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Dutasteride, the Dual 5α–Reductase Inhibitor, Inhibits Androgen Action and Promotes Cell Death in the LNCaP Prostate Cancer Cell Line

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BACKGROUND. Reduction of T to DHT by 5αR in the prostate enhances androgenic activity for most targets. Inhibition of 5αR activity with finasteride attenuates androgen action in men and animal models. The objective of this study was to compare and contrast the effects of a potent new 5αR inhibitor, dutasteride, with finasteride in the LNCaP prostate cancer cell line.
METHODS. LNCaP cells were incubated for varying times with T or DHT in steroid-free medium in the absence or presence of increasing doses of dutasteride or finasteride and the effects on 5αR activity, PSA accumulation in the medium, and on cell proliferation were determined. Drug effects on apoptosis were investigated using Annexin V staining and a cell death ELISA assay. Effects of the drugs on AR ligand-binding activity and on AR protein levels were determined.
RESULTS. Dutasteride inhibited ^{3}H-T conversion to ^{3}H-DHT and, as anticipated, inhibited T-induced secretion of PSA and proliferation. However the drug also inhibited DHT-induced PSA secretion and cell proliferation ($IC_{50} \sim 1$ μM). Finasteride also inhibited DHT action but was less potent than dutasteride. Dutasteride competed for binding the LNCaP cell AR with an $IC_{50} \sim$ 1.5 μM. High concentrations of dutasteride (10–50 μM), but not finasteride, in steroid-free medium, resulted in enhanced cell death, possibly by apoptosis. This was accompanied by loss of AR protein and decreased AR ligand-binding activity. Occupation of AR by R1881 partly protected against cell death and loss of AR protein. PC-3 prostate cancer cells, which do not contain AR, also were killed by high concentrations of dutasteride, as well as by 50 μM finasteride.
CONCLUSIONS. Dutasteride exhibited some inhibitory actions in LNCaP cells possibly related to 5αR inhibition but also had antiandrogenic effects at relatively low concentrations and cell death-promoting effects at higher concentrations. Finasteride also was antiandrogenic, but less than dutasteride. The antiandrogenic effects may be mediated by the mutant LNCaP cell AR. Promotion of cell death by dutasteride can be blocked, but only in part, by androgens.
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KEY WORDS: **5α reductase; androgen; prostate cancer cells**

INTRODUCTION

T, the principal serum androgen, is converted in the prostate to the more active androgen, DHT, by the enzyme 5αR. Two isozymes of 5αR (type 1 and 2) have been characterized. Each is encoded by a separate gene [1]. The type 2 selective 5αR inhibitor, finasteride, has been widely used in the treatment of BPH, alopecia, and in clinical research studies in prostate cancer [2]. It is now clear that the human prostate contains both type 1 and 2 5αR isozymes and that inhibition of both is more effective in lowering DHT than inhibition of a

Abbreviations: 5αR, 5α–reductase; T, testosterone; DHT, dihydrotestosterone; PSA, prostate-specific antigen; AR, androgen receptor; CSS, charcoal-stripped serum; CSSM, medium containing 2% CSS.

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single isozyme [3–10]. A potent 5αR inhibitor with dual specificity, dutasteride, shows promising characteristics in pharmacokinetic studies in rodent, dogs, and men [11,12], and has recently been approved for treatment of benign prostatic hyperplasia. Dutasteride is a 4-azasteroid (like finasteride) but has a longer half-life, is more potent, and results in more profound inhibition of serum DHT in men, in both single and multi-dose regimens, than does finasteride. These characteristics of dutasteride have potentially important application in androgen ablation protocols in prostate cancer treatment as well as in possible prevention strategies [13,14].

In this study, we compare the behavior of dutasteride with finasteride in the androgen-responsive LNCaP human prostate cancer cell line, the most widely used model for androgen action in prostate cancer [15]. Although LNCaP cells contain only type 1 5αR [6,9], high doses of finasteride can inhibit the enzyme [16]. The Ki values for dutasteride inhibition of human 5αR type 1 and 2 isozyme activities are both in the low nM range (6 and 7 nM, respectively) [11]. Finasteride, on the other hand, has a Ki of 325 nM for the type 1 isozyme and 12 nM toward type 2 5αR [16]. Both of these drugs exhibit typical competitive inhibition behaviors in short term kinetic experiments. In longer term experiments, they appear to be functionally irreversible inhibitors due to formation of intermediates that dissociate only very slowly from the enzyme [16].

We show here that dutasteride inhibits 5αR activity in LNCaP cells and, possibly as a result, inhibits T-induced proliferation and PSA accumulation in the medium. However, dutasteride at 0.5–1 μM inhibits DHT-induced proliferation and PSA accumulation, which obviously does not result from its 5αR inhibitory activity. The antiandrogenic actions of dutasteride may be related to binding to the mutant AR that is present in these cells [17]. Furthermore, higher doses of dutasteride have some cell death-promoting effects in prostate cell lines which are partly, but not completely, blocked by androgen treatment.

MATERIALS AND METHODS

Cell Culture and Growth Studies

LNCaP (passage 25–50) or PC-3 cells (passage 22–40) from American Type Culture Collection (Rockville, MD) were seeded at a density of 2×10^5 cells/well in poly-L-lysine coated 6-well plates and left to adhere for 48 hr in routine medium (RPMI-1640, phenol red-free, supplemented with 2 mM glutamine and 10% FBS for LNCaP cells and Ham's F12K supplemented with 2 mM glutamine and 10% FBS for PC-3 cells). The monolayers were washed twice with PBS and incubated for the designated time in CSSM [18] with or without dutasteride, obtained from GlaxoSmithKline (Research Triangle Park, NC) at concentrations of 0.1–50 μM or finasteride, obtained from Merck Research Laboratories (Rahway NJ) at concentrations of 1–50 μM. Medium was changed every 2 days. Each treatment was carried out in triplicate for each timepoint. Cells were counted by Coulter counter (model ZF, Coulter Electronics, Inc., Hialeah, FL) and medium was assessed for PSA on days 5 and 9. Dutasteride, was dissolved in ethanol (10 mM) and added after further dilution to cells such that the final ethanol volume was 0.5% or less. An equivalent amount of ethanol was added to control cells. The very high concentrations of dutasteride (10–50 μM) showed the presence of some crystals a day or two after incubation with cell monolayers, but finasteride did not. Other solvents such as DMSO were assessed for use but gave the same results as ethanol.

Microsome Assay for 5αR Activity

LNCaP cells were grown to 70% confluency in routine medium. The monolayers were washed once and scraped off in PBS. Cells were pelleted, homogenized in 5 volumes of sodium phosphate, pH 7.0, and microsomes were prepared as previously described [19]. 5αR activity in microsomes was assayed according to the method of Klus et al. [18]. Incubations were set up by adding 300,000 cpm of purified ^{3}H-T (specific activity 80 Ci/mmol), 30 nM unlabeled T, and inhibitor in concentrations ranging from 0.1 μM to 10 μM for dutasteride and at concentrations of 5 and 50 μM for finasteride, to sample tubes containing 10 μl of propylene glycol. The ethanol was evaporated and the tubes were preincubated with an NADPH generating system (6.5 mM $NADP^+$, 71 mM glucose-6-phosphate, and 2.5 I.U. of glucose-6-phosphate dehydrogenase in phosphate buffer, pH 7.4) for 15 min at 37°C. LNCaP microsomes (600 μg of protein) or BPH microsomes (100 μg of protein) were added in 500 μl of 0.1 M phosphate buffer, pH 7.4, and incubations were carried out at 37°C for 10 min. Steroids were ether-extracted and separated by celite chromatography [20]. Fractions were analyzed for radioactivity by liquid scintillation spectrometry.

PSA Assay

PSA was assayed in the culture medium obtained after incubation of LNCaP cells under varying conditions as described above, using the ELISA-PSA kit (Medicorp, Inc., Montreal, QC) according to the manufacturer's protocol.

AR Ligand-Binding Assays

AR ligand-binding activity in whole cells was measured by a modification of a method described

earlier by Hseih et al. [21]. Cells were seeded at a density of 3×10^5 cells/well in poly-L-lysine coated 6-well plates, left to adhere for 48 hr in FBS-containing medium, washed with PBS, and treated overnight with CSSM prior to incubation for 2 hr with 1 nM ^{3}H-R1881 in the absence or presence of increasing concentrations of potential competitors (0.3–50 μM). Cells were washed with PBS, scraped and washed twice with ice-cold PBS prior to ethanol extraction of pellet, and scintillation counting. For the experiments involving pretreatment of the cells prior to AR assay, cells at 70% confluence were washed twice with PBS, treated overnight with dutasteride, finasteride, or ethanol vehicle in CSSM, and assayed by the method of Hsieh et al. [21] or Culig et al. [22]. For the first method, the medium was replaced with CSSM prior to incubation of the monolayers with 5 nM ^{3}H-R1881 in the presence or absence of unlabeled R1881 or dutasteride, and scraping of the cells [21]. For the second method, the treated monolayers were washed twice with PBS prior to preparation of the cell suspension and incubation with 5 nM ^{3}H-R1881 in the presence or absence of unlabeled R1881 or dutasteride [22]. For both approaches, washing and ethanol extraction was carried out as outlined above.

