Growth Inhibition of Human Prostate Cells in Vitro by Novel Inhibitors of Androgen Synthesis

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ABSTRACT

The long-standing strategy for the treatment of metastatic prostate cancer has been to reduce androgenic stimulation of tumor growth by removal of the tests, the primary site of testosterone synthesis. However, a low level of androgenic stimulation may continue, even after castration, by the conversion of adrenal androgens to 5α-dihydrotestosterone (DHT) in the prostate tumor cells. Two important enzymes of the androgen biosynthetic pathway are 17α-hydroxylase/C17,20-lyase, which regulates an early step in the synthesis of testosterone and other androgens in both the testes and adrenal glands, and 5α-reductase, which converts testosterone to the more potent androgen, DHT, in the prostate. We have identified new inhibitors of these enzymes that may be of use in achieving a more complete ablation of androgens in the treatment of metastatic prostate cancer. Three derivatives of androstane were shown to inhibit 17α-hydroxylase/C17,20-lyase with potencies 2-20-fold greater than that of ketoconazole, a previously established inhibitor of this enzyme. Derivatives of pregnane and pregnene displayed activities against 5α-reductase that were comparable to that of N-(1,1-dimethyl-ethyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide. All of the 5α-reductase inhibitors were able to at least partially inhibit the mitogenic effect of testosterone in either histocultures of human benign prostatic hypertrophic tissue or in cultures of the LNCaP human prostatic tumor cell line. For these compounds, it appears that this inhibition can be attributed to a reduction of DHT synthesis in these cultures, because no inhibitory effect was observed in DHT-treated cultures, and none of the compounds had a cytotoxic effect. Surprisingly, one of the inhibitors of 17α-hydroxylase/C17,20-lyase, 17β-(4-imidazolyl)-5-pregnen-3β-ol, was also able to inhibit the mitogenic effect of testosterone in both the histoculture and cell culture assays and had an effect against DHT as well. In transcriptional activation assays, it was found that this compound is an antagonist of both the wild-type androgen receptor and the mutant androgen receptor, which is present in LNCaP cells. In conclusion, the abilities of these compounds to inhibit androgen synthesis and, in some cases, to exert antianrogen activity, did in fact translate to an inhibitory effect on the growth of human prostatic tissue in vitro, suggesting their potential utility in the treatment of prostate cancer.

INTRODUCTION

The androgens testosterone and DHT play a key role in the maintenance of cell proliferation in the prostate gland. Accord-ingly, human prostatic tumors are often androgen dependent, and treatments designed to reduce androgenic stimulation of these tumors frequently produce therapeutic responses. Testosterone, the major circulating androgen in the human male, is synthesized primarily in the testes but also in the adrenal glands in much smaller amounts. A key regulatory enzyme of the testosterone biosynthetic pathway is steroidal 17α-hydroxylase/C17,20-lyase (EC 1.14.99.9), which catalyzes both the 17α-hydroxylation and the cleavage of the C17,20 side chain during the conversion of the 21-carbon steroids pregnenolone and progesterone to the 19-carbon androgens dehydroepiandrosterone and androsteronenedione, respectively (Fig. 1). It has been reported that a single active site on this enzyme catalyzes both reactions (2), and that the 17α-hydroxylase/C17,20-lyases present in human testes and adrenal glands have identical amino acid sequences (3). In the prostate, testosterone is converted by 5α-reductase to DHT (4), a more potent androgen (5, 6) which is apparently the primary trophic hormone of the prostate (7). The growth effects of DHT and testosterone on the prostate are mediated by the AR, a M, 120,000 protein (8) that is expressed in both the epithelial and stromal cells of both normal and cancerous prostatic tissue (9).

The beneficial effect of androgen ablation therapy in patients with metastatic prostate cancer was first established by Huggins et al. (10) in 1941 when they demonstrated that castration could often produce an improvement in clinical status. Castration, performed either surgically or medically by the administration of an luteinizing hormone-releasing hormone analogue, remains a standard treatment for metastatic prostate cancer. More recently, Labrie et al. (11) proposed that androgen ablation therapy could be substantially improved by administration of an AR antagonist to castrate patients to block the effects of residual androgens produced by the adrenal glands. The merit of this approach is supported by a large clinical trial conducted by the National Cancer Institute in which combination treatment with the luteinizing hormone-releasing hormone analogue leuprolide and the antiandrogen flutamide was compared to treatment with leuprolide only (12). Patients treated with the combination therapy had a longer median progression-free period (16.5 months versus 13.9 months) and longer median length of survival (35.6 months versus 28.3 months). In a small subset of patients in this study who had minimal disease at the initiation of therapy, the advantage of combination therapy was particularly impressive; the progression-free period was 58 months with combination therapy versus 19 months with monotherapy (13). The benefit of combination therapy was also observed in a similar study conducted by the European Organization for Research and Treatment of Cancer (14). Thus, efforts to block the effects of adrenal androgens apparently result in worthwhile therapeutic gains.

