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Pregnenolone stimulates LNCaP prostate cancer cell growth via the mutated androgen receptor

Dmitry N. Grigoryev [1], Brian J. Long, Vincent C.O. Njar, Angela H.M. Brodie *

Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Health Science Facility, 685 W. Baltimore Street, Baltimore, MD 21201, USA

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Abstract

Pregnenolone (P_5), a common precursor of many steroids, is present in the blood of normal adult men at concentrations of 1–3 nM. In vitro, P_5 was found to stimulate LNCaP-cell proliferation 7–8-fold at a physiological concentration (2 nM), and 3–4-fold at a subphysiological concentration (0.2 nM). Growth stimulation at the 2-nM concentration was comparable with that of the androgen, dihydrotestosterone at its physiological concentration (0.5 nM; 9–10-fold increase in cell number). To determine whether P_5 or its metabolites were mediating this growth response, LNCaP cells were incubated with [^{3}H]P_5 and high-performance liquid chromatography (HPLC) was performed. After a 48-h exposure, two unidentified metabolites were detected. Although, the P_5 metabolites slightly increased LNCaP-cell growth in vitro, their effect was significantly less than P_5 alone, suggesting that the growth stimulation was mediated by P_5 itself. We further showed that P_5 sustained its proliferative activity in vivo and stimulated the growth of LNCaP-tumor xenografts in intact male SCID mice as well as in castrated animals. In order to determine whether P_5 was binding to a specific site in LNCaP cells, receptor binding studies were performed. Scatchard analysis predicted for a single class of binding sites with $K_d = 1.4$ nM. Studies were performed to determine the effects of P_5 on transcription mediated by wild-type and LNCaP androgen receptors. P_5 was shown to activate transcription through the LNCaP androgen receptor (AR), but not the wild-type AR. This implies that P_5 most likely stimulates LNCaP-cell proliferation through binding to the cellular mutated AR present in LNCaP cells. We have also demonstrated that drugs designed to be antagonists of the androgen, progesterone and estrogen receptors, and one of our novel compounds designed to be an inhibitor of androgen synthesis, were potent inhibitors of the AR-mediated transcriptional activity induced by P_5, and were able to inhibit LNCaP-cell proliferation. These findings suggest that some prostate cancer patients who appear to become hormone-independent may have tumors which are stimulated by P_5 via a mutated AR and that these patients could benefit from treatment with antiestrogens, antiprogestins, or with some of our novel androgen synthesis inhibitors.

Keywords: Pregnenolone; LNCaP prostate cancer cell line; Androgen receptor; Novel androgen synthesis inhibitors; Growth inhibition

1. Introduction

LNCaP cells are prostatic cancer cells derived from a patient with lymph-node metastases. LNCaP cells are the only androgen receptor (AR) positive prostate cancer cell line that can be readily grown in tissue culture [1]. These cells respond to androgens with increased cell proliferation and elevated expression of prostate-specific antigen (PSA) [2]. Although LNCaP cells have a mutated AR [3], this cell line has been used extensively in research on the causes, treatment, and prevention of prostate cancer [4]. In our laboratory, we have been utilizing LNCaP cells for evaluating novel inhibitors of

Abbreviations: ACN, acetonitrile; AR, androgen receptor; DHEA, dehydroepiandrostene; DHT, dihydrotestosterone; DPBS, dulbecco's phosphate-buffered saline; DTT, dithiothreitol; ER, estrogen receptor; FBS, fetal bovine serum; HPC, hydroxypropyl cellulose; HPLC, high-performance liquid chromatography; P_5, pregnenolone; PAGE, polyacrylamide gel electrophoresis; PR, progesterone receptor; P/S, penicillin/streptomycin; P_5-S, pregnenolone-sulfate; PSA, prostate-specific antigen; *r.t.*, retention time; SDS, sodium dodecyl sulfate; TRA, triamcinolone acetonide.

* Corresponding author. Tel.: +1-410-7063137; fax: +1-410-7060032.

E-mail address: abrodie@umaryland.edu (A.H.M. Brodie).

[1] Present address: Department of Veterans Affairs Medical Center, Baltimore, MD, USA.

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androgen synthesis. During these studies, it was found that the growth of LNCaP cells can be stimulated by physiological levels of the major androgen substrate, P_5 [5]. There have been previous reports on the effects of a number of steroids on LNCaP-cell growth [5–9], and significant increases in cell growth have been observed in the presence of estrogen and progesterone. In a publication by Sonnenschein et al. [8], some minor stimulation by P_5 of proliferation of LNCaP cells grown (1% of maximum stimulation with testosterone) in medium supplemented with human serum was noted, but data was not included. It was also reported that progesterone and the Δ5-steroid dehydroepiandrostene (DHEA) interact with the LNCaPAR that has been transfected into CV-1 cells, and activate transcription from the AR-response element of the reporter vector [9]. These effects were attributed to the missense mutation in the LNCaPAR [3]. Subsequently, amino acid substitutions were described in organ-confined as well as metastatic tumors [10]. Culig et al. [11] reported that a mutant AR, detected in advanced-stage prostatic carcinoma, is activated by adrenal androgens and progesterone. In addition, mutated human ARs were detected in a prostatic-cancer patient that were activated by estradiol [12]. Of the several other AR mutants that have been characterized in prostate-cancer patients so far, the majority are functional and can mediate androgen-induced cell growth [13–16].