Cytosol fractions for measurement of AR ligand-binding activity were prepared from LNCaP cells grown on 10 cm dishes in routine medium, harvested and washed with PBS, homogenized and subjected to centrifugation at 100,000*g* essentially as described earlier [23]. Ligand-binding activity was assessed by incubation of the cytosol in a total volume of 300 μl with ^{3}H-R1881 (5 nM) in the absence or presence of 1 μM radioinert R1881 or increasing concentrations of dutasteride (5 nM–50 μM) or other potential competitors. Incubation was carried out for 30 min at 37°C. Samples were cooled on ice, treated with charcoal-dextran suspension (0.5% charcoal, 0.05% dextran in TE buffer pH 7.5) and samples of the supernatant counted in a liquid scintillation spectrometer.

Western Blot Analysis

Whole cell lysates of treated or control LNCaP cells were prepared by washing the monolayers and scraping cells in RIPA lysis buffer [24]. The lysates were passed through a 21G needle 5-times then incubated on ice for 30 min. Lysates were centrifuged at 15,000*g* for 20 min and supernatants were carefully decanted and immediately frozen at −35°C, after an aliquot was taken for protein assay. Twenty micrograms of protein from each lysate were separated on a 10% gel by SDS–PAGE. After transfer to nitrocellulose and blocking with 10% skim milk powder in TTBS, the blots were incubated overnight in 4 μg/ml of rabbit anti-AR (Affinity Bioreagents, Boulder, CO), washed in TTBS and incubated with donkey anti-rabbit-HRP (Amersham, Oakville, ON). After washing, the blots were covered with Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed to X-ray film for 1–5 min.

Sulforhodamine Blue (SRB)/Trypan Blue Assays

LNCaP cells were seeded in poly-L-lysine-coated 6-well plates at a density of 3×10^5 cells/well. They were left to adhere in routine medium for 48 hr then washed twice and treated for 4 days with dutasteride or finasteride at doses of 1 and 50 μM in CSSM. Controls were incubated in CSSM containing ethanol vehicle. Six-wells per group were seeded. Media were changed on day 2 and viable cells were assessed on day 4. One triplicate set from each group was trypsinized and counted by the Coulter counter. An aliquot was also assessed for viability by the trypan blue exclusion assay [25]. Coulter counts were corrected by the viability index. The second triplicate set from each group was assayed for biomass by staining the monolayers with SRB using a Tox 6 kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Quantification was carried out by spectrophotometry.

Apoptosis ELISA Assay

LNCaP or PC-3 cells were grown in the same manner as for the SRB assay. Cells were harvested by trypsinization after 4 days of treatment by different concentrations of dutasteride, finasteride, or vehicle, and were prepared for assay as previously reported [25]. Briefly, 1×10^4 cells were incubated for 30 min in lysis buffer then sedimented by centrifugation at 200*g* for 15 min. The Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Laval, QC) was used to measure DNA fragments in the supernatants by a quantitative sandwich-enzyme-immunoassay using anti-histone and anti-DNA conjugates for capture and detection. Absorbance at 405 nm was measured on a microplate reader, using 490 nm as a reference wavelength, and results were expressed as apoptotic enrichment relative to CSSM controls (apoptotic index).

Annexin V Staining

LNCaP cells were grown on 22 mm^2 glass coverslips by placing the coverslips in 35 mm dishes and seeding cells at a density of 2×10^5 cells/dish. They were left to adhere in routine medium for 48 hr then washed twice and incubated overnight in medium containing 2% CSS with or without dutasteride at concentrations of 1, 10, or 50 μM, or R1881 at a concentration of 10 or 100 nM, or

mixtures of the two compounds as indicated in the figure legend. The cells were stained for phosphatidylserine using the Annexin-V-Fluos staining kit (Roche) according to the manufacturer's protocol. Propidium iodide was applied simultaneously as a DNA stain to allow detection of necrotic cells. After staining, the cells were immediately observed by fluorescence microscopy and photographed.

Statistical Analysis

Results are expressed as the mean ± SEM. Statistics were performed using StatView (Abacus Concepts, Berkeley, CA) on a Macintosh computer. Group means were compared using analysis of variance followed by Fisher's protected least significant difference test. $P < 0.05$ was considered statistically significant. For the binding assays, IC_{50} and SEM determinations were made using GraphPad Prism, Macintosh version 3.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Inhibition of 5αR Activity in LNCaP Cell Fractions

In order to demonstrate complete inhibition of the type 1 5αR activity in LNCaP cells, we examined the effects of dutasteride at concentrations of 0.1–10 μM and of finasteride at concentrations of 5 and 50 μM on ^{3}H-T reduction in microsomal preparations. The cell fractions were incubated at 37° for 10 min with 30 nM ^{3}H-T, and ^{3}H-DHT formation was assessed by celite column chromatography. Eighty-three percent inhibition was obtained after incubation with 0.1 μM dutasteride and greater than 99% inhibition of 5αR activity was obtained after incubation with 0.5–10 μM dutasteride. Finasteride at 5 or 50 μM also completely inhibited 5αR activity in the LNCaP cell fractions. 5αR activity in the LNCaP cells was relatively low, compared to BPH microsome preparations; routinely we found about 1% conversion/300 μg protein/10 min in LNCaP microsomes compared to at least 50-times that in BPH microsomes.

Dutasteride Inhibits T- and DHT-Induced PSA Accumulation

To test the effects of dutasteride on T- and DHT-induced PSA production, LNCaP cells were subcultured, incubated for 2 days in medium containing 10% FBS, then washed and incubated for 5–9 days in CSSM and T (0.5 or 100 nM) or DHT (0.1 or 100 nM) in the presence or absence of 1 μM dutasteride. Culture medium was changed every 48 hr. PSA was measured in the culture medium. Figure 1 shows that exposure to 1 μM dutasteride for 5 days clearly inhibited the ability of both 0.5 and 100 nM T to stimulate PSA accumulation. This is consistent with inhibition of 5αR activity. However, in the same experiment, 1 μM dutasteride also inhibited the ability of 0.1 and 100 nM DHT to induce PSA accumulation, which is clearly not due to 5αR inhibition. Similar results were observed for T- and DHT-induced cell proliferation, where 1 μM dutasteride resulted in inhibition of the effects of both hormones on proliferation after 9 days of culture (data not shown).

This apparent anti-androgenic action of dutasteride was examined further in a dose-response experiment in which LNCaP cells were incubated in CSSM plus 0.1 nM DHT for 5 days, in the absence or presence of increasing amounts of dutasteride (0.1–50 μM; Fig. 2A). Significant inhibition was seen in the range of dutasteride concentrations of 0.5–50 μM. The apparent IC_{50} for inhibition of the DHT-induced PSA production was about 1 μM. Finasteride was also inhibitory towards PSA accumulation, but less so than dutasteride. No effect of finasteride at a concentration of 1 μM on DHT-induced PSA accumulation was seen.

Inhibition of DHT-induced PSA accumulation was also seen in an experiment in which LNCaP cells were incubated in CSSM for 3 days, followed by 18 hr incubation with 0.1 nM DHT, in the absence or presence of increasing doses of dutasteride. The IC_{50} was 0.5 μM. In the same experiment, dutasteride had no effect on cell number (data not shown).

Dutasteride Inhibits DHT-Induced LNCaP Cell Proliferation

Effects of dutasteride on LNCaP cell proliferation were measured under the same conditions used to assess effects on PSA accumulation in Figure 2. Proliferation induced by 0.1 nM DHT after 9 days was significantly inhibited by 1 μM and higher concentrations of dutasteride. The highest dose of dutasteride tested, 50 μM, resulted in significant cell loss, compared to control cells cultured in CSSM in the absence of DHT (Fig. 2B). Finasteride gave significant inhibition of DHT-induced proliferation at a concentration of 25 μM, but no cell loss compared to DHT-free controls.