An inhibitor of steroidal 17α-hydroxylase/C17,20-lyase could potentially produce the same effect as the combination of leuprolide and flutamide because it could eliminate the contribution of both testicular and adrenal androgens to the growth of the neoplastic prostate. Ketoconazole, an imidazole antifungal agent, is one such inhibitor of this enzyme, and it has been used to treat metastatic prostate cancer. Some clinical success has been achieved with ketoconazole, both as a
MATERIALS AND METHODS

Materials

The LNCaP (human prostatic carcinoma) and CV1 (monkey kidney) cell lines were obtained from the American Type Culture Collection (Rockville, MD). Eagle's MEM without phenol red was prepared from MEM amino acid solution, MEM vitamins, and Earle's balanced salt solution and supplemented with penicillin/streptomycin/ampicillin B, all of which were purchased from Life Technologies, Inc. (Grand Island, NY). RPMI 1640, DMEM (with high glucose, without phenol red), trypsin/EDTA (0.25%/0.02%), and PBS were provided by Sigma Chemical Co. (St. Louis, MO) except where otherwise stated.

Candidate Inhibitors of Androgen Synthesis. L-133, L-34, flutamide, and ketoconazole were purchased from Sigma Chemical Co. 1-41, 1-47, and 1-49 were synthesized in our laboratory as described by Li et al. 6 L-10 was synthesized as described by Ling et al. 7 Finasteride and 4MA were kindly provided by Dr. Linda Rhodes of Merck Research Laboratories (Rahway, NJ). Hydroxyflutamide was kindly provided by Dr. Rudolph Neri of Schering Plough Research Institute (Kenilworth, NJ).

Preparation of Charcoal-stripped FBS

Steroids were removed from FBS by stirring with activated charcoal (4 mg/ml) for 16 h (all steps were performed at 4 °C), followed by centrifugation for 1 h at 1600 × g. The supernatant was then centrifuged for 1 h at 100,000 × g, and the resulting supernatant was either immediately sterilized by passage through a cellulose acetate filter with a 0.22-μm pore size (Corning Glass Works, Corning, NY) or, when preparing triple-charcoal-stripped serum, subjected to the above procedure two more times and then filtered.

Human Tissue Specimens

Fresh specimens of testis (from untreated prostatic cancer patients undergoing orchiectomy) and BPH tissue were kindly provided by Dr. Stephen Jacobs (Division of Urology, Department of Surgery, University of Maryland Medical Center). The preparation of microsomes from these tissues was performed as described by Li et al. (20).

Enzyme Inhibition Studies

17α-Hydroxylase/C17,20-Lyase Assay. Incubations were carried out in a total volume of 1.01 ml. Sample tubes were first supplied with propylene glycol (10 μl) and ethanol solutions containing [7-3H]pregnenolone (280,000 dpm), pregnenolone (0.063 μg; final concentration in incubation of 200 nM), and the indicated candidate inhibitors. The ethanol solutions were evaporated under air, and then 850 μl of 0.1 M sodium phosphate buffer (with DTT at 78 μM, pH 7.4) and a NADPH generating system (50 μl of phosphate substitute (15) and adjuvant (16) for castration. However, the majority of the clinical experience with this compound indicates that it has limited usefulness in the treatment of metastatic prostate cancer because of its relative lack of efficacy and specificity, undesirable side effects, and short plasma half-life, which necessitates an inconvenient three-times-daily dosing schedule (17). New, more potent inhibitors of 17α-hydroxylase/C17,20-lyase could potentially overcome these shortcomings of ketoconazole.

Alternatively, compounds that possess dual activity against 17α-hydroxylase/C17,20-lyase and 5α-reductase or against one of these enzymes and the human AR might also produce a therapeutic effect that is comparable to or greater than that produced by the leuprolide/flutamide combination. The success of combination therapy with flutamide and the 5α-reductase inhibitor finasteride in preliminary animal and human studies (18, 19) lends support to the concept that other combination therapies may also be of clinical use.

Here we report on the identification of novel inhibitors of 17α-hydroxylase/C17,20-lyase and 5α-reductase, some of which exhibit potencies against these enzymes that are comparable to or greater than that of ketoconazole and finasteride, and some which additionally display antiandrogen activity. Also presented are the antiproliferative effects of selected compounds on histocultures of human BPH tissue and on cultures of the human prostatic tumor cell line, LNCaP.
buffer containing 6.5 mM NADP⁺, 71 mM glucose-6-phosphate, 1.25 IU of glucose-6-phosphate dehydrogenase) were added to each tube, and the tubes were preincubated for 10 min at 34°C. The reaction in each tube was initiated by adding human testicular microsomes (100 μg of protein in 100 μl of phosphate buffer), and the incubation was carried out for 5 min under oxygen with shaking in a water bath at 34°C.

