J. steroid Biochem. Vol. 36, No. 5, pp. 385–389, 1990
Printed in Great Britain.

0022-4731/90 $3.00 + 0.00

ANTIANDROGEN ICI 176,334 DOES NOT PREVENT DEVELOPMENT OF ANDROGEN INSENSITIVITY IN S115 MOUSE MAMMARY TUMOUR CELLS

PHILIPPA D. DARBRE* and R. J. B. KING[1]

Cellular Endocrinology and Hormone Biochemistry Laboratories[1], Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England

(*Received* 25 *January* 1990)

Summary—Many forms of endocrine therapy for steroid-sensitive tumours involve regimes of steroid agonist deprivation by administration of steroid antagonists. The partial or short-lived response to such therapy results from the inevitable progression of the tumour cells to a state of steroid insensitivity. Several cell culture systems have shown that steroid ablation results in loss of steroid sensitivity and we have used an *in vitro* model here to study the influence of steroid antagonists on this progression. Growth of androgen-responsive S115 mouse mammary tumour cells in the long-term absence of steroid results in a loss of androgen-sensitivity. We have studied here the effects of the pure antiandrogen ICI 176,334 on the growth of S115 cells and on their progression to steroid autonomy. Although a pure antiandrogen in its action on these cells with very low toxicity, it had no protective effect against loss of cellular or molecular androgen-responsive parameters. The clinical implications for endocrine therapy are discussed.

INTRODUCTION

Endocrine manipulation provides an effective treatment for many steroid-sensitive tumours but only on a temporary basis since the tumour regression is inevitably followed by growth of hormone-independent tumours resistant to such therapy. Since the major form of endocrine therapy involves administration of steroid antagonists, it is important to ascertain whether this treatment influences the development of steroid resistance.

Our approach to this question has been to study the behaviour of cloned steroid-responsive breast cancer cells when deprived of steroid *in vitro*. Growth of cloned S115 + A mouse mammary tumour cells is responsive to androgen and glucocorticoid *in vitro* [1]. Short-term removal of androgen results in a change from fibroblastic to epithelial morphology [2], reduced proliferation rate [3], increased density regulation [4] and loss of ability to grow in suspension culture [5]. At a molecular level, RNA from mouse mammary tumour virus (MMTV) is a marker of post-receptor androgen action [6]. However, long-term growth of these cells in the absence of steroid results in an ordered, reproducible series of phenotypic changes culminating in loss of both cellular and molecular steroid-sensitive parameters [7]. Steroid-regulated growth responses are lost, such that the unresponsive cells grow at the same rate and to the same saturation density as the steroid-stimulated responsive cells in both monolayer [7] and suspension [8] culture. Production of MMTV RNA is lost and is followed by increased methylation of MMTV-LTR sequences in the DNA [7]. This loss of response is not accompanied by any loss of steroid receptor function [9] demonstrating that it results from removal of ligand rather than loss of receptor. However, there are interactions between steroids in that either androgen alone or glucocorticoid alone will protect against loss of response to both steroids [10]. In view of these cross-protective effects between different steroids, the question arises as to which ligand–receptor complexes can protect against loss of response especially as antagonist–receptor complexes as well as agonist ones can bind to specific DNA sequences [11]. We describe here the effects on loss of steroid-sensitive parameters when the S115 + A cells are grown long-term in the presence of the pure antiandrogen ICI 176,334 [12].

*To whom correspondence should be addressed.

EXPERIMENTAL

Nomenclature of S115 cells

Stock S115 cells are a cloned cell line maintained in androgen that exhibit a positive proliferative response to androgen. These cells are called +A cells. Long-term maintenance of such cells in the absence of androgen results in the cells becoming unresponsive to androgens, and such cells are then called −A cells. Experimental growth of S115 cells in the presence or absence of testosterone is indicated as +T or −T, respectively.

Culture of stock S115 cells

Stock +A S115 cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DME) supplemented with 2% dextran-charcoal-treated fetal calf serum (DC-FCS) (FCS from Gibco-Biocult, Glasgow, Scotland), 40 mM HEPES buffer (Sigma Chemical Co., Poole, England), and 3.5×10^{-8} M testosterone, DC-treated serum was used in all cultures and this treatment has been described previously [13]. Testosterone (Steraloids Ltd, Croydon, England) was dissolved in ethanol and added such that the final concentration was <0.01% ethanol in culture medium. Cells were seeded at 0.1×10^5 cells/ml in 16-ml aliquots in 9-cm plastic tissue culture dishes (Nunc, Denmark) and placed in a humidified atmosphere of 10% carbon dioxide in air at 37°C.