Androgen Agonist Activity of Dutasteride

The antiandrogenic activity of dutasteride led to investigation of its androgen agonist activity when used alone. Figure 3A shows that dutasteride incubated with LNCaP cells in CSSM had significant androgen agonist activity towards PSA accumulation in the culture medium. A bell-shaped dose-response curve was obtained, with a peak at a dutasteride

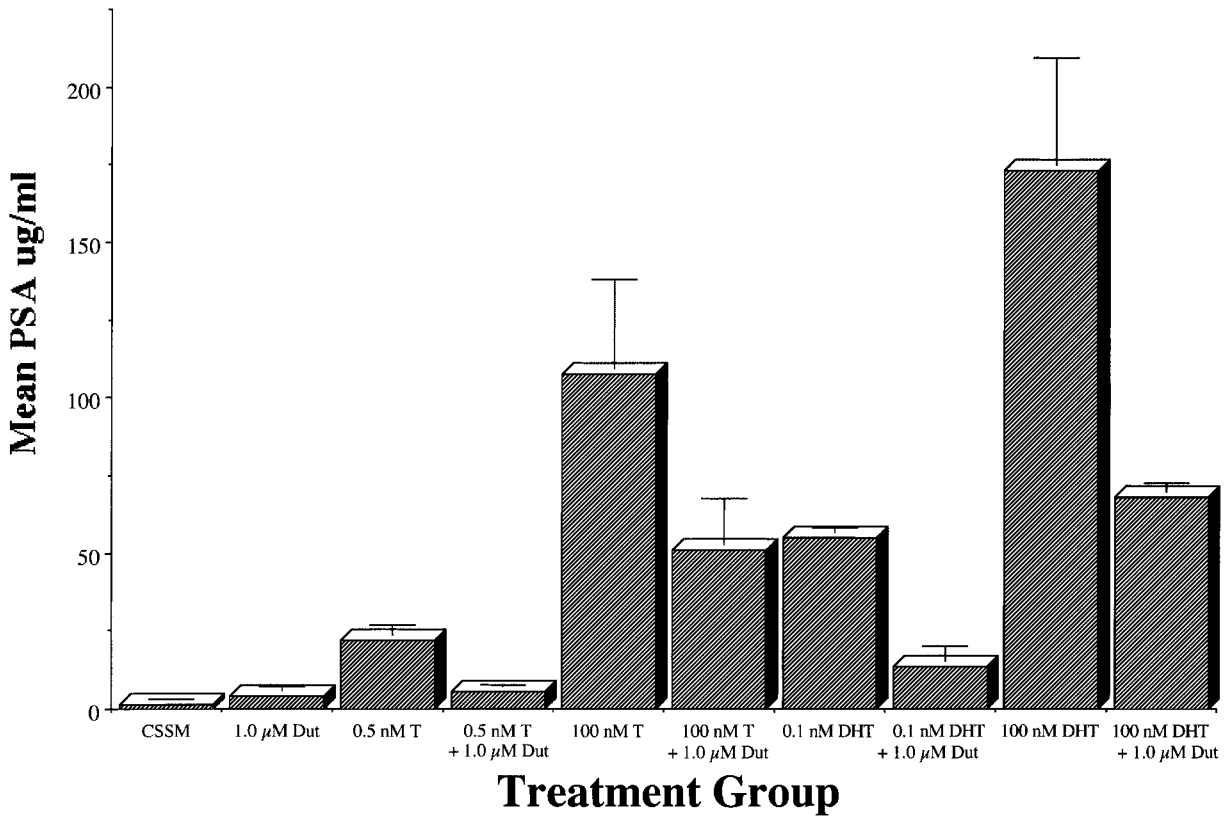


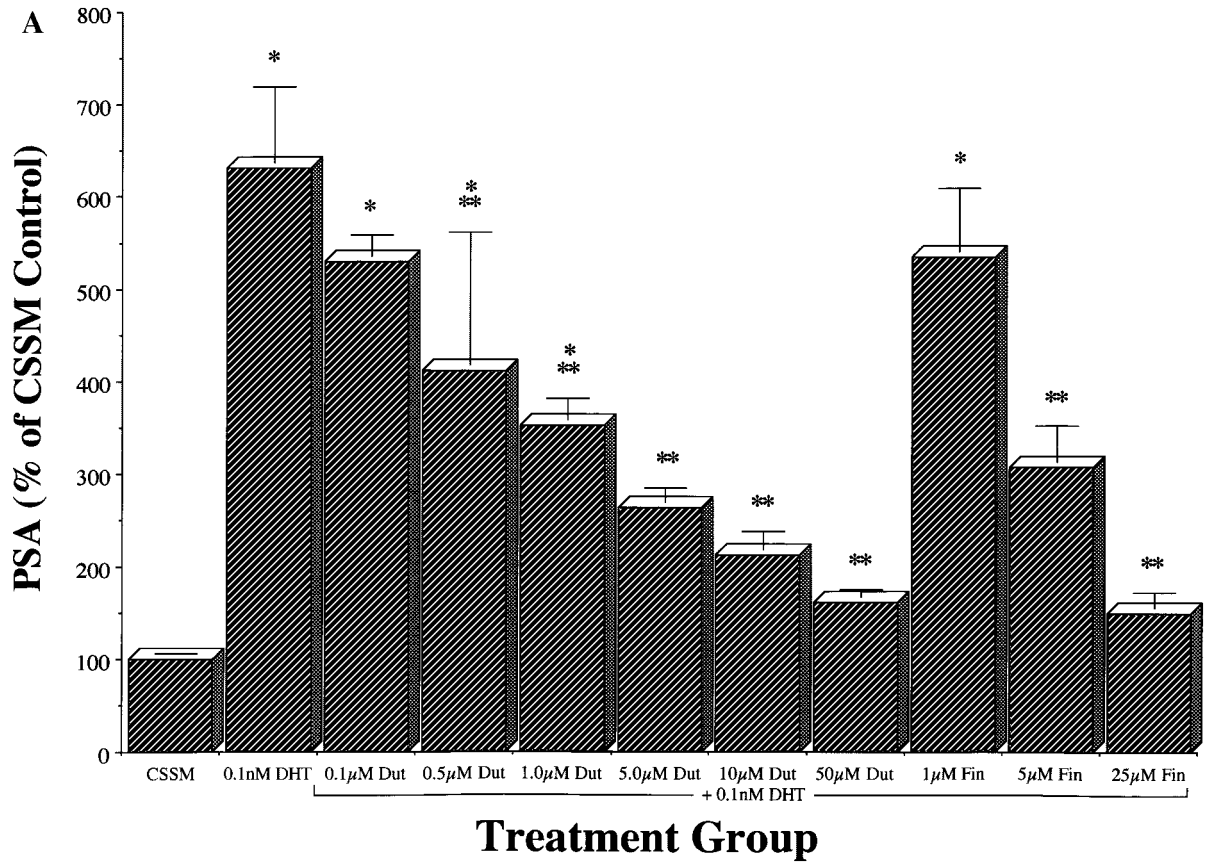
Fig. 1. Dutasteride inhibition of bothT- and DHT-induced PSA accumulation in LNCaP cell medium. LNCaP cells were incubated with CSSM alone or with CSSM plusTor DHT in the absence or presence of I μM dutasteride (Dut) for 5 days, with the medium being changed every 48 hr. PSA levels in the medium were measured by the ELISA–PSA technique described in the "Materials and Methods." The results represent the mean ± range for two independent experiments, each carried out in duplicate.

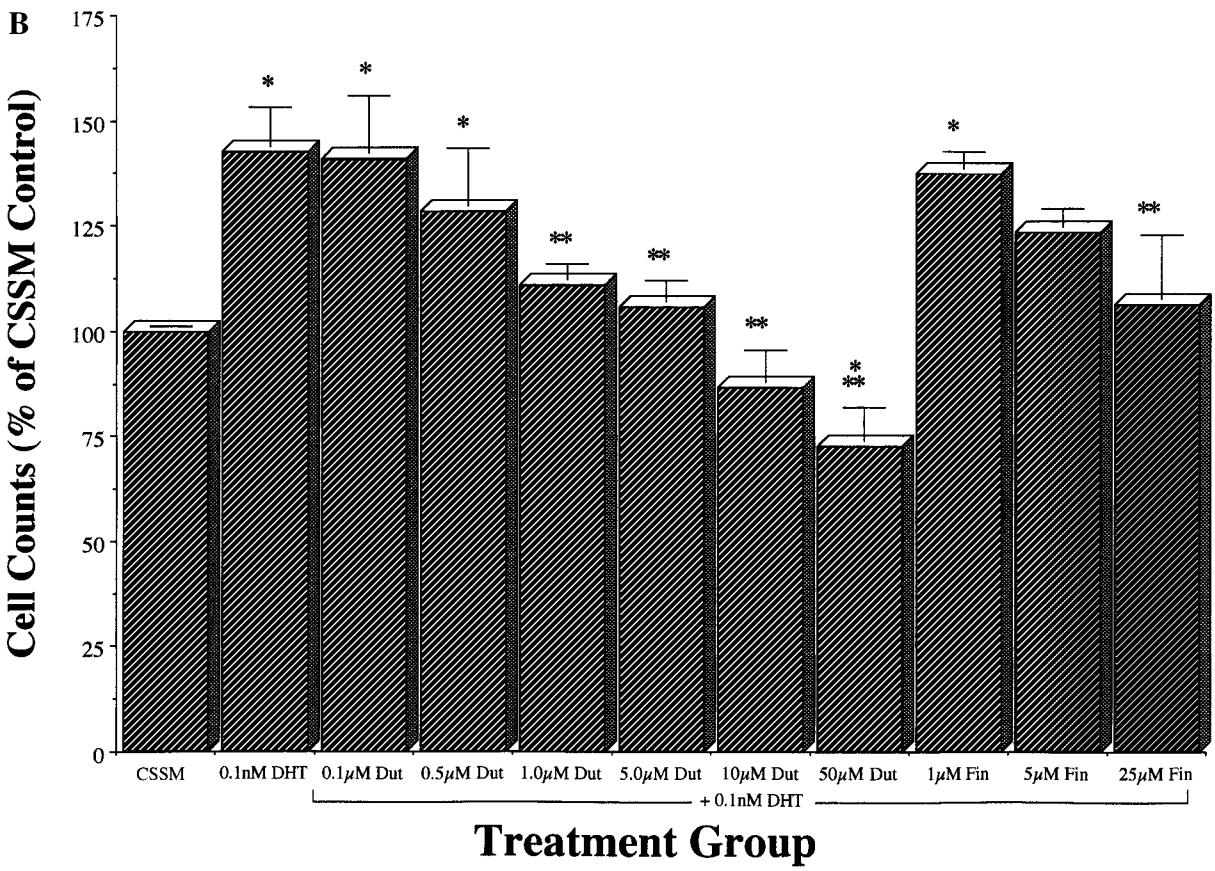
concentration of 1 μM, reflecting about an 8.8× increase in PSA accumulation over 5 days. Higher doses were less effective in promotion of PSA production. Note that the apparent agonist activity of dutasteride is weak compared to DHT: at an optimal DHT dose in LNCaP cells (100 nM), 60 fold higher accumulation of PSA is found (Fig. 1). With regard to the effect of dutasteride on proliferation, Figure 3B shows that after 9 days of incubation, dutasteride alone had no agonist effects but high concentrations (10 and 50 μM) resulted in cell loss. Comparison of these data with those in Figure 2 shows that DHT prevented cell loss seen with 10 μM but not with 50 μM dutasteride.