At the end of the incubation, authentic markers of 17α-hydroxyprogrenolone and dehydroepiandrosterone were added to each tube, the steroids were extracted with ether, and the ether evaporated. To remove unwanted lipids and particulate matter, which could interfere with the subsequent HPLC separation, the samples were resuspended in acetonitrile and drawn by suction sequentially through a C18 Sep-Pak Vac cartridge (100-mg mass; Waters Corp., Milford, MA) and a Teflon filter (0.22-μm pore size; Micron Separations, Inc., Westboro, MA). After evaporation of the acetonitrile, the samples were resuspended in 80 μl of acetonitrile:methanol (50:50), mixed with 80 μl of the HPLC initial mobile phase of water:acetonitrile:methanol (60:30:10), and injected into the HPLC system (Waters). HPLC was performed with a Nova-Pak C18 reverse phase column (Waters) and a total flow rate of 1 ml/min. Two pumps were used to produce the mobile phase: pump A, acetonitrile; and pump B, water:acetonitrile: methanol (60:30:10). For the elution of each sample, the initial condition of 1% pump A and 99% pump B was maintained for the first 15 min. Conditions were then changed as follows: from initial conditions at 15 mm to 32 mm at 15°C. A constant rate of change in pumping conditions was maintained over the course of any single transition. The UV absorbance at 216 nm and the radioactivity of each 0.1 ml of eluate were measured by an in-line spectrophotometer (Waters) and an in-line radioactivity flow detector (Packard Instrument Co., Meriden, CT), respectively. The UV absorption peaks of the authentic standards were used to identify the corresponding peaks of radioactivity. The 17α-hydroxyxylene was determined from the percentage of [7-3H]pregnenolone converted to [3H]17α-hydroxyprogrenolone and [3H]dehydroepiandrosterone. C17,20-lyase activity was determined from the percentage conversion of substrate to [3H]dehydroepiandrosterone.

5α-Reductase Assay. Incubations were carried out in a total volume of 1.01 ml. Ethanol solutions containing [7-3H]testosterone (600,000 dpm), testosterone (6.1 ng; final total concentration of testosterone in incubation of 30 nm), and the indicated candidate inhibitors were added to 0.01 ml propylene glycol in sample tubes. The ethanol was evaporated, and then 400 μl of 0.1 M sodium phosphate buffer (with DTT at 77 μM, pH 7.4) and a NADPH generating system (100 μl of phosphate buffer containing 6.5 mM NADP⁺, 71 mM glucose-6-phosphate, and 2.5 IU of glucose-6-phosphate dehydrogenase) were added to each tube; the tubes were then preincubulated for 15 min at 37°C. The reaction in each tube was initiated by adding human BPH microsomes (600 μg protein in 500 μl of phosphate buffer), and the incubation was carried out for 10 min under oxygen with shaking in a water bath at 37°C. At the end of the incubation, approximately 3000 dpm of [14C]DHT and 50 μg of nonradioactive DHT were added to each tube to serve, respectively, as a recovery standard and visualization marker for thin layer chromatography. Extracted steroids were then separated by thin layer chromatography (chloroform:chloroform:ether, 80:20) and visualized in iodine vapor. The DHT spot of each sample was scraped from the thin layer chromatography plate, the DHT was extracted with ether, the ether evaporated, and the extract was analyzed for radioactivity by liquid scintillation spectrometry. The 5α-reductase activity was determined from the percentage conversion of [7-3H]testosterone to [3H]DHT. IC₅₀ values were determined from plots of enzyme activity at four different concentrations of inhibitor (each sample in duplicate).

Histoculture

Human BPH tissue was collected in surgery and then washed in HBSS (Sigma) and divided into pieces 1–2 mm in diameter and 0.5–1.0 mm thick. Four to five pieces were placed on each of several absorbent gelatin sponges (Upjohn, Kalamazoo, MI). Each sponge was placed in a separate well of a 24-well plate and incubated (37°C, 5% CO₂) for 7 days in 1 ml of Eagle’s MEM (without phenol red, with penicillin, streptomycin, and amphotericin B) containing 5% charcoal-stripped FCS and the desired test compounds. (Testosterone, DHT and candidate inhibitors were delivered in ethanol vehicle; final ethanol concentration was 0.2%). The tissue pieces were then transferred on their sponges to a fresh 24-well plate and incubated for 3 days in a fresh preparation of the same medium/treatment containing 2 μCi/ml/well [3H]testosterone. The tissue pieces on each sponge were then transferred to a 1.5-ml microtube and incubated overnight at 37°C in 0.5 ml of collagenase solution [10 mM Tris (pH 7.8), 0.1 mg/ml collagenase, and 5 mM EDTA]. Next, tissue pieces were transferred to a fresh microtube tube and incubated for 2–3 h at 37°C in 0.5 ml of proteinase K solution [10 mM Tris (pH 10) and 1 mM EDTA] was added to each tube, and the contents were mixed gently until an emulsion was formed. The tube was then centrifuged at 1600 × g at room temperature for 15 min or until the organic and aqueous phases had separated well. Approximately 0.45 ml of the aqueous phase was transferred to a fresh microtube tube, and 0.45 ml of chloroform:isoamyl alcohol (24:1, v/v) was added. The tube was centrifuged at 12,000 × g for 15 min at room temperature, and then 0.4 ml of the supernatant was transferred to a fresh microtube tube and mixed vigorously with 0.04 ml of 3 M sodium acetate. This was followed by an addition of 1.25 ml of ice-cold ethanol, with gentle mixing. After storage of the tube at −70°C for at least 1 h, it was centrifuged at 12,000 × g at room temperature for 10 min. The pellet was washed in 70% ethanol, and the tube was centrifuged again. Finally, the pellet was resuspended in 0.4 ml of 10 mM Tris (pH 7.8) and 5 mM EDTA. One-half of the 0.4 ml was used for a spectrophotometric determination of DNA concentration (A₂₆₀), and the other one-half was used for measurement of the incorporation of [3H]testosterone into DNA by liquid scintillation spectrometry.

