmann A. O. and Mulder E.: A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem. Biophys. Res. Commun.* **173** (1990) 534–540.
16. Veldscholte J., Berrevoets C. A., Brinkmann A. O., Grootegoed J. A. and Mulder E.: Antiandrogens and the mutated receptor of LNCaP cells: Differential effects on binding affinity, heat-shock protein interaction, and transcription activation. *Biochemistry* **31** (1992) 2393–2399.
17. Schüle R., Muller M., Kaltschmidt C. and Renkawitz R.: Many transcription factors interact synergistically with steroid receptors. *Science* **242** (1988) 1418–1420.
18. Nielsen D. A., Tsu-Chung Chang T.-C. and Shapiro D. J.: A highly sensitive, mixed-phase assay for chloramphenicol acetyl transferase activity in transfected cells. *Analyt. Biochem.* **179** (1989) 19–23.
19. Veldscholte J., Berrevoets C. A., Zegers N. D., Van der Kwast T. H., Grootegoed J. A. and Mulder E.: Hormone-induced dissociation of the androgen receptor-heat-shock protein complex: Use of a new monoclonal antibody to distinguish transformed from nontransformed receptors. *Biochemistry* **31** (1992) 7422–7430.
20. Zegers N. D., Claassen E., Neelen C., Mulder E., Van Laar J. H., Voorhorst M. M., Berrevoets C. A., Brinkmann A. O., Van der Kwast T. H., Ruizeveld de Winter J. A., Trapman J. and Boersma W. J. A.: Epitope prediction and confirmation for the human androgen receptor: Generation of monoclonal antibodies for multi-assay performance following the synthetic peptide strategy. *Biochim. Biophys. Acta* **1073** (1991) 23–32.
21. Green S.: Modulation of oestrogen receptor activity by oestrogens and anti-oestrogens. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 747–751.
22. Reese J. C. and Katzenellenbogen B. S.: Differential DNA-binding abilities of estrogen receptor occupied with two classes of antiestrogens: studies using human estrogen receptor overexpressed in mammalian cells. *Nucleic Acids Res.* **19** (1991) 6595–6602.
23. Gronemeyer H., Benhamou B., Berry M., Bocquel M. T., Gofflo D., Garcia T., Lerouge T., Metzger D., Meyer M. E., Tora L., Vergezac A. and Chambon P.: Mechanism of antihormone action. *J. Steroid Biochem. Molec. Biol.* **41** (1992) 217–221.
24. Kemppainen J. A., Lane M. V., Sar M. and Wilson E.: Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation: Specificity for steroids and antihormones. *J. Biol. Chem.* **267** (1992) 968–974.