Cells were subcultured at weekly intervals. Cells were suspended by treatment in 5 ml of 0.06% trypsin/0.02% EDTA (pH 7.3) and added to 5 ml of culture medium. The cells were pelleted by centrifugation, resuspended in culture medium alone, counted on a haemocytometer, and repeated as above.

Stock cultures for loss of response experiments

At the start of each experiment, a new vial of cells was thawed, which ensured that cells were always of the same clone and passage generation. Freshly thawed cells were grown for 2 weeks as stock +A cultures and then switched to medium containing 1% DC-FCS with no steroid, testosterone, ICI 176,334, or dexamethasone at the appropriate concentration. Cells were assayed from these stock cultures for androgen-responsive cellular and molecular parameters.

Cell growth experiments

For monolayer culture, cells were suspended from stock plates by treatment with 5 ml 0.06% trypsin/0.02% EDTA (pH 7.3), added to 5 ml DME/2% DC-FCS/40 mM HEPES buffer, and counted on a haemocytometer. Cells were added to the overall required volume of medium DME/1% DC-FCS/40 mM HEPES buffer at a concentration of 0.1×10^5 cells/ml and plated in monolayer in 5-ml aliquots into 5-cm plastic tissue culture dishes for 24 h. The medium was then changed so that dishes contained no steroid, testosterone, antiandrogen ICI 176,334 (ICI Pharmaceuticals, Macclesfield, England) or dexamethasone at the appropriate concentraton. Culture medium was changed routinely every 3–4 days (Sigma, Poole, England). For suspension culture, cells were grown in the same medium as for monolayers but in 5-cm plastic bacteriological dishes (Sterilin, Teddington, England).

Cell counting

Cells in monolayer were washed with saline *in situ*. Cells in suspension were harvested in PBS and pelletted by centrifugation. Cells were then lysed in 2 ml 0.01 M HEPES buffer/1.5 mM $MgCl_2$ plus four drops of Zaponin (Coulter Electronics Ltd, Harpenden, England) for 5 min (monolayers) or 1 h (suspensions). The nuclei released were counted in Isoton (Coulter Electronics Ltd) in triplicate on a model ZB1 Coulter Counter. All cell counts were done on triplicate dishes and results were calculated as the mean ± SE.

Preparation of RNA

A minimum of 3 × 15 cm dishes of stock cells were used for each RNA preparation. Cells were washed *in situ* with PBS, harvested with a rubber policeman into ice-cold PBS and pelleted by centrifugation. Whole cell RNA was prepared by the guanidinium-caesium chloride method [14].

Analysis of RNA by Northern blotting

RNA was subjected to electrophoresis in 1.5% agarose-formaldehyde gels at 30 μg total RNA per track. The RNA was transferred to nitrocellulose (Sartorius GmbH, Göttingen, Federal Republic of Germany) by blotting in 20 × standard saline-citrate buffer (SSC) and hybridized to 10^6 cpm of ^{32}P-labelled probe per ml. Hybridization was in 1 × SSC, 10 × Denhardt's 0.1% SDS, 50 μg/ml single-stranded calf thymus DNA, and 10% dextran sulphate at 65°C for 16 h. The DNA probe was the cloned 1.4 kb pair Pst I fragment of MMTV containing the LTR region. The fragment was cloned into the plasmid pAT 153 for preparation of large quantities and cut out and isolated from the plasmid before use as a probe. It was labelled with ^{32}P by random priming (Amersham International, England). Blots were washed at a stringency of 0.2 × SSC, 0.1% SDS for 1 h at 65°C and autoradiographed.