Growth Effects on LNCaP Cell Cultures

Cultures of the human prostatic cancer cell line LNCaP were maintained in RPMI 1640/5% FBS. Growth assays were performed in six-well plates (Nunc, Inc., Naperville, IL). LNCaP cells adhere only weakly to untreated tissue culture plastic. Thus, to enhance adherence, each well was exposed prior to seeding to 0.5 ml of a solution of poly-L-lysine (0.067 mg/ml of sterile double-distilled water) for 30 min; then the poly-L-lysine solution was removed, and the well was washed with 1 ml sterile water and allowed to dry completely. Growth assays were then initiated by seeding six-well plates with 60,000 LNCaP cells/well (passage number 20–28) in incubation medium (RPMI 1640/2% triple-charcoal-stripped FBS, with penicillin, streptomycin, and amphotericin B). After 48 h, the medium of each well was replaced with fresh incubation medium containing the inhibitors and androgens indicated in Fig. 4 (added as concentrated ethanol solutions). Control wells were treated with ethanol only (final concentration, 0.25%), which was shown in several previous experiments to have no effect on growth. Medium/treatments were changed every 3 days. After 9 days of treatment, cells were removed from the wells with trypsin/EDTA and counted in a Coulter counter (Coulter Electronics, Hialeah, FL).

Transcriptional Activation Assays

Agonist and antagonist properties of I-33, I-47, hydroxyflutamide, and finasteride with human wild-type AR and with the mutant AR with coding modifications. Briefly, CV1 cells (monkey kidney cells) were plated in DMEM containing 10% calf serum were added to each plate and incubated for 4.5 h at 37°C. DNA was precipitated in 4-ml aliquots and incubated for 20 mm at room temperature prior to the addition of 250 μl plate. Four ml of DMEM/10% calf serum were added to each plate and incubated for 4.5 h at 37°C. The medium of each plate was then replaced with DMEM/0.2% calf serum, and the new medium was incubated for 16 h at 37°C. Finally, plates were incubated for 30 h at 37°C with serum-free phenol red-free DMEM containing the indicated concentrations of inhibitors, with refreshment of the medium/treatment after 24 h. Cells were harvested in lysis buffer (Ligand Pharmaceuticals, San Diego, CA), and luciferase activity was determined by adding ATP and luciferin and...
RESULTS

Enzyme Inhibition. Compounds were first evaluated for their ability to inhibit 17α-hydroxylase/C_{17,20}^-lyase and 5α-reductase. Over 70 derivatives of pregnene or androstene were tested. The structures of compounds that exhibited strong inhibition of at least one of these enzymes are shown in Fig. 2, and their relative potencies are listed in Table 1. The inhibitory activities of the previously described 5α-reductase inhibitors finasteride and 4MA and the 17α-hydroxylase/C_{17,20}^-lyase inhibitor ketoconazole were also included in Table 1 to serve as standards for comparison. The compounds I-49, I-47, and I-41 all displayed greater inhibition of 17α-hydroxylase/C_{17,20}^-lyase than did ketoconazole, with I-49 producing an inhibition that was 20-fold more potent than that of ketoconazole. Compounds I-33, I-34, I-41, and L-10 were effective inhibitors of 5α-reductase. The relative potencies of I-33 and L-10 were similar to those of the Merck compounds, finasteride and 4MA. It is noteworthy that compounds I-34 and I-41 inhibit both 17α-hydroxylase/C_{17,20}^-lyase and 5α-reductase, indicating that they could potentially block androgen synthesis in all three tissues targeted in androgen ablation therapy: the testes, adrenals, and prostate.

Inhibition of DNA Synthesis in Androgen-treated Histocultures of Human BPH Tissue. Because the primary trophic hormone of the prostate is DHT rather than testosterone (7), we hypothesized that the compounds with activity against 5α-reductase might be able to inhibit the stimulatory effect of testosterone on DNA synthesis in prostatic tissue as a result of their inhibition of the conversion of testosterone to DHT. In addition, compounds possessing antagonist activity for the AR would inhibit the growth-stimulatory effect of both testosterone and DHT. Experiments were, therefore, conducted to determine the effect of the various inhibitors on the rate of DNA synthesis in androgen-treated histocultures of human BPH tissue (Fig. 3). In cultures treated with 1 μM testosterone only or 10 nM DHT only, the rates of DNA synthesis over a 3-day treatment period were, respectively, 2- and 3-fold that of cultures that were exposed only to the ethanol vehicle (data not shown). These control values served as reference standards for cultures treated with a combination of testosterone and inhibitor or DHT and inhibitor. Fig. 3 shows that at 1 μM, I-33, I-34, I-47, 4MA, and flutamide produced almost complete inhibition of the stimulatory effect of testosterone, and I-49 was partially effective. I-33 and 4MA were also highly effective at 0.3 μM, and I-34 was partially effective. Only I-47 and flutamide at 1 μM demonstrated a strong inhibition of DHT-stimulated DNA synthesis. None of the compounds had an independent effect on DNA synthesis in the absence of added androgens (data not shown). Thus, in no case was the inhibitory effect on DNA synthesis in androgen-stimulated DNA synthesis the result of a direct toxic effect of the compound.