Interaction of Dutasteride With the LNCaP Cell AR

Inhibitory effects of dutasteride on DHT-stimulated PSA accumulation and on cell proliferation could be due to direct antiandrogenic effects involving the AR. It has been reported that dutasteride at concentrations up to 10 μM does not bind the wild-type human AR [12], but the mutation known to be present in the ligand-binding domain of the LNCaP AR affects the binding affinity for a variety of hormonal and anti-hormonal ligands [17,23]. We, therefore, assessed the ability of dutasteride to compete for binding to the LNCaP AR using the synthetic androgen ^{3}H-R1881. Table I

Fig. 2. **A**: Dutasteride inhibition of DHT-stimulated PSA accumulation in LNCaP cell medium in a dose-dependent manner. LNCaP cells were incubated with CSSM alone or with CSSM and 0.1 nM DHT in the absence or presence of increasing concentrations of dutasteride (Dut) or finasteride (Fin), for 5 days. The medium was changed every 48 hr. PSA levels were measured by ELISA. The results represent the mean ± SEM for four independent experiments, each carried out in triplicate. *, Significantly different from the CSSM group ($P \leq 0.05$); **, significantly different from the 0.1 nM DHT group ($P \leq 0.05$). **B**: Dutasteride inhibition of DHT-induced cell proliferation in a dose-dependant manner. LNCaP cells were incubated with CSSM alone or with CSSM and 0.1 nM DHT in the absence or presence of increasing concentrations of dutasteride or finasteride, for 9 days. The medium was changed every 48 hr. Cells were counted in a Coulter counter. The results represent the mean ± SEM for four independent experiments each carried out in triplicate. *, Significantly different from the CSSM group ($P \leq 0.05$); **, significantly different from the 0.1 nM DHT group ($P \leq 0.01$).


A
PSA (% of CSSM Control)
800
700
600
500
400
300
200
100
0
CSSM
0.1nM DHT
0.1μM Dut
0.5μM Dut
1.0μM Dut
5.0μM Dut
10μM Dut
50μM Dut
1μM Fin
5μM Fin
25μM Fin
+ 0.1nM DHT
Treatment Group


B
Cell Counts (% of CSSM Control)
175
150
125
100
75
50
25
0
CSSM
0.1nM DHT
0.1μM Dut
0.5μM Dut
1.0μM Dut
5.0μM Dut
10μM Dut
50μM Dut
1μM Fin
5μM Fin
25μM Fin
+ 0.1nM DHT
Treatment Group

shows IC_{50} values derived from competition curves comparing the ability of increasing concentrations of dutasteride or finasteride or unlabeled R1881 to compete for binding of 1 nM ^{3}H-R1881 to AR in LNCaP cell monolayers [21]. The IC_{50} for dutasteride under these conditions was about 1.5 μM. Finasteride showed somewhat weaker competition (IC_{50} 3.8 μM). AR ligand-binding activity in cytosol preparations was also measured and dutasteride competed with 5 nM ^{3}H-R1881 for binding with an IC_{50} of 1.3 μM (Table I).

In another set of experiments, LNCaP cells in monolayers were incubated with dutasteride for 16 hr prior to assay of whole cell AR. The results showed a decrease in AR-binding activity, using two different assays. With the method of Hsieh et al. [21], dutasteride-pretreated cells were incubated in monolayers for 2 hr at 37°C with 5 nM ^{3}H-R1881 in the absence or presence of excess radioinert R1881 and specific binding determined. This method showed a significant decrease in available AR due to pretreatment with 1 μM dutasteride (Table II). With the second method [22], pretreated cells were washed and then incubated for 90 min at 20°C in suspension with 5 nM ^{3}H-R1881 in the presence or absence of 1 μM radioinert R1881 and specific AR-binding was determined. Table II shows that the pretreatment reduced AR available for binding ^{3}H-R1881, with half-maximal inhibition at a dutasteride concentration between 10 μM and 50 μM. The mechanism of the loss in AR after dutasteride pretreatment could reflect occupation of AR ligand binding sites by dutasteride (or a metabolite), an indirect inhibitory mechanism, or turnover of AR protein. It seems likely that the second method, in which cells were thoroughly washed before AR assay, mainly involves loss of AR protein, while the first method, without washing, may also involve competition for binding.

The possibility of AR protein loss was investigated by Western blot analysis, which showed no change in the levels of AR protein after 16 hr treatment with 1 μM dutasteride alone but significant loss after treatment with 50 μM dutasteride (Fig. 4). However treatment of the cells with 10 nM R1881 in addition to 10 or 50 μM dutasteride appeared to partially protect the AR from destruction. These results suggest that occupation of the AR by R1881 (10 nM), results in a receptor conformation that is resistant to a proteolysis pathway targeted by high doses of dutasteride.

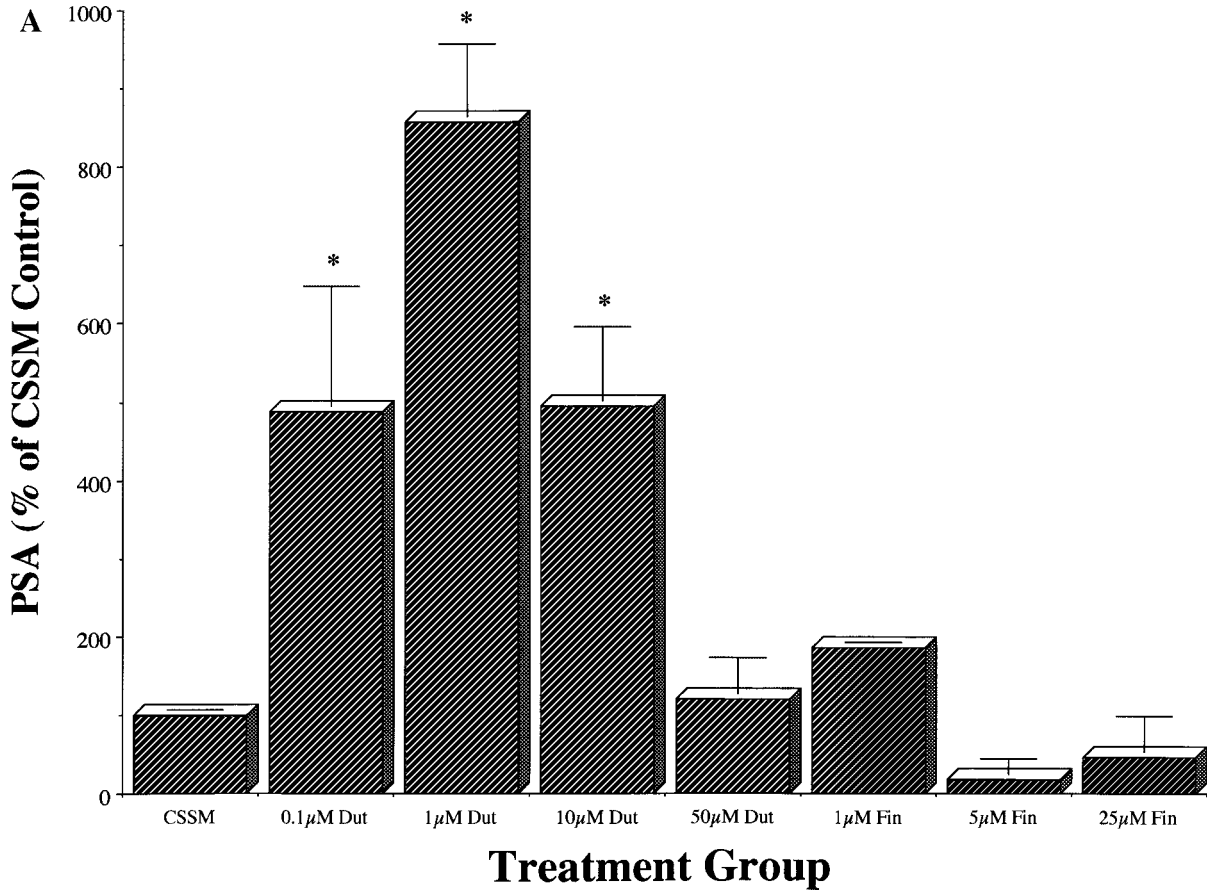
Dutasteride Promotes Cell Loss in Both LNCaP and PC-3 Cell Cultures

The cell loss noted by Coulter counting after incubation of LNCaP cells with dutasteride was confirmed by the SRB assay for cell biomass, and by trypan blue staining for viable cells (Fig. 5). No cell death was apparent after incubation for 4 days with a dutasteride concentration of 1 μM, but significant cell death was obvious with 50 μM dutasteride. In contrast, finasteride at a concentration of 50 μM did not result in any cell death.