Preparation of DNA

A minimum of 2 × 15 cm dishes of stock cells were used for each DNA preparation. Cells were washed *in situ* with PBS, harvested with a rubber policeman into PBS, spun down, and resuspended in 10 mM Tris/10 mM EDTA/100 μg/ml proteinase K (BDH Chemicals Ltd)/0.5% SDS at 37°C for at least 4 h. The viscous solution was extracted with phenol-chloroform and the DNA was precipitated in ethanol. The DNA was dissolved in 10 mM Tris/10 mM EDTA/RNase A 20 μg/ml (Millipore Corp., Bedford, Mass) at 37°C for 2 h. The solution was then made 0.2 M NaCl/0.1% SDS/100 μg/ml proteinase K and incubated for another 2 h. The DNA was purified by phenol-chloroform extraction, precipitated in ethanol, and redissolved in sterile 1 mM Tris/1 mM EDTA pH 8.

Methylation patterns of DNA

High molecular weight DNA was digested with the restriction enzymes Hpa II or Msp I (Biolabs) and the fragments were separated on 0.8% agarose gels (5 μg DNA per track) and transferred to nitrocellulose as described in detail elsewhere [14]. Blots were

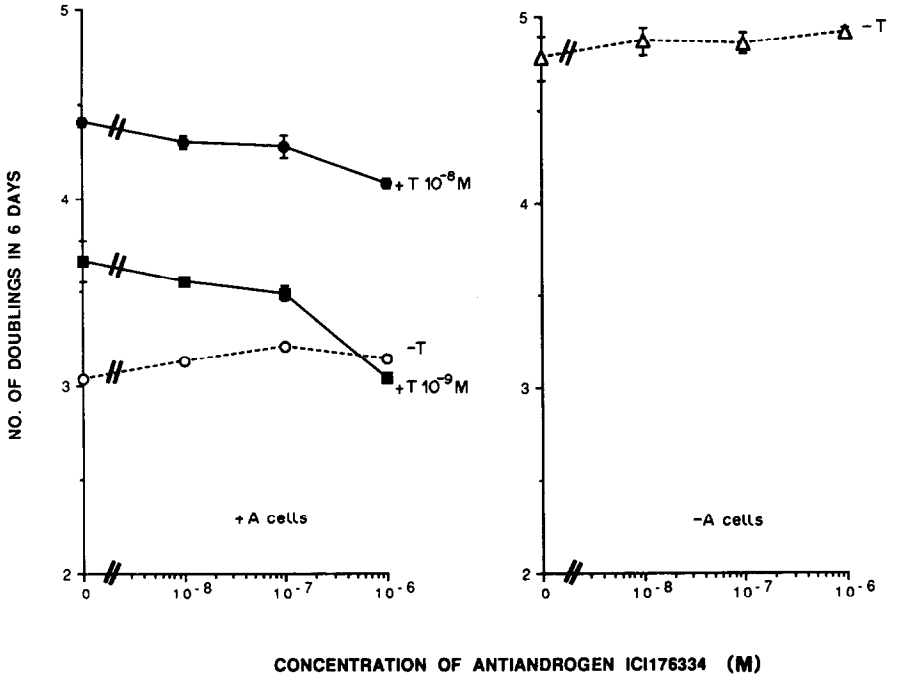

Fig. 1. Effects of antiandrogen ICI 176,334 on growth of androgen responsive (+A) and unresponsive (−A) S115 mouse mammary tumour cells in monolayer culture. Cells were grown in 1% DC-FCS with varying concentrations of antiandrogen without steroid (−T ○----○; △----△) or with testosterone at 10^{-8} M (●—●) or 10^{-9} M (■—■). Bars, SE of triplicate dishes. Where no bars are shown, variation was too low for visual display.

hybridized to ^{32}P-labelled MMTV-LTR and washed as for the RNA analysis.

RESULTS

Antiandrogenic action of ICI 176,334 on S115 cells

On its own, up to concentrations of 10^{-6} M, ICI 176,334 had no agonist activity on growth of S115 + A mouse mammary tumour cells in monolayer culture (Fig. 1). The cells remained epithelial in morphology in the presence of ICI 176,334 and not fibroblastic as seen with testosterone. In addition, ICI 176,334 did not promote growth in suspension culture (data not shown). Loss of steroid sensitivity in long-term (48 weeks) steroid-deprived S115-A cells did not result in any acquired sensitivity to ICI 176,334 (Fig. 1).