![Fig. 3. Effect of compounds on DNA synthesis in histocultures of human BPH tissue cotreated with androgens. The ability of selected compounds to inhibit the stimulatory effect of 1 μM testosterone or 10 nM DHT on DNA synthesis in histocultures of human BPH tissue was tested as described in "Materials and Methods." Briefly, fresh human BPH tissue was cut into small pieces, placed on gelatin sponges in 24-well plates, and cultured for 7 days in phenol red-free MEM/5% charcoal-stripped FBS with the indicated medium/treatment of each well. The medium/treatment of each well was discarded, the tissue was digested, and DNA was extracted. For each sample, the incorporation of [3H]thymidine into DNA was normalized to DNA content and expressed as a percentage of the [3H]thymidine incorporation in all experiments; bars, SE. Each experiment was performed with BPH tissue from a different patient. Where no number is present, the column indicates the number of experiments in which that treatment was tested, and the data are represented as the means of the [3H]thymidine incorporation in all experiments; bars, SE. Each experiment was performed with BPH tissue from a different patient. Where no number is present, the column indicates the mean incorporation in duplicate wells of only one experiment. Flut., flutamide.](image-url)
The inhibition of testosterone-stimulated DNA synthesis by I-33, I-34, and 4MA may be attributable to their inhibition of 5α-reductase. These compounds failed to inhibit the stimulation of DNA synthesis in DHT-treated cultures (Fig. 3), ruling out the possibility that they are AR antagonists. I-47, however, had no activity against 5α-reductase (Table 1) and yet still inhibited testosterone-stimulated DNA synthesis. This suggests that I-47 has antagonist activity in addition to its activity against 17α-hydroxylase/C17-20-lyase, a possibility which is supported by its almost complete inhibition at 1 μM of DHT-stimulated DNA synthesis in histoculture. Of the tested compounds, only flutamide, a well-known antagonist of the AR, also had effect against DHT.

**Inhibition of Growth in Androgen-treated Cultures of the LNCaP Human Prostatic Cancer Cell Line.** The compounds effective at reversing the stimulatory effect of testosterone in histocultures of human BPH tissue were further tested in a similar assay using the LNCaP human prostatic tumor cell line (22). Cells were cultured for 9 days in a steroid-free medium (RPMI 1640/2% triple-charcoal-stripped serum) so that the maximal stimulatory effect of androgen treatment on cell growth could be observed. In preliminary experiments (data not shown), it was determined that the concentrations at which testosterone and DHT produce their optimal effects on the stimulation of LNCaP growth are 0.1 and 0.03 nM, respectively, and therefore, these were the concentrations used in all subsequent experiments. The lower concentrations of androgens required to stimulate LNCaP cell cultures as opposed to histocultures of human BPH tissue may result from greater access of the compounds to the cell culture monolayers.

Testosterone at 0.1 nM stimulated cell proliferation by approximately 2-fold compared to control cultures treated with drug vehicle only (Fig. 4A). At a concentration of 5 μM, I-47, I-49, L-10, finasteride, and 4MA all completely reversed the stimulatory effect of testosterone on cell proliferation. I-47, finasteride, and 4MA were the most effective compounds, all producing at 1 μM at least a 50% reduction in the growth effect of testosterone. The compounds I-33, I-34, and hydroxyflutamide, which is the potent metabolite of flutamide in vivo (23), were not only ineffective at reversing the action of testosterone but actually contributed their own stimulatory effects on growth, all of which were much stronger than that of testosterone. This may be attributable to a previously described mutation of the AR of LNCaP cells (24) that increases their responsiveness to progestins (see “Discussion”).

DHT at 0.03 nM stimulated LNCaP growth by 2.8-fold (Fig. 4B), and again, I-47, I-49, L-10, finasteride, and 4MA were all at least partially effective at reversing this stimulatory effect. At a concentration of 1 μM, all of these compounds reduced the growth effect of DHT by more than 50%.

To determine whether any of the above compounds reverse the growth-stimulating effects of testosterone or DHT on LNCaP cells by exerting a direct toxic effect, a cytotoxicity assay was performed. The number of cells in LNCaP cultures treated for 9 days with candidate inhibitors at concentrations of up to 20 μM, but not with added androgens, were compared with the cell number in control cultures treated only with drug vehicle (Fig. 5). The data show that only I-49 is toxic to LNCaP cells in this assay system and only at concentrations above 2.5 μM. I-47, finasteride, and 4MA have no independent effect on LNCaP growth; thus, their ability to reverse the growth stimulation produced by testosterone and DHT does not result from their intrinsic toxicity. At a concentration of 1 μM, LNCaP growth was increased 9-fold by hydroxyflutamide and, interestingly, 2.5-fold by L-10. Thus, although L-10 is by itself mitogenic, it has an overall negative effect on growth when administered in combination with testosterone or DHT in the LNCaP system (compare with Fig. 4).