The Cell Death Detection ELISA^{PLUS} Kit (which measures DNA–histone release from chromatin) was used to determine whether apoptosis or necrosis prevailed as the mechanism of cell loss provoked by high doses of dutasteride [25]. Results show that the enrichment factor for apoptosis in LNCaP cells in CSSM treated with 50 μM dutasteride for 4 days was enhanced 4× compared to control cells in CSSM alone (Fig. 6). The culture medium examined for dead cells by this method showed no enrichment for necrotic cells compared to control cell medium.

Apoptosis in LNCaP cells was also examined by Annexin-V-Fluos (Roche) and propidium iodide staining after 18 hr incubation in the absence or presence of dutasteride (1, 10, or 50 μM). Figure 7 shows increased Annexin-V staining, reflecting binding to phosphatidylserine externalized by apoptotic cells in the presence of the higher concentrations of dutasteride compared to CSSM controls or to 1 μM drug. An infrequent necrotic cell was noted by its orange propidium iodide staining. Addition of R1881 (10 or 100 nM) along with dutasteride had a partial effect in preventing the apparent apoptosis. At the same time, the presence of 10 nM R1881 partially prevented the loss of LNCaP cell AR protein (Fig. 4). The results suggest that maintenance of the AR partly, but not completely, protects LNCaP cells against promotion of cell death by dutasteride. In other words, there may be two apoptotic pathways involved, only one of which is blocked by androgen treatment.

The PC-3 cell line, the androgen-independent prostate cancer cell line which does not contain AR, was also used to examine the effects of dutasteride on cell death. Figure 8 shows PC-3 cell counts after incubation in CSSM for 9 days with increasing concentrations of dutasteride or in CSSM alone. A significant decrease in cell numbers was found with 10 or 50 μM dutasteride, and with 50 μM finasteride. Similar results were obtained by the trypan blue method. Annexin V staining showed an increase 5 hr after dutasteride treatment, but longer times were not studied because of pronounced lifting of apoptotic cells from the coverslip, unlike with LNCaP cells (data not shown). Nevertheless, these results with PC-3 cells suggest that cell death caused by high concentrations of the 4-azasteroids can occur in the absence of the AR. Both finasteride and dutasteride at 50 μM caused cell loss in PC-3 cells, in contrast to LNCaP cells where only dutasteride had such an effect (Figs. 3 and 5).


A
PSA (% of CSSM Control)
1000
800
600
400
200
0
*
*
*
CSSM
0.1μM Dut
1μM Dut
10μM Dut
50μM Dut
1μM Fin
5μM Fin
25μM Fin
Treatment Group

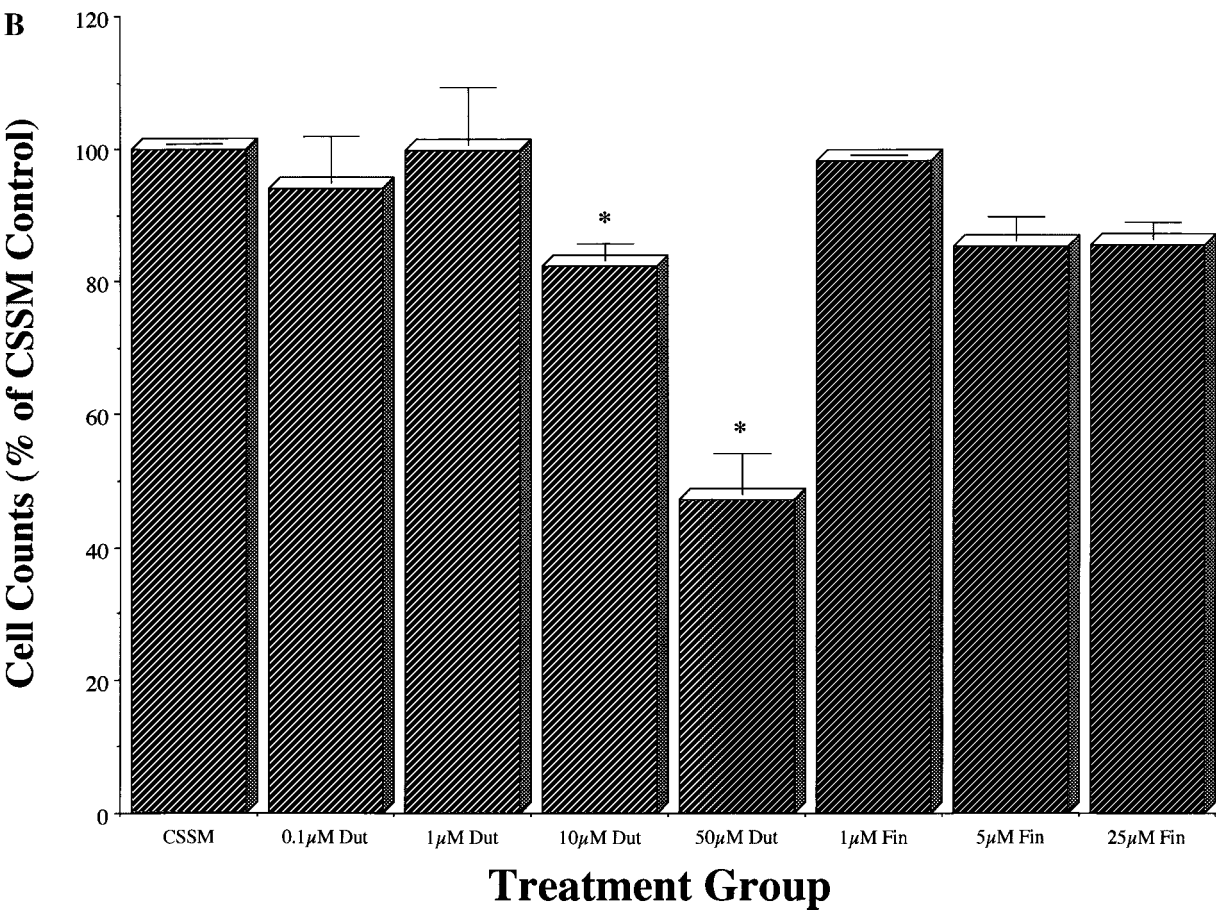

B
Cell Counts (% of CSSM Control)
120
100
80
60
40
20
0
*
*
CSSM
0.1μM Dut
1μM Dut
10μM Dut
50μM Dut
1μM Fin
5μM Fin
25μM Fin
Treatment Group

TABLE I. Competition of ^{3}H-R1881-Binding to the LNCaP Cell AR

Competitor	IC_{50}
Whole cell AR (1 nM ^{3}H-R1881)	
R1881	0.6 ± 0.1 nM
Dutasteride	1.5 ± 0.2 μM
Finasteride	3.8 ± 0.8 μM
Cytosol AR (5 nM ^{3}H-R1881)	
R1881	10.8 ± 0.8 nM
Cyproterone	11.3 ± 2.0 nM
Dutasteride	1.3 ± 0.3 μM
Finasteride	3.9 ± 2.2 μM

IC_{50} values were based on the average for three independent experiments in which competition for ^{3}H-R1881 by six increasing concentrations of radioinert compounds was determined as described in the "Materials and Methods" (whole cells [21], cytosol [22]).

DISCUSSION

In this study we have shown that treatment of LNCaP cells with dutasteride results in inhibition of both T- and DHT-induced PSA accumulation and cell proliferation. The inhibition of T action in principle may be due, at least in part, to 5αR inhibition. Since 0.1 μM dutasteride gave about 80% inhibition of microsomal 5αR activity, we expected to find that this dose would significantly inhibit T action. Instead we found only a small effect (data not shown). The explanation may be that LNCaP cells contain very little 5αR activity or that dutasteride in intact cells is not as effective a 5αR inhibitor as it is in isolated microsomes. Nevertheless, it is clear that inhibition of DHT action does not involve 5αR.