Functioning as an antagonist of androgen action 10^{-6} M ICI 176,334 inhibited all growth stimulation of S115 + A mouse mammary tumour cells by 10^{-9} M testosterone in monolayer culture (Fig. 1). Partial inhibition was noted at 10^{-6} M ICI 176,334:10^{-8} M testosterone (Fig. 1) but a ratio of 1:1,000 was necessary for full inhibition. The same ratio was noted in suspension culture growth (data not shown). At a molecular level, ICI 176,334 had no agonist activity on stimulating MMTV RNA production whereas androgen-stimulated MMTV RNA accumulation was prevented at a ratio of 10^{-6} M ICI 176,334:10^{-9} M testosterone (Fig. 2).

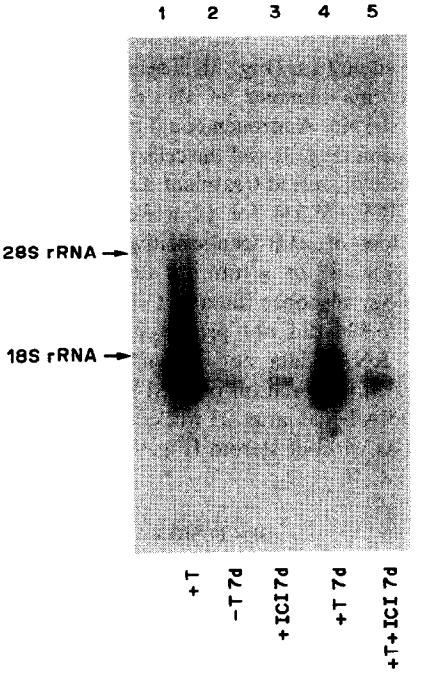

Fig. 2. Effects of antiandrogen ICI 176,334 on androgen-sensitive regulation of MMTV-LTR RNA from S115 + A mouse mammary tumour cells. Northern blots of whole cell RNA from S115 + A cells grown with 10^{-8} M testosterone (1), without steroid (2), 10^{-6} M ICI 176,334 (3), 10^{-9} M testosterone (4) or 10^{-9} M testosterone + 10^{-6} M ICI 176,334 (5) for 7 days.

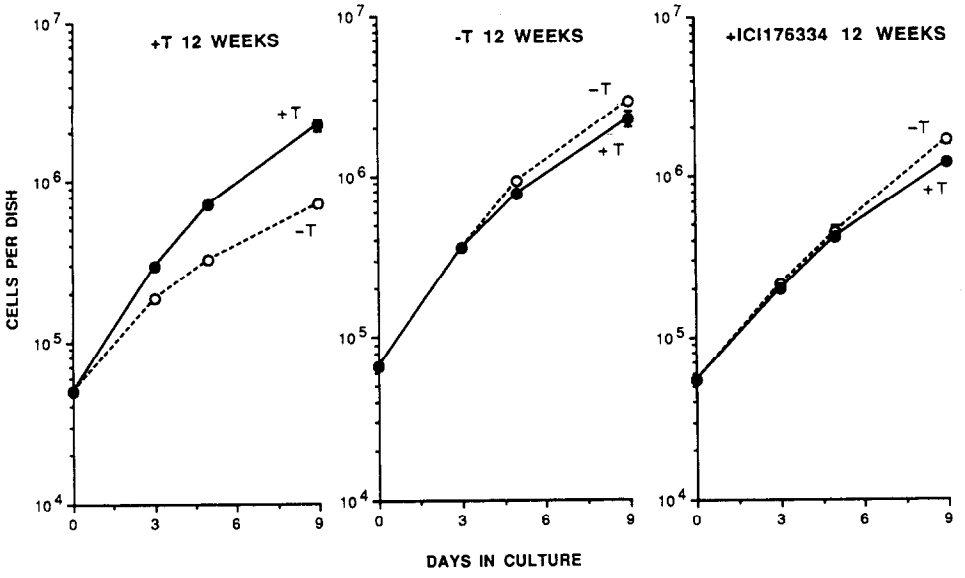

Fig. 3. Antiandrogen ICI 176,334 does not protect against loss of androgen-sensitive growth in monolayer culture for S115 + A mouse mammary tumour cells. Growth curves in monolayer in 1% DC-FCS in the short-term presence (●—●) or absence (○----○) of 10^{-8} M testosterone (T) following maintanence in stock monolayer cultures in 1% DC-FCS for 12 weeks either with or without 10^{-8} M testosterone or with 10^{-6} M antiandrogen ICI 176,334. Bars, SE of triplicate dishes. Where no bars are shown, variation was too low for visual display.