The effectiveness of the 5α-reductase inhibitors L-10, finasteride, and 4MA in reversing the effect of testosterone on LNCaP growth was expected, due to their inhibitory effect on DHT synthesis. However, as in the histoculture experiments, it was unexpected that I-47, which

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**Table 1. Growth inhibition of LNCaP cells by candidate compounds.**

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<thead>
<tr>
<th>Compound</th>
<th>Inhibitory Effect</th>
<th>IC50 (μM)</th>
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<tr>
<td>T (0.1 nM)</td>
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<tr>
<td>DHT (0.03 nM)</td>
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<td>Testosterone</td>
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<tr>
<td>DHT</td>
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<tr>
<td>I-33</td>
<td>Approximately 2</td>
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<tr>
<td>I-34</td>
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<td>I-47</td>
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<td>I-49</td>
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<td>L-10</td>
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<td>Finasteride</td>
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<tr>
<td>OH-FL</td>
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8 Note that cell cultures were treated with hydroxyflutamide (Figs. 4–6), whereas histocultures were treated with flutamide (Fig. 3).
producing a 46% reduction in transcriptional activity at a concentration of 0.1 μM. However, unlike I-47, neither I-33 nor hydroxyflutamide behaved as antagonists of the LNCaP AR. Interestingly, although finasteride did not show an antagonist effect on the wild-type AR, it did antagonize the LNCaP AR in a dose-dependent manner similar to that of I-47; both compounds reduced transcriptional activity by approximately 40% at 0.3 μM and by 50% at 1 μM.

In an assay for agonist activity using the wild-type AR, none of the compounds induced transcriptional activity to more than 25% of the level of activity induced by 0.1 nm DHT (Fig. 6B). In cultures transfected with the LNCaP AR sequence, however, I-33 and hydroxyflutamide displayed agonist activity that was similar, and, at some concentrations, greater than that of DHT. This agonist effect explains their lack of antagonism against LNCaP AR in the previous assay (Fig. 6A). I-47 and finasteride generally displayed minimal agonist activity with the LNCaP AR, with the exception of finasteride at 0.3 μM, which produced an agonist effect that was 46% of the

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**Fig. 5.** Cytotoxicity assay of compounds in LNCaP cell cultures. LNCaP cells were seeded into six-well plates at a density of 180,000 cells/well. Beginning 48 h later, each of the indicated compounds, at concentrations ranging from 0.3—20 μM, was applied to duplicate wells for 9 days in RPMI 1640/2% triple-charcoal-stripped FBS, with medium/treatment changes every 3 days. In contrast to the experiments presented in Fig. 4, there was no co-addition of testosterone or DHT in this assay. Control wells were treated with ethanol vehicle only (final concentration, 0.25%). At the end of the 9-day treatment, cells were removed from the wells with trypsin/EDTA and counted in a Coulter counter. The mean cell number of duplicate wells of each treatment, expressed as a percentage of the mean cell number in the control wells, is plotted versus inhibitor concentration; bars, SE. Similar results were obtained in a repeat experiment. OH-FL, hydroxyflutamide.

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**Fig. 6.** Antagonist and agonist effects of compounds on the wild-type and LNCaP AR. CV1 cells were transiently transfected with either wild-type human AR expression vector DNA, pCMVhAR, or an expression vector with the mutant human AR coding sequence present in LNCaP cells, pCMVLNCaPAR. Cells were also transfected with a reporter vector comprised of the luciferase coding sequence under the control of the androgen response element of the mouse mammary tumor virus promoter. Stimulation of AR-mediated transcriptional activity by the various treatments was determined using a luminescence assay as described in “Materials and Methods.” A, antagonist effects of the compounds were assessed by comparing transcriptional activities observed in cultures treated with 0.1 nm DHT with or without the indicated concentrations of compounds. The data represent the means of 3—5 experiments; bars, SE. Included in each experiment as negative controls were cultures not treated with DHT and transfected with either wild-type pCMVhAR, pCMVLNCaPAR, or the parent expression vector lacking AR sequence, pCMV5 (p5). B, agonist effects were determined by comparing the transcriptional activity in cultures treated with the indicated compounds (without cotreatment with DHT) with the activity in cultures treated with 0.1 nm DHT. The data represent the means of three experiments; bars, SE. For the agonist experiments, the relative transcriptional activities observed in the negative control cultures were similar to those presented in A and are not shown. WT, wild-type; OH-FL, hydroxyflutamide; Finast, finasteride.
magnitude of the effect of DHT. In summary, I-33 and hydroxyflutamide are antagonists of the wild-type AR but are agonists of the LNCaP AR, whereas I-47 is an antagonist of both receptors. At concentrations up to 5 μM, finasteride is an antagonist of the LNCaP AR but not of the wild-type AR. Although it inhibits the stimulatory effect of DHT on the LNCaP AR, finasteride itself exerts a small agonist effect on the LNCaP AR (Fig. 6B).

