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# Effect of anti-estrogens on the androgen receptor activity and cell proliferation in prostate cancer cells

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**Abstract** Although some anti-estrogens have been reported to inhibit the proliferation of prostate cancer cells, few studies on the mechanism by which they suppress the growth of prostate cancer have been reported. We investigated, for the first time, whether anti-estrogens modulate the transactivation activity of the androgen receptor (AR) in prostate cancer cells. In DU-145 cells transfected with AR, the transactivation activity of AR was inhibited by tamoxifen and toremifene, even in the presence of 10 nM of DHT. On the other hand, in LNCaP cells having an endogenous AR mutation at codon 877, the activity of AR was suppressed by faslodex in the presence of 10 nM DHT, whereas it was not inhibited by tamoxifen nor toremifene. In PC-3 cells, both the cell growth and the AR activity were remarkably inhibited by tamoxifen at 50 μM. Faslodex and toremifene inhibited AR activity to some extent, but they seemed to function as agonists at higher concentrations. In PC-3 cells, the inhibition of cell growth by flutamide, faslodex and toremifene was much less than their suppression of AR activity. We also demonstrated that a synthetic estrogen diethylstilbestrol and progesterone-related drugs such as chlormadinone acetate and allylestrenol dose-dependently inhibited the activity of AR in DU-145 and PC-3 cells. These results highlight the anti-androgenic aspect of anti-estrogens and estrogens in regard to the AR-mediated transcription of the relevant genes in prostate cancer.

**Keywords** Androgen receptor · Anti-estrogens · Estrogen · Prostate cancer · Estrogen receptors

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## Introduction

Although the most striking characteristic of prostate cancer is its androgen dependence, estrogen seems to play an important role in the development of prostatic diseases. In the human prostate, the estrogen receptor alpha (ERα) is primarily localized in stromal tissue. Stromal cell hyperplasia, i.e. BPH, has been shown to be related to an enhanced estrogenic status after middle age, with a decreased androgen level and an increased conversion of adrenal androgens to estrogen by aromatase [1]. Because both the enhanced estrogen/androgen ratio and the incidence of prostate cancer increase with age, an involvement of estrogen in the pathogenesis of prostate cancer has also been suggested [1]. An increased expression of ERα is associated with the progression, metastasis and the hormone-refractory phenotype of prostate cancer [2].

Recently, a new isoform of estrogen receptor, termed ERβ, was cloned from the prostate gland [3], and shown to be localized predominantly in the epithelium [4]. Although its role and involvement in the pathogenesis and progression of prostate cancer require further study, the expression of ERβ in prostate cancer cell lines and clinical specimens has been investigated in several key studies. ERβ was reported to be expressed in LNCaP and DU145 cells while both ERα and ERβ were expressed in PC-3 cells [5]. The expression level of ERβ in both clinically localized and hormone-refractory tumors was shown to be lower compared with normal prostate tissue by real-time quantitative RT-PCR [6]. Of particular interest is the report that telomerase has an estrogen responsive element (ERE) in its promoter region, and that its transcription and activity are induced through activated estrogen receptors in normal and malignant human prostate epithelium, suggesting that this signaling is associated with the tumorigenesis of prostate cancer [7].

Because of the notion that the estrogen receptor-mediated pathway seems to be involved in prostatic disease progression and its tumorigenesis, an experimental

attempt to use anti-estrogens as therapeutic drugs for prostate cancer was made from a chemopreventive point of view [8, 9]. The inhibitory effects of anti-estrogens, 4-hydroxy-tamoxifen and ICI 182,780, as well as estrogens on cell proliferation of prostate cancer cell lines have been reported, although the mechanism has not yet been analyzed [5]. Among studies on the mechanism by which anti-estrogen-induced inhibition of prostate cancer cell growth occurs, is the report that raloxifene induces apoptosis in androgen-independent prostate cancer cells through caspase activation [10] and the investigation that tamoxifen inhibits prostate cancer cell growth by inhibiting protein kinase C followed by the induction of p21 (waf1/cip1) [11]. Clinically, a phase II study of high-dose tamoxifen in patients with hormone-refractory prostate cancer has shown that the drug is well tolerated but produces only limited objective responses [12].

In order to clarify the possible mechanism of anti-estrogen-induced inhibition of prostate cancer cell proliferation, we investigated whether anti-estrogens modulate the transactivation activity of the androgen receptor (AR) or not, because AR plays a central role in the progression of prostate cancer. In the present paper, we report the effects of anti-estrogens as well as estrogenic hormones on the transactivation activities of AR in several prostate cancer cell lines.