Effect of ICI 176,334 on development of steroid insensitivity

S115 + A cells maintained as stock monolayer cultures in the presence of androgen showed a proliferative response to 10^{-8} M testosterone in short-term monolayer culture (Fig. 3). This response was lost following maintenance of the cells for 12 weeks without steroid. Androgen could no longer stimulate proliferation (Fig. 3) and the cells remained epithelial irrespective of steroid treatment. Growth of cells with 10^{-6} M ICI 176,334 for 12 weeks did not protect against loss of androgen-sensitive proliferative response (Fig. 3) or saturation density response or morphology response (data not shown).

ICI 176,334 did not protect against the loss of MMTV RNA (data not shown) and in addition, increases in methylation of MMTV-LTR sequences in the DNA began after 23 weeks of culture with ICI 176,334 as without steroid (Fig. 4).

DISCUSSION

Steroid deprivation is a widely used, effective treatment for tumours of the prostate, breast and endometrium. However, when used for advanced disease it is palliative, not curative and responses are eventually followed by resurgence of growth resistant to such treatment; prevention or delay of that progression to insensitivity is a desirable clinical objective. Our studies [1, 15] and those of others [16, 17] indicate that steroid withdrawal contributes to loss of sensitivity in several cell culture models. The loss of sensitivity does not involve loss of receptor [9] and we have further shown that with S115 cells, there are cross-protective effects between steroids in that glucocorticoids can protect against loss of androgen sensitivity and vice versa [10]. These and other data have led us to suggest that loss of response is due to inactivation of steroid response elements (SRE) in the genome without change of the receptors themselves; if a steroid–receptor complex is bound to the SRE, inactivation does not occur and the cells remain hormone sensitive [9]. As oestrogen antagonists, in association with receptor, bind to oestrogen response elements (EREs), albeit in an unproductive manner [11] we tested the possibility that an antiandrogen might inhibit progression by such a mechanism. Unfortunately this was not the case, which suggests that a fully activated receptor complex is required for protection. Although one study has reported some protection in short-term experiments with flutamide [18], our longer-term studies with ICI 176,334 showed no hint of any protection.

Clinically, therefore, pure antagonists like ICI 176,334 [12] probably would not delay progression but partial agonists might be more successful. Alternatively, it may be possible to utilise the broad specificity of androgen response elements (ARE) to prevent progression in prostate tumours. ARE's bind receptors for androgens, glucocorticoids, progestagens and mineralocorticoids [19]. Thus, glucocorticoids or progestagens in cells containing the appropriate receptors might prevent loss of androgen sensitivity.

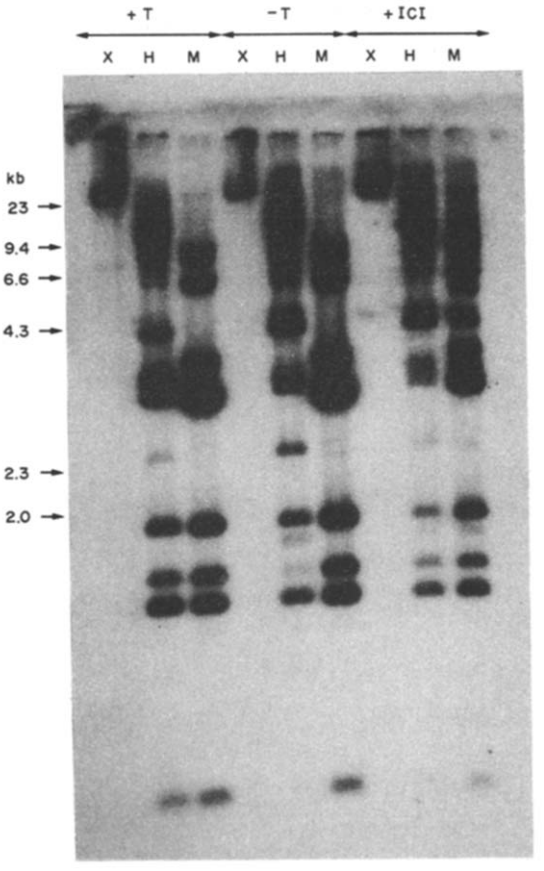

Fig. 4. Restriction endonuclease analysis of MMTV-LTR sequences in the DNA of S115 + A cells following growth for 23 weeks with 10^{-8} M testosterone (+T), no steroid (−T) or 10^{-6} M antiandrogen ICI 176,334 (+ICI). DNA was either undigested (×) or digested with Hpa II (H) or Msp 1 (M) and probed with ^{32}P-labelled MMTV-LTR. Molecular weight standards were provided by a Hind III digest of bacteriophage λ DNA and their positions are indicated by arrows.