**DISCUSSION**

It has been recognized that adrenal androgens may play an important role in the progression of prostatic tumors since the early 1940s, when Huggins and Scott first performed bilateral adrenalectomies on patients who had relapsed after an initial response to castration therapy (25). Thirty years later, Harper et al. (26) demonstrated that dehydroepiandrosterone and androstenedione, the major adrenal androgens, are in fact metabolized in the prostate to other androgens, including DHT. Although this study showed that the percentage of adrenal androgens converted to DHT is low, the authors proposed that the quantities of DHT produced in the prostate in this way are physiologically significant because of the high plasma concentration of dehydroepiandrosterone. The influence of adrenal androgens has been confirmed in the past few years by two large clinical trials that demonstrated the therapeutic advantage of maximal androgen blockade therapy (12–14) in which castration is supplemented with an antiandrogen to block the effects of adrenal androgens. The importance of maximal androgen ablation has been further verified in a recent study by Visakorpi et al. (27) in which AR gene amplification was found in 7 of 23 human prostatic tumors that had occurred during androgen deprivation therapy. This suggests that some prostatic tumors recur not by becoming truly androgen independent but rather by undergoing an adaptation that permits them to respond to very low levels of androgen.

These findings have prompted our efforts to develop new inhibitors of 17α-hydroxylase/C17,20-lyase and 5α-reductase. Compounds with activity against 17α-hydroxylase/C17,20-lyase may be of particular value in that they can potentially suppress the synthesis of both testicular and adrenal androgens. Inhibitors of 5α-reductase may be useful when used in combination with treatments designed to reduce extraprostatic androgen synthesis, since these treatments usually fail to produce 100% inhibition. By preventing the conversion to DHT of the small amount of androgens that remain in spite of these treatments, 5α-reductase inhibitors may be able to produce a more complete reduction of the androgenic stimulation of prostatic tumor cells.

We have identified several compounds with activity against these enzymes. I-33, I-34, I-41, and L-10, as well as the reference compounds finasteride and 4MA, all displayed activity against 5α-reductase (Table 1). Because DHT has a stronger mitogenic effect on the prostate than does testosterone, it was expected that all of these compounds would be able to diminish the stimulatory effect of testosterone on histocultures of human BPH tissue by virtue of their abilities to block the conversion of testosterone to DHT. Of the compounds that were tested in the histoculture assay (L-10 and finasteride were not tested), all did in fact at least partially reduce the mitogenic effect observed in testosterone-treated cultures (Fig. 3). The compounds L-10, finasteride, and 4MA produced a similar effect in testosterone-treated cultures of LNCaP cells. At a concentration of 5 μM, these compounds caused almost complete reduction of the growth effect of testosterone (Fig. 4A). This result is not entirely unexpected, given the fact that the AR of LNCaP cells contains a mutation at amino acid 868 in the ligand-binding domain (24) that increases the affinity of the receptor for estrogens, progesterins, and even some antiandrogens (24, 28). Moreover, this increased binding affinity is associated with a greatly enhanced agonist effect on the receptor, even for the antiandrogens. It is well documented that the growth rate of LNCaP cells can be stimulated by a variety of steroidal and nonsteroidal compounds, such as estradiol, progesterone, and the antiandrogens cyproterone acetate, nilutamide, and hydroxyflutamide (28–31). Activation of the LNCaP AR can apparently account for the growth effect on LNCaP cells of some of these compounds, but not all. Although several earlier studies had failed to detect steroid receptors other than the AR in LNCaP cells (32–34), it has recently been reported that both estrogen and progesterone receptors are present in LNCaP, and furthermore, that the mitogenic effect of estradiol on these cells is mediated by their estrogen receptors (35). Unlike the mechanism of estradiol stimulation, however, it remains unclear whether the stimulation of LNCaP growth by progesterone is mediated by progesterone receptors or by the mutant AR of LNCaP or by a combination of both. Using a transcriptional activation assay, we have verified that I-33 is an agonist of the LNCaP AR (Fig. 6B). Although we have not tested I-34, it is likely that it is also an agonist of the LNCaP AR, as it differs from I-33 only in the orientation of the hydroxyl group at the C20 position. Thus, the stimulation of LNCaP growth by I-33 and I-34 may be attributable, at least in part, to an agonist effect on the LNCaP AR.

An interesting aspect of the LNCaP experiments is that L-10 and the reference compounds finasteride and 4MA were also able to reduce the mitogenic effect not only of testosterone but also of DHT in LNCaP cultures (Fig. 4B), suggesting that these compounds may possess antagonist activity for the LNCaP AR. Transcriptional activation assays verified that finasteride is an antagonist of the LNCaP AR but not of human wild-type AR (Fig. 6). The lack of antagonist activity for the human wild-type AR in our assay is consistent with the previous finding by Liang et al. (36) that finasteride does not bind the AR of rat prostatic cytosol, because the rat and human AR have identical hormone-binding domains (8). However, to our knowledge, it has not been reported previously that finasteride is an antagonist of the LNCaP AR. Because finasteride is not toxic to LNCaP cells at concentrations up to 20 μM (Fig. 5), this antagonist effect probably accounts for the effectiveness of finasteride in diminishing the mitogenic effect of DHT in LNCaP cultures. This antagonist activity probably also contributes, together with the inhibition of 5α-reductase, to the effectiveness of finasteride against testosterone (Fig. 4A).