## Materials and methods

### Materials

The dual-luciferase reporter assay system was purchased from Promega (Madison, Wis., USA) and the SuperFect transfection reagent was obtained from Qiagen (Chatsworth, Calif., USA). The prostate cancer cell lines, PC-3, DU-145 and LNCaP, were purchased from the American Type Culture Collection (Rockville, Md., USA). Mouse mammary tumor virus (MMTV)-luciferase (MMTV-luc) reporter plasmid and allylestrenol were generous gifts from N.V. Organon (Oss, The Netherlands); a human androgen receptor expression plasmid pSG5AR was kindly provided by Dr. Chawnshang Chang, University of Rochester (Rochester, N.Y., USA). An anti-androgen, flutamide, and an anti-estrogen, toremifene, were generous gifts from the Nippon Kayaku (Tokyo, Japan). Chlormadinone acetate was a gift from the Teikoku Hormone. Diethylstilbestrol (DES), 5α-dihydrotestosterone (DHT) and tamoxifen were purchased from Sigma-Aldrich (Saint Louis, Mo., USA) and a pure anti-estrogen faslodex (ICI182,780) was obtained from Tocris (Ellisville, Mo., USA).

### Cell culture and transient transfection

Human prostate cancer cell lines, DU-145 and PC-3, were maintained in Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin. LNCaP cells were maintained in RPMI1640 supplemented with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin. A total of $2\times10^5$ cells per well were plated onto six-well culture plates, incubated for

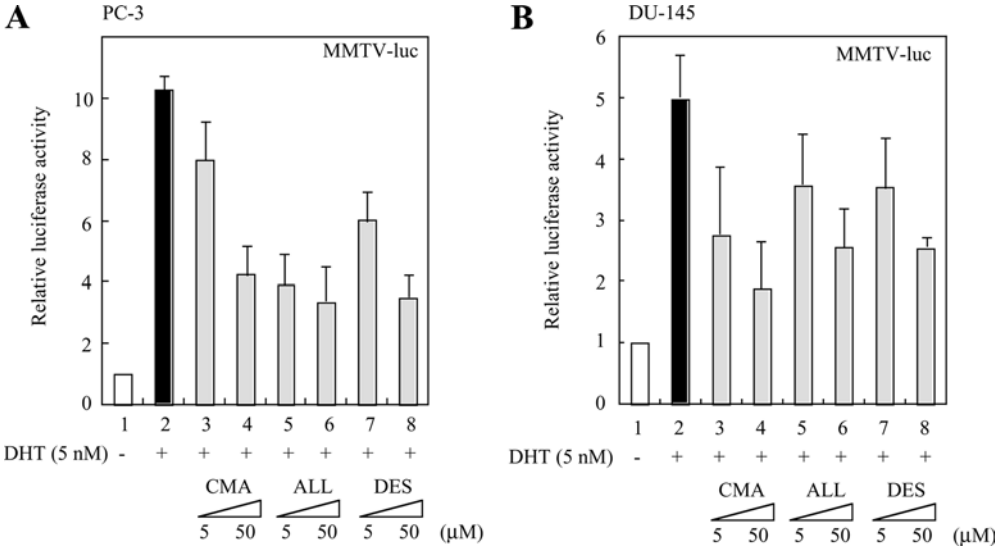


**Fig. 1** Effects of diethylstilbestrol, chlormadinone acetate and allylestrenol on the transactivation activity of AR in: **A** PC-3 and **B** DU-145 cells. **A** PC-3 cells were transfected with a DNA mixture containing pMMTV-luc reporter (1 μg), pSG5AR (960 ng), and pRL-TK (40 ng), and grown for 24 h. Then, 5 nM of DHT with/without diethylstilbestrol, chlormadinone acetate or allylestrenol was added. The cells were incubated for another 24 h and luciferase activities were measured. The values represent the means ± SE from three independent experiments. *CMA*, chlormadinone acetate; *ALL*, allylestrenol; *DES*, diethylstilbestrol. **B** DU-145 cells were transfected with pMMTV-luc reporter (1 μg), pRL-TK (40 ng), and pSG5AR (960 ng), and the transactivation activity of AR as well as the inhibitory effects of diethylstilbestrol, chlormadinone acetate or allylestrenol on AR activity was examined as above. The values represent the mean ± SE of three independent transfections