Acknowledgement—We thank Dr B. J. A. Furr of ICI Pharmaceuticals (Alderley Park, Macclesfield, Cheshire, England) for providing the antiandrogen ICI 176334.

REFERENCES

1. Darbre P. D. and King R. J. B.: Steroid hormone regulation of cultured breast cancer cells. In *Breast Cancer: Cellular and Molecular Biology* (Edited by M. E. Lippman and R. Dickson). Kluwer Academic, Boston (1988) pp. 307–341.
2. Yates J. and King R. J. B.: Correlation of growth properties and morphology with hormone responsiveness of mammary tumor cells in culture. *Cancer Res.* **41** (1981) 258–262.
3. King R. J. B., Cambray G. J. and Robinson J. H.: The role of receptors in the steroidal regulation of tumour cell proliferation. *J. Steroid Biochem.* **7**, (1976) 869–873.
4. Yates J. and King R. J. B.: Multiple sensitivities of mammary tumour cells in culture. *Cancer Res.* **38** (1978) 4135–4137.
5. Yates J., Couchman J. R. and King R. J. B.: Androgen effects on growth, morphology and sensitivity of S115 mouse mammary tumor cells in culture. *Prog. Cancer Res. Therapy* **14** (1980) 31–39.
6. Darbre P. D., Moriarty A., Curtis S. A. and King R. J. B.: Androgen regulates MMTV RNA in the short-term in S115 mouse mammary tumour cells. *J. Steroid Biochem.* **23** (1985) 379–384.
7. Darbre P. and King R. J. B.: Progression to steroid autonomy in S115 mouse mammary tumor cells: role of DNA methylation. *J. Cell Biol.* **99** (1984) 1410–1415.
8. Darbre P. D. and King R. J. B.: Differential effects of steroid hormones on parameters of cell growth. *Cancer Res.* **47** (1987) 2937–2944.
9. Darbre P. D. and King R. J. B.: Progression to steroid insensitivity can occur irrespective of the presence of functional steroid receptors. *Cell* **51** (1987) 521–528.
10. Darbre P. D. and King R. J. B.: Interaction of different steroid hormones during progression of tumour cells to steroid autonomy. *Int. J. Cancer* **40** (1987) 802–806.
11. Webster N. J. G., Green S., Jin J. R. and Chambon P.: The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. *Cell* **54** (1988) 199–207.
12. Furr B. J. A., Valcaccia B., Curry B., Woodburn J. R., Chesterson G. and Tucker H.: ICI 176334: a novel non-steroidal peripherally selective antiandrogen. *J. Endocr.* **113** (1987) R7–R9.
13. Darbre P., Yates J., Curtis S. and King R. J. B.: Effect of estradiol on human breast cancer cells in culture. *Cancer Res.* **43** (1983) 349–354.
14. Maniatis T., Fritsch E. F. and Sambrook J.: *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, New York (1982).
15. Darbre P. D. and Daly R. J.: Effects of estrogen on human breast cancer cells in culture. *Proc. R. Soc. Edinb.* **95B** (1989) 119–132.
16. Katzenellenbogen B. S., Kendra K. L., Norman M. J. and Berthois Y.: Proliferation, hormonal responsiveness and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res.* **47** (1987) 4355–4360.
17. Welshons W. V. and Jordan V. C.: Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *Eur. J. Cancer Clin. Oncol.* **23** (1987) 1935–1939.
18. Luthy I. and Labrie F.: Development of androgen resistance in mouse mammary tumor cells can be prevented by the antiandrogen flutamide. *Prostate* **10** (1987) 89–94.
19. Evans R. M.: The steroid and thyroid hormone receptor superfamily. *Science* **240** (1988) 889–895.