The antiandrogenic effects of dutasteride may be mediated by its interaction with AR directly. LNCaP cell AR contains a mutation, T877A, in the ligand-binding domain, which broadens receptor-binding specificity compared to wild-type hAR [17,23,26]. Recent crystallographic analysis of the ligand-binding domains of wild-type and T877A mutant AR clearly demonstrate differences in the binding pocket of the

TABLE II. Effect of 16 hr Pretreatment of LNCaP Cells With Dutasteride on AR-Binding Activity

Pretreatment	Specific-binding (%)
Method 1	
CSSM	100
Dutasteride 1 μM	76 ± 5[c]
Finasteride 1 μM	77 ± 3[d]
Method 2	
CSSM	100
Dutasteride	
1 μM	79 ± 16
10 μM	59 ± 16[a]
50 μM	43 ± 13[b]

[a] $P = 0.06$; [b] $P = 0.02$; [c] $P = 0.002$; [d] $P = 0.0002$. Each value for specific binding is the average of three independent experiments, each carried out in duplicate. Ligand-binding activity in whole cells was determined by the method of Hsieh et al. [21] (method 1) or Culig et al. [22] (method 2).

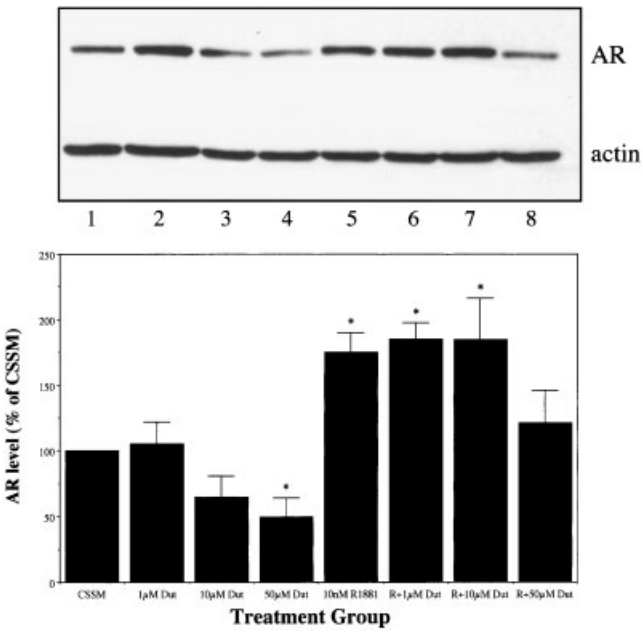


Fig. 4. AR protein levels in LNCaP cell lysates after treatment of cells with dutasteride in the absence or presence of R1881. LNCaP cell monolayers were incubated for 18 hr with CSSM alone (1) or CSSM plus 1 μM dutasteride (Dut) (2) or 10 μM dutasteride (3) or 50 μM dutasteride (4) or 10 nM R1881 (5) or 10 nM R1881 plus 1 μM dutasteride (6) or 10 nM R1881 plus 10 μM dutasteride (7) or 10 nM R1881 plus 50 μM dutasteride (8). Cell lysates (20 μg protein) were prepared and subjected to electrophoresis and Western blotting as described in the "Material and Methods." Bands were scanned and quantitated by densitometry. Results are shown from five independent experiments (four for lanes 3 and 7). *, Significantly different from lane 1 ($P \leq 0.05$).

Fig. 3. **A**: Androgen agonist activity on PSA accumulation of dutasteride given alone. LNCaP cells were incubated in CSSM alone or with CSSM plus increasing concentrations of dutasteride (Dut) or finasteride (Fin) for 5 days. Medium was changed every 48 hr. The results are the mean ± SEM for three independent experiments, each carried out in triplicate. *, Significantly different from the CSSM group ($P \leq 0.01$). **B**: Cell loss after incubation of LNCaP cultures with high doses of dutasteride. Cells were incubated in CSSM alone or with CSSM plus increasing concentrations of dutasteride or finasteride for 9 days, changing the medium every 48 hr. Cells were counted with a Coulter counter. The results are the mean ± SEM for three independent experiments, each carried out in triplicate. *, Significantly different from the CSSM group ($P \leq 0.05$).

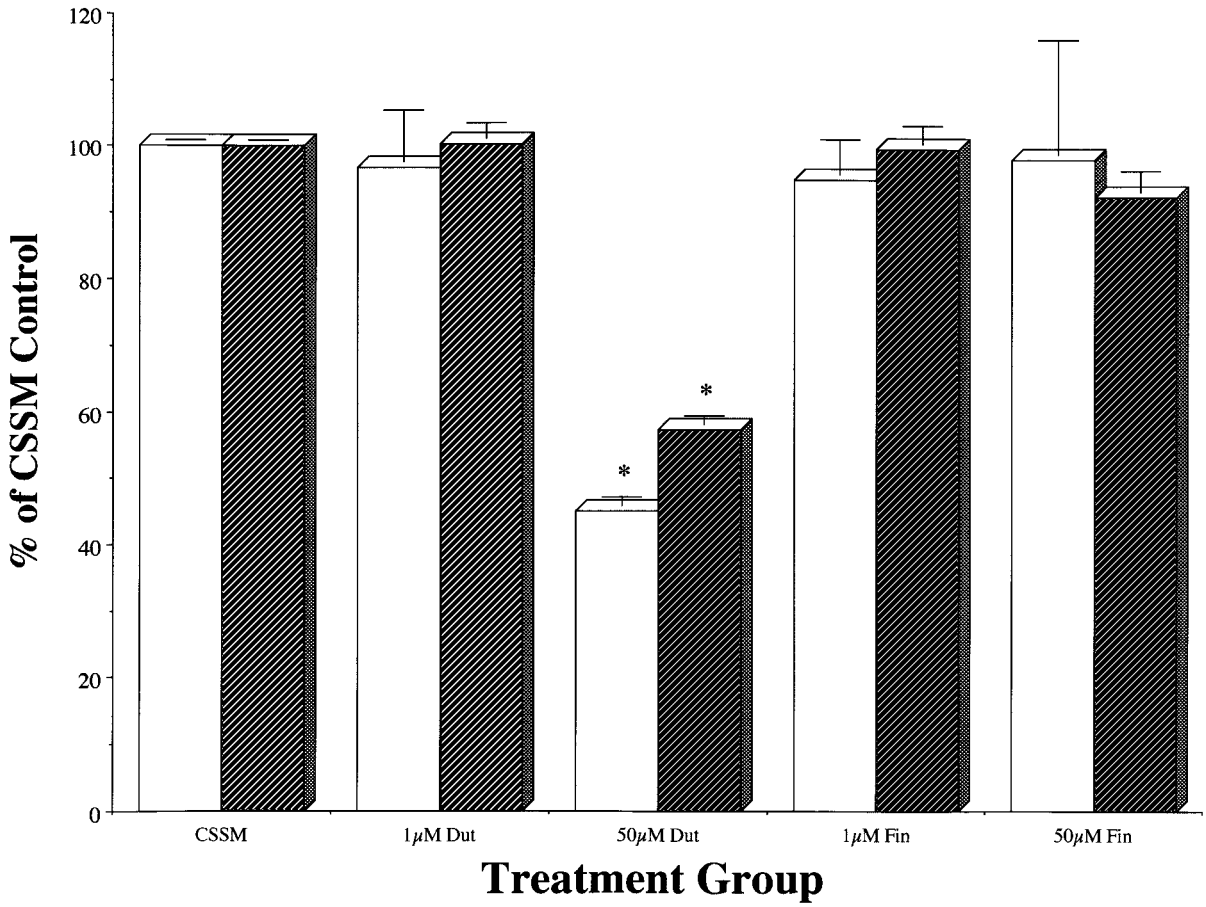


Fig. 5. Assessment of cell viability by trypan blue exclusion (open bars) or by the sulforhodamine blue (SRB) assay (hatched bars). LNCaP cell monolayers were incubated in CSSM alone or with 1 or 50 μM dutasteride (Dut) or finasteride (Fin) for 4 days. The medium was changed after 2 days. The results are the mean ± SEM for three independent experiments, each carried out in triplicate. *, Significantly different from the CSSM group ($P = 0.007$, Trypan blue method; and $P < 0.0001$, SRB assay).

two ARs which explain the relaxed specificity of the mutant [26]. Our ligand-binding assays show significant, but limited, affinity of dutasteride for the LNCaP AR. The IC_{50} for the antiandrogenic effects of dutasteride on PSA accumulation is about 0.5–1 μM, and the IC_{50} for dutasteride binding to the LNCaP AR is about 1.5 μM. This slight discrepancy might be explained by differences in the cell treatment and assay conditions for measurement of the two parameters. If the AR does mediate the inhibitory effects of dutasteride directly, the AR bound to dutasteride might assume a conformation that is inefficient in transcription activation at a number of different promoters, leading to inhibitory effects in specific gene expression and in cell proliferation. It is also possible that the apparent antiandrogenic effects of dutasteride are indirect, and could involve inhibition of AR action by cross-talk with other signaling pathways. Numerous recent reports suggest cross talk between signaling pathways affecting androgen-induced PSA expression or proliferation in LNCaP cells. These may involve other nuclear receptor superfamily members such as the calcitriol or retinoic acid receptors and/or a variety of phosphorylation cascades signaling from growth factor, cytokine, or other membrane receptors [27–29].