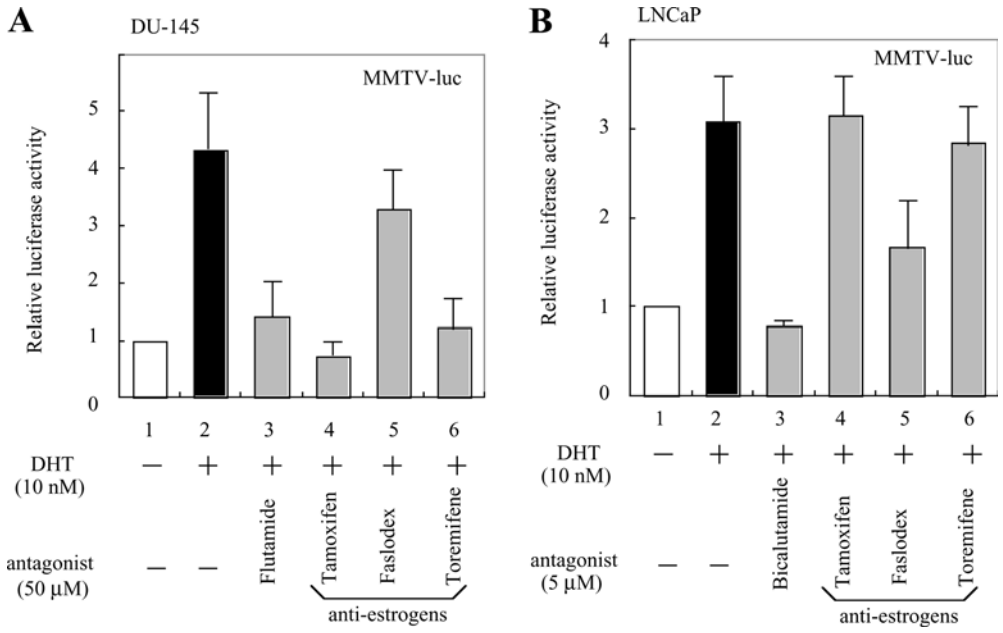


**Fig. 2** Difference in the effects of anti-estrogens on AR activity between DU-145 and LNCaP cells. **A** DU-145 cells were transiently transfected with a DNA mixture containing pMMTV-luc reporter (1 μg), pSG5AR (960 ng), and pRL-TK (40 ng), and grown for 24 h. Then, 10 nM of DHT with/without flutamide or anti-estrogens was added. The cells were incubated for another 24 h and luciferase activities were measured. The values represent the means ± SE from three independent experiments. **B** LNCaP cells were co-transfected with pMMTV-luc reporter (1 μg) and pRL-TK (40 ng), and 24 h after transfection, DHT with/without bicalutamide or anti-estrogens was added. The relative luciferase activities were measured using a dual-luciferase reporter assay system. The values represent the mean ± SE ($n = 4$)

24 h, and used for the assay. PC-3 and DU-145 cells, plated onto six-well culture plates, were transiently transfected using the SuperFect transfection reagent (Qiagen) with 2 μg of DNA mixture containing pMMTV-luc reporter (1 μg), pSG5AR (960 ng), and pRL-TK (40 ng) in accordance with the manufacturer's instructions and grown for an additional 24 h in D-MEM with 10% fetal bovine dialyzed (10,000 MW cut-off, Sigma) serum, penicillin and streptomycin. LNCaP cells plated onto six-well culture plates were transiently transfected using the SuperFect transfection reagent with pMMTV-luc (1 μg) and pRL-TK (40 ng) according to the manufacturer's instructions, and grown for an additional 24 h in D-MEM with 10% fetal bovine dialyzed (10,000 MW cut-off, Sigma) serum, penicillin and streptomycin. Next, DHT with/without estrogenic hormones or anti-estrogens was added and cells were cultured for another 24 h.