Whether this is also true for 4MA and L-10 remains to be determined. 4MA has been shown previously to bind to rat prostate AR (35), but it has not yet been determined whether 4MA and L-10 are antagonists of the LNCaP AR.

As a 5α-reductase inhibitor, I-47 has minimal activity, and I-49 has not been tested. However, both of these compounds are potent inhibitors of 17α-hydroxylase/C17,20-lyase. Although these compounds were not expected to reduce the mitogenic influence of testosterone on the prostate, they were tested in the histoculture assay and the LNCaP cell culture assay to determine if they might have antagonist activity. I-47 was effective against both testosterone and DHT in the histoculture assay, whereas I-49 was ineffective against DHT and was only partially effective against testosterone. This suggested that I-47 could be an antagonist. The data for I-49 was harder to interpret because of the lack of effect against DHT. In the LNCaP assays, I-47 continued
to exhibit the characteristics of an antagonist, and I-49 also displayed antagonistic properties, because both compounds were effective against both testosterone and DHT. However, it was subsequently shown that the behavior of I-49 could be explained by its cytotoxic effect on LNCaP cells at concentrations above 2.5 μM (Fig. 5). In contrast, I-47 is not toxic, and transcriptional activation assays confirmed that it is an antagonist of both the wild-type AR and the LNCaP AR.

A wide variety of compounds that are agonists of the LNCaP AR, but not of the wild-type AR, will stimulate the growth of LNCaP cells (29-31). If tested only in this system, compounds that would have a negative effect on the growth of prostatic tumor cells containing a wild-type AR would be erroneously classified as having either no effect or possibly even a stimulatory effect on growth if they happen to activate the LNCaP AR (e.g., flutamide). However, the LNCaP system is not without use. LNCaP is the only androgen-responsive human prostatic tumor cell line that will grow continuously in culture. The mutant AR responsible for its aberrant hormonal responsiveness has been found in a number of other human prostatic tumors (37-39).

One study reports finding this mutation in the tumors of 6 of 24 prostate cancer patients (38). With the LNCaP system, the opportunity exists to test whether a candidate drug would stimulate the growth of this subset of tumors. The importance of this concept is evident when one considers the phenomenon of the flutamide withdrawal syndrome. Patients who have relapsed after an initial period of positive response to the combination therapy of castration and flutamide often experience significant clinical improvement upon the discontinuation of flutamide (40-42).

It has been proposed that in these patients, the prolonged exposure to flutamide has resulted in the selective proliferation of tumor cells containing a mutant AR, such as the LNCaP AR, that recognizes flutamide and its metabolites as agonists (40). The withdrawal of flutamide from these patients would, therefore, equate to removal of a tumor growth factor. Thus, in the evaluation of new compounds as potential treatments for prostatic cancer, an assessment of their effects on the LNCaP AR or on the growth of LNCaP cells clearly now has new relevance. In this regard, I-47 may offer an advantage over hydroxyflutamide. Although hydroxyflutamide is somewhat more potent than I-47 as an antagonist of the wild-type AR, I-47 is also an antagonist of the LNCaP AR, whereas hydroxyflutamide is actually an agonist of this mutant receptor. For the treatment of prostatic cancer patients with tumors that possess the LNCaP AR, which may be a substantial percentage (38), a compound that is an antagonist of both the LNCaP AR and the wild-type AR, such as I-47, may be preferable to hydroxyflutamide. Relapsed patients who experience a positive response to the withdrawal of flutamide from their treatment regimen might further benefit from the concurrent addition of a compound such as I-47.

Another clinical situation in which a compound such as I-47 may find special use is in the treatment of prostatic tumors that display an increased responsiveness to adrenal androgens and their metabolites. Recent studies have identified specific mutations in the AR of human prostate tumors that confer this increased responsiveness (43, 44). If these mutations do indeed contribute to the relapse of some patients during androgen deprivation therapy, an inhibitor of adrenal androgen synthesis may be useful in reestablishing disease remission.

In conclusion, we have identified several compounds that could potentially achieve a more complete ablation of androgens in patients with metastatic prostate cancer. The compound I-47 is particularly promising in that it has the potential to inhibit testosterone synthesis in both the testes and adrenal glands and also to exert an androgenic effect in tumors containing the LNCaP AR as well as the wild-type AR. I-47 may be useful in the treatment of tumors containing the LNCaP AR or a similar mutant AR that recognizes hydroxyflutamide as an agonist. Alternatively, by helping to produce a more thorough androgen ablation, I-47, and possibly also I-41 and L-10, may prove useful in the treatment of tumors that may have recurred as a result of AR overexpression, which allows them to respond to very low levels of androgens, or as a result of an AR mutation, which enhances their mitogenic response to adrenal androgens. The data and screening strategy presented here demonstrate the importance of evaluating potential new treatments by several methods, including enzyme assay, histoculture of patient tissue, cell culture, and transcriptional activation assay with both wild-type and mutant AR.

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Growth Inhibition of Human Prostate Cells \textit{in Vitro} by Novel Inhibitors of Androgen Synthesis

Gregory T. Klus, Junji Nakamura, Ji-song Li, et al.


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