Androgen regulation of cell proliferation and of PSA accumulation in LNCaP cells are obviously complex phenomena and dutasteride may act at independent sites in each process. Inhibition of cell cycle could involve induction of p21/WAF, the cyclin dependent kinase inhibitor, or induction of insulin-like growth factor binding protein-3 (IGFBP-3), among other mechanisms [24,30] possibly mediated by blockade or loss of AR. We showed here by Western blot analysis that no loss of AR protein occurred after treatment with 1 μM dutasteride, but that AR loss was obvious after high doses of drug. Similar results have been reported with finasteride [31]. The mechanisms involved in receptor turnover by high concentrations of the 4-azasteroids are unknown, but could involve actions at

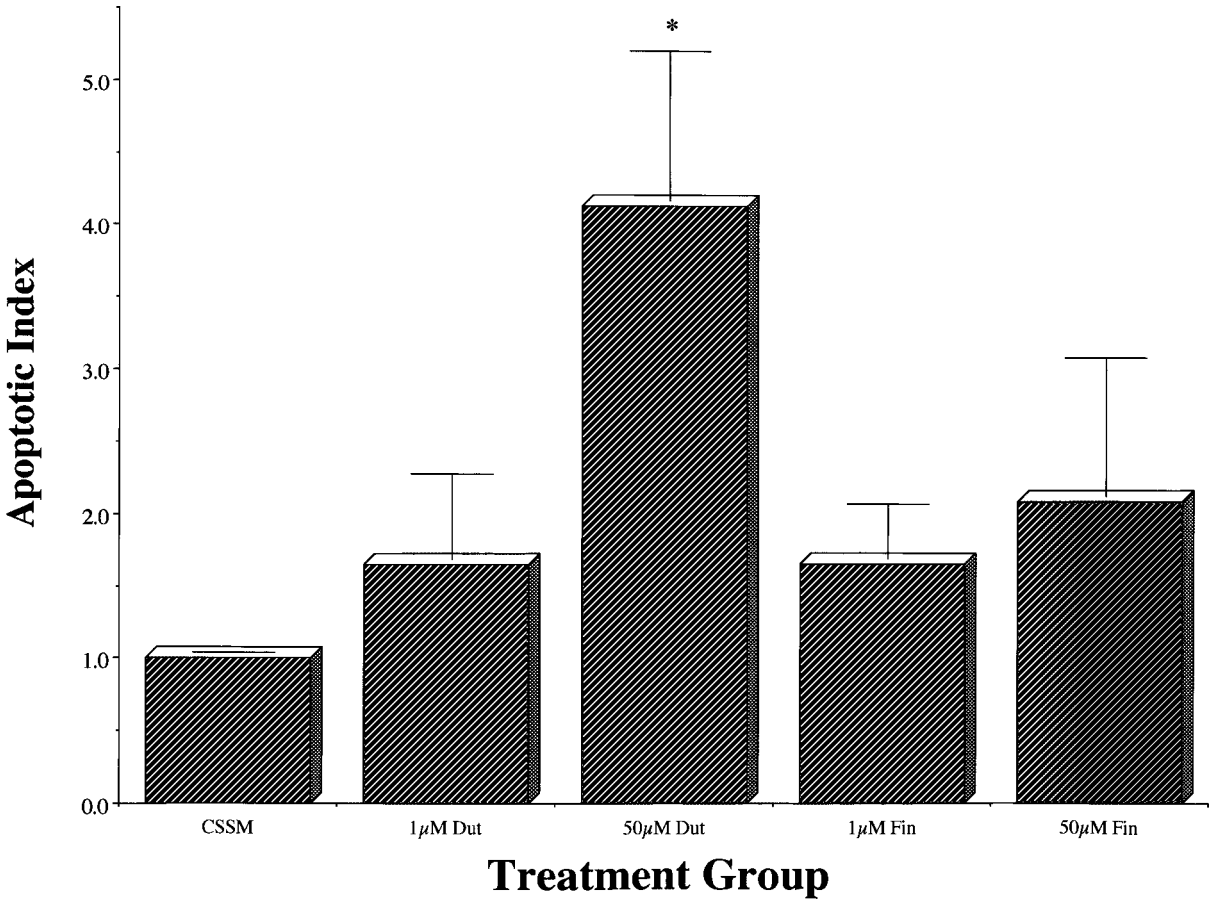


Fig. 6. Effect of dutasteride on cell death in LNCaP cells. LNCaP cell monolayers were incubated in CSSM alone or with 1 or 50 μM dutasteride (Dut) or finasteride (Fin) for 4 days. The medium was changed after 2 days. Cells and medium were harvested and assayed using the Roche Cell Death ELISA^{PLUS} assay as described in the "Materials and Methods." The results are the mean ± SEM for four to six separate experiments, each carried out in duplicate. *, Significantly different from the CSSM group ($P = 0.0013$).

any of a number of different sites ranging from transcription to the proteasome. Inhibition of PSA accumulation by dutasteride at doses of 1 μM and less could occur at transcriptional or posttranscriptional levels, including enhanced turnover or decreased secretion. At the transcription level, dutasteride bound to the AR might result in an AR conformation with altered binding to the androgen response elements in the large PSA promoter, or it might prevent correct recruitment of transcription coregulators and assembly of the transcription initiation complex [32,33].

Although antiandrogens and gonadotropin analogs are often used to decrease PSA production and inhibit cell proliferation in prostate cancer, in many cases progression of disease results in androgen-independent PSA production and cell growth [34]. This state sometimes reflects loss of AR, but there is growing evidence that unliganded-AR may play a role in PSA transcription in some cases. Many advanced prostate cancers still contain AR, which may be mutated in a number of different ways [35,36]. Possible stimuli for AR ligand-independent activity include PKA activators, IL-6, various growth factors, and differentiation agents such as butyrate [29,37,38]. It would be of interest to determine if dutasteride can still inhibit PSA production in androgen-independent prostate cancer cell lines which still contain AR, particularly in view of the recent report that several different androgen-refractory cell lines require AR for proliferation, even in the absence of androgens [39].

The effect of dutasteride to increase cell death occurs at considerably higher doses (10–50 μM) than its antiandrogenic effects (IC_{50} 0.5–1 μM). The cytotoxic effect may not be relevant clinically, because of the high concentration required. However, the mechanisms involved in the cell death are of interest, as are the structural and metabolic features of dutasteride that make it more active in this regard than finasteride. The pathway(s) involved in LNCaP cell death with 10–50 μM dutasteride have not been worked out but appear to involve phosphatidylserine externalization, as shown