### Luciferase assay

The luciferase assay was carried out using a dual-luciferase reporter assay system (Promega) according to the methods recommended by the manufacturer. In short, cells were rinsed with PBS and harvested following the addition of 250 μl lysis buffer per well by scraping with a rubber baton. The cells were subjected to two freeze/thaw cycles to accomplish complete lysis, and the lysate was cleared by centrifugation for 1 min. After preparation of the Luciferase Assay Reagent II and the Stop and Glo Reagent supplied in the kit, 20 μl of the cell lysate was added to 100 μl of the Luciferase Assay Reagent II in a luminometer tube and mixed. The firefly luciferase activity was measured using a luminometer programmed for a 2-s premeasurement delay followed by a 10-s measurement period for each reporter assay. Next, 100 μl of Stop and Glo Reagent was added and the second measurement of the renilla luciferase activity was performed. The results of the luciferase assay were normalized using the activity of the renilla expression vector pRL-TK co-transfected with the reporter plasmid. For counting cell numbers, a small sample (40 μl) was taken from each well, and the cells were counted using a Coulter counter.

## Results and discussion

Although the synthetic estrogen DES has been effectively used as a therapeutic drug for patients with hormone-refractory prostate cancer [13], the mechanism of growth inhibition of prostate cancer cells by estrogen is not fully understood, except for its action mediated via the blockade of the pituitary-testicular axis. We therefore evaluated the effects of DES on the transactivation activity of AR in the prostate cancer cell lines PC-3 and DU-145. As shown in Fig 1A and B, the transactivation activity of AR was dose-dependently inhibited by DES, as well as by the therapeutic drugs for BPH, chlormadinone acetate and allylestrenol. Because the estrogen receptor-mediated pathway seems to be primarily involved in pathogenesis and the development of prostatic

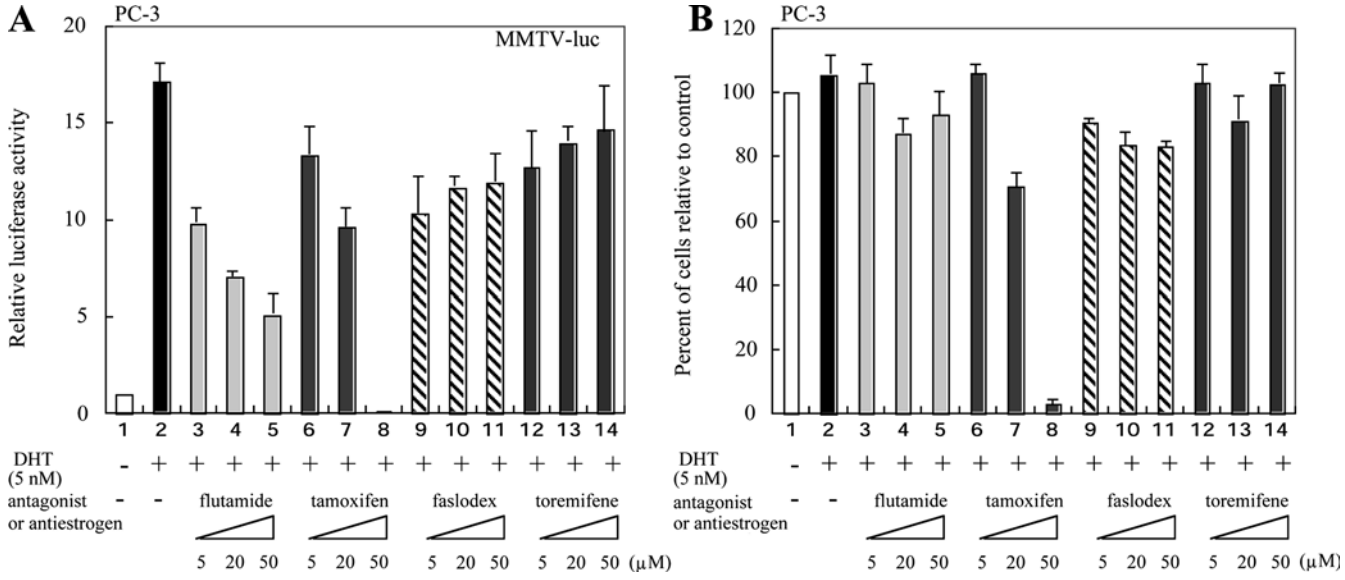


**Fig. 3** Effect of anti-estrogens such as tamoxifen, faslodex and toremifene on the transactivation activity of the **A** AR and **B** cell proliferation in PC-3 cells. **A** PC-3 cells were co-transfected with pMMTV-luc reporter (1 μg), pSG5AR (960 ng), and pRL-TK (40 ng), and 24 h after transfection the ligand, with/without flutamide, tamoxifen, faslodex or toremifene, was added. The relative luciferase activities were measured using a dual-luciferase reporter assay system. The values represent the mean ± SE ($n = 4$). **B** A small sample (40 μl) was taken from each well, and the cells were counted using a Coulter counter

diseases such as BPH and prostate cancer, the somewhat confusing phenomenon that estrogens inhibit the progression of prostate cancer might be mediated not through activating the ERs but through suppressing the AR pathway. The above results highlight the anti-androgenic property of DES in the AR-mediated transcription in prostate cancer.