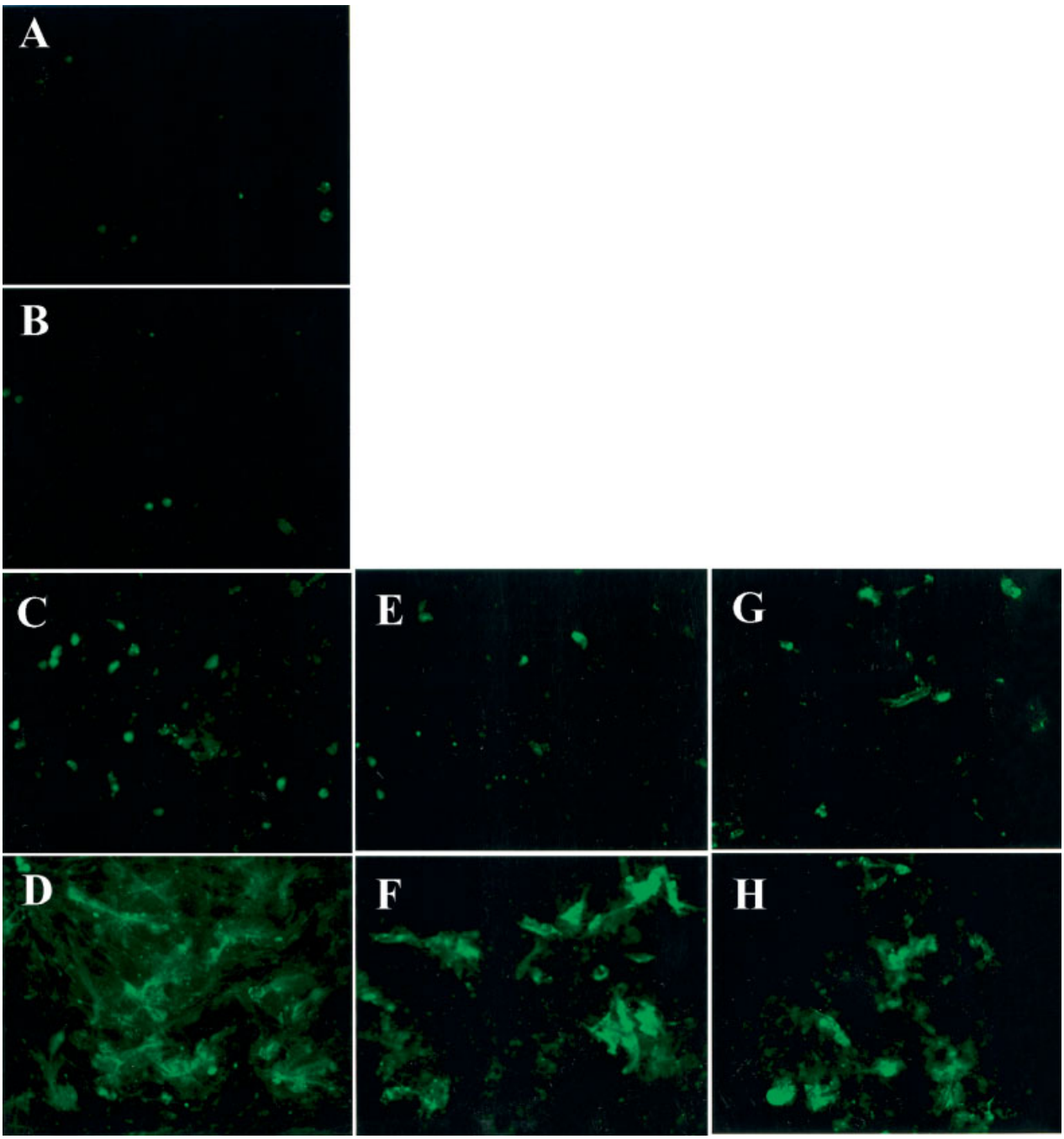


Fig. 7. Visualization of dying cells using AnnexinV-Fluos (Roche) staining. Cells grown on coverslips were incubated for 18 hr in CSSM alone (**A**) or with 1 μM dutasteride (**B**), 10 μM dutasteride (**C**), or 50 μM dutasteride (**D**), or with10 nM R1881 plus 10 μM dutasteride (**E**), or with 10 nM R1881 plus 50 μM dutasteride (**F**), or with 100 nM R1881 plus 10 μM dutasteride (**G**), or with 100 nM R1881 plus 50 μM dutasteride (**H**). The photographs of panels A–F are representative of results obtained in three to four independent experiments with at least six fields examined per group in each experiment. The photographs of panels G and H are representative of a single experiment with 10 fields examined per group.

by Annexin-V binding with the relative absence of propidium iodide staining, and DNA-histone release, as shown by the ELISA^{PLUS} assay. Although both of these observations suggest that apoptosis is the mechanism of cell death, we have been unable to demonstrate the involvement of caspase-3 or -8 activation after dutasteride treatment (data not shown). It is possible that a variant apoptotic pathway is activated [40].

LNCaP cells do not undergo apoptosis on androgen withdrawal, but do actively commence one or more particular apoptotic pathways on exposure to different

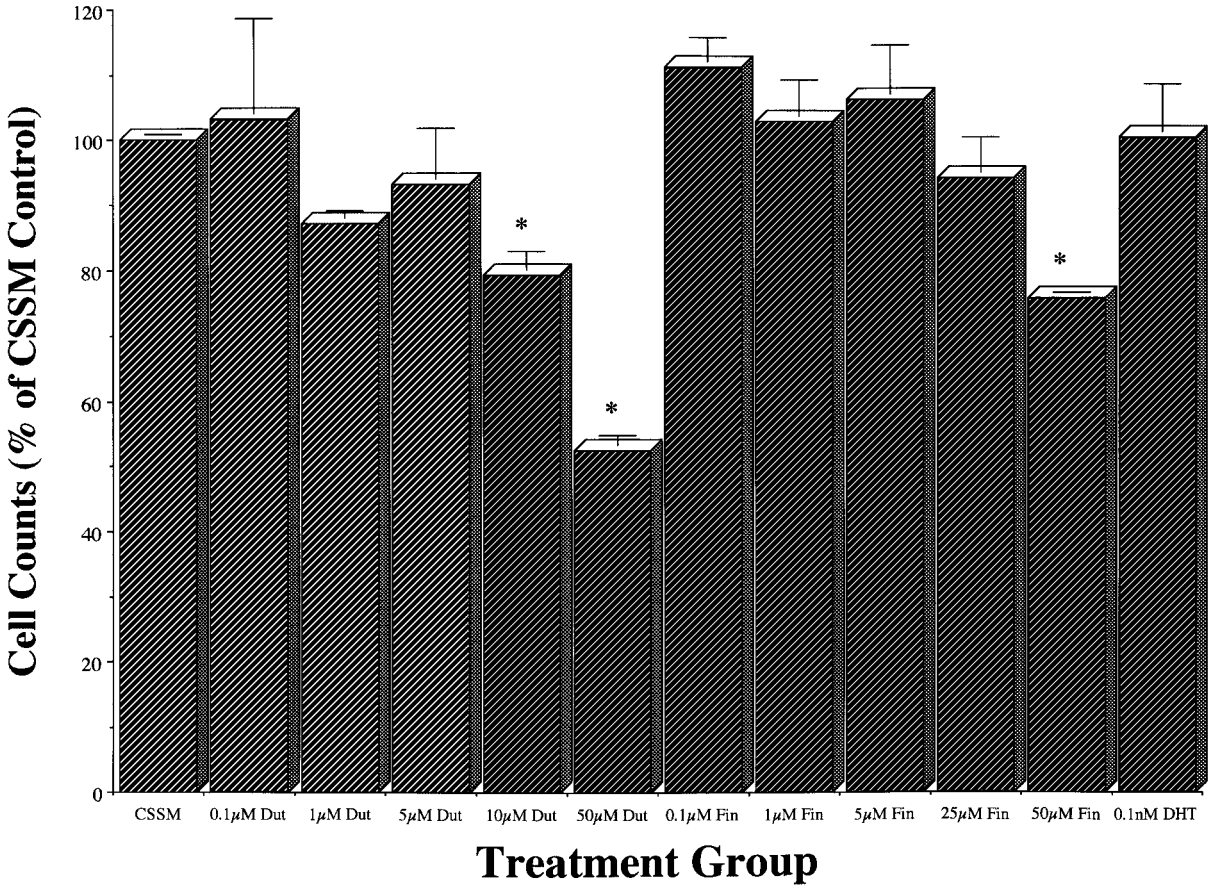


Fig. 8. Cell loss in PC-3 cells after incubation with high doses of dutasteride or finasteride. PC-3 cell monolayers were incubated for 9 days with CSSM alone or with CSSM plus increasing concentrations of dutasteride (Dut) or finasteride (Fin), or with 0.1 nM DHT. Cell number was determined using a Coulter counter. The results are given for three independent experiments, each carried out in duplicate. *, Significantly different from the CSSM group ($P \leq 0.05$).

agents, including cytokines such as TNFα, PI3 kinase inhibitors and other signaling cascade inhibitors, a wide variety of antitumor agents, or various nuclear receptor ligands such as calcitriol and some steroidal antagonists [25,41–43]. In the context of the present study, the apoptotic mechanisms of the latter group of compounds may be particularly relevant. Mifepristone, the glucocorticoid/progesterone antagonist, at 15 μM in LNCaP cells, promotes DNA fragmentation and downregulation of Bcl-2 typical of apoptosis. This seems unlikely to involve the glucocorticoid or progesterone receptor because apoptosis still occurs when these receptors are occupied by their native ligands [25]. With dutasteride, cell death occurs both in LNCaP and PC-3 cells, but the effects are partly blocked by androgen in the former, but not the latter, cell line. Muenchen et al. [44] have recently shown that PC-3 and LNCaP cell lines activate different apoptotic pathways in response to the same drug (docetaxel), illustrating the diversity of apoptotic control in the different prostate cancer cell lines.

CONCLUSIONS

In summary, although dutasteride and finasteride have inhibitory effects on T action in LNCaP cells that may be related to inhibition of 5αR, both drugs also inhibit DHT-induced PSA accumulation, which is obviously unrelated to 5αR. Dutasteride is more potent than finasteride in this regard and also has weak androgen agonist activity maximal at a concentration of about 1 μM. While the antiandrogenic effects of dutasteride may be related to its ability to bind the mutant LNCaP AR, higher concentrations of the drug in the absence of androgen promote loss of AR protein as well as cell death. In contrast, finasteride has no androgen agonist effects in the LNCaP model and has little effect on cell viability, even at a high concentration. It may be useful to determine if the antiandrogenic or cell death-promoting effects of dutasteride can be reproduced in animal prostate cancer models. Partial attenuation of tumor growth by 5αR inhibition has already been reported in PC-82 xenograft and Dunning

rat models [45]. This suggests that further studies should be carried out with dutasteride, with its unique advantage of potent dual 5αR inhibition and antiandrogenic actions.

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