Recently, much attention has been paid to ER$\beta$ in terms of its expression and role in the prostate gland. Contrary to ER$\alpha$, which is primarily expressed in stromal cells and seems to be involved in the pathogenesis of BPH and prostate cancer, ER$\beta$, through activation by its putative ligand 5$\alpha$-androstane-3$\beta$, 17$\beta$-diol, was reported to suppress the growth of the ventral prostate and to decrease AR content in rodents [14, 15]. Accordingly, there have been several reports showing that loss of ER$\beta$ expression in high-grade prostate intraepithelial neoplasia and high-grade dysplasia compared with normal prostate epithelium expressing ER$\beta$ [16, 17, 18]. Thus, prostatic carcinogenesis seems to be characterized by a loss of ER$\beta$ expression. However, conflicting results have also been reported on the frequency of the ER$\beta$ expression and its association with tumor grade in prostate cancer [17, 18, 19]. Further study is necessary to elucidate the role of ERs in prostate cancer cells by exploring whether endogenous ERs will actually be activated by their ligands followed by the ER-mediated induction of the relevant proteins.

We also investigated the effects of anti-estrogens on the transactivation activities of AR in DU-145 cells transfected with pSG5AR and in LNCaP cells with an AR mutation. In DU-145 cells, as shown in Fig. 2A, the transactivation activity of AR was inhibited by tamoxifen and toremifene even in the presence of 10 nM DHT, while it was not inhibited by faslodex. On the other hand, in LNCaP cells having an endogenous AR mutation at codon 877, the activity of the mutated AR was suppressed by faslodex whereas it was not inhibited by tamoxifen or toremifene (Fig. 2B). In LNCaP cells, bicalutamide was used as an inhibition control because flutamide is an agonist for the mutated AR at codon 877. The inhibitory effect of each anti-estrogen on AR activity was different between these two cell lines, reflecting the difference in the structure of the ligand-binding domains of AR in each cell line.

Mutation in the ligand-binding domain of AR is one of the mechanisms involved in the hormone-refractory progression of prostate cancer. A mutation at codon 877 has been well studied [20, 21] and seems to be selected by combined androgen blockade using flutamide [22]. The mutated AR at codon 877 is activated by flutamide, progesterone and estrogen and antagonized by bicalutamide [20]. Another AR mutation at codon 741 has been found in the LNCaP cells cultured in androgen-depleted medium with bicalutamide. AR with this mutation at was activated by bicalutamide, whereas hydroxyflutamide worked as an antagonist [23]. These facts, together with the results in the present study, indicate that estrogens, anti-estrogens and non-steroid anti-androgens can be both agonist and antagonist to AR, depending on the site of the mutation.

We further examined effects of anti-estrogens on the transactivation activities of AR in PC-3 cells transfected with pSG5AR with the corresponding changes in cell proliferation. The transactivation activity of AR was inhibited by tamoxifen dose-dependently (Fig. 3A). Both cell growth and AR activity were remarkably inhibited by tamoxifen at 50 μM (Fig. 3A, B). Cell death by tamoxifen at 50 μM may not be mediated through

suppressing the AR pathway but might be caused by other reported mechanisms such as apoptosis through the activation of caspase [10] or the inhibition of protein kinase C [11], because the PC-3 cells not transfected with AR also died in response to a high concentration (50 μM) of tamoxifen (data not shown). As shown in Fig. 3A, faslodex and toremifene inhibited the AR activity to some extent, but it seems they functioned as agonists at higher concentrations. Figure 3B shows that the inhibition of cell growth by flutamide, faslodex and toremifene was much less than their suppression of AR activity, indicating that the AR-mediated pathway does not have a strong influence on the cell proliferation in PC-3 cells transfected with AR.

In conclusion, we have shown that some of anti-estrogens as well as DES suppress the transactivation activity of AR in prostate cancer cells, emphasizing the need to take the anti-androgenic aspect of estrogens and anti-estrogens into consideration as one of the mechanisms of their inhibitory effect on prostate cancer growth.

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