In this report, we describe finding that P_5 stimulates LNCaP-cell proliferation at physiological concentrations. Although P_5 is readily available to tissue, it is generally considered to be an inert steroid and its role is thought to be confined to that of a precursor for conversion to other steroids. This is the first report that physiological concentrations of P_5 stimulate LNCaP-tumor-cell growth. We determined that P_5 stimulates cell proliferation through binding to the mutated LNCaPAR and that this receptor has an unusually high affinity for P_5. Given that mutations in the AR are considered to be relatively common in prostate cancer patients, these findings may have important implications for the treatment of the disease.

2. Materials and methods

2.1. Chemicals and biochemicals

The antiprogesterone triamcinolone acetonide (TRA) was purchased from Sigma Chemical Co. (St Louis, MO). The antiandrogen casodex and the antiestrogen ICI 182,780 were kindly supplied by Zeneca Pharmaceuticals Inc. (Macclesfield, England). VN/63-1, an inhibitor of androgen synthesis with high antiandrogenic activity, was synthesized in our laboratory [17].

[7-^{3}H]Pregnenolone (19.9 Ci/mmol), [1,2,6,7-^{3}H]dehydroepiandrosterone (83.2 Ci/mmol), [1,2,6,7-^{3}H]testosterone (96.5 Ci/mmol), and metribolone [17α-methyl-^{3}H]-R1881 ([^{3}H]-R1881, specific activity 86 Ci/mmol) were obtained from DuPont NEN (Boston, MA). [7-^{3}H]Progesterone (23.1 Ci/mmol), and [7-^{3}H]17α-hydroxypregnenolone (11.2 Ci/mmol) were purchased from Amersham, Co. (Arlington Heights, IL).

The steroids, pregnenolone (P_5), pregnenolone-sulfate (P_5-S), progesterone, dihydrotestosterone (DHT), dexamethasone, estradiol, and the solvent hydroxypropyl cellulose (HPC), were purchased from Sigma. High-performance liquid chromatography (HPLC)-grade chloroform, acetonitrile, water, and methanol were purchased from J.T. Baker, Inc. (Phillipsburg, NJ). VN-63 was prepared in our laboratory [7]. Casodex and ICI 182,780 was kindly provided by Dr Alan Wakeling (Arstra Zeneca, Maccelsfield, UK). Ketoconazole was a gift from Professor Y-Z Ling, Peking University, Beijing, China.

LNCaP and PC-3 human prostate cancer-cell lines, green monkey kidney CV-1 cells, and MCF-7 breast cancer-cell line were obtained from the American Type Culture Collection (Rockville, MD). RPMI 1640 medium, trypsin/EDTA (0.25/0.02%), penicillin/streptomycin (P/S), geniticin (G418), and LipofectAMINE-Plus were from Gibco-BRL (Grand Island, NY). Phenol red free IMEM and trypsin/versine were purchased from Biofluids Inc. (Rockville, MD). Fetal bovine serum (FBS) was obtained from Summit Biotechnology Inc. (Fort Collins, CO) and steroid-free FBS was prepared, as described previously [18]. Matrigel was generously supplied by Dr Hynda Kleinman (NIH, Bethesda, MD).

The luciferase reporter construct pMAM*neo*LUC was purchased from Clontech (Palo Alto, CA). The luciferase assay kit was from Promega (Madison, WI). Wild-type human androgen receptor (AR) expressing vector pCMV5-hAR, and the LNCaPAR expressing vector pCMV5-LNCaPAR were generous gifts from Dr Elisabeth Wilson (University of North Carolina).

2.2. Cell-growth studies

LNCaP, PC-3, and MCF-7 cells were transferred into steroid-free medium 3 days prior to the start of all experiments. Steroid-free medium consisted of phenol red-free IMEM supplemented with 5% steroid-free FBS and 1% P/S. Cells were then plated into 24-well culture plates (15 000 cells/well) in 1 ml of the same medium. After a 24-h attachment period, the vehicle (ethanol) or selected steroids (Fig. 1A, B) at concentrations of $\times 0.1 \times 1 \times 10$-times their physiological plasma concentrations [18–20] were added to triplicate wells. Medium and treatments were changed every 72 h. After 9 days of treatment, the cells were removed with trypsin/

EDTA and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). The effects of different antagonists (5 μM) of steroid receptors on P_5-stimulated LNCaP-cell growth were evaluated by the same method.

2.3. HPLC

LNCaP cells, prepared as described above, were plated into 6-well plates at a density 5×10^5 cells/well and exposed to [7-^{3}H]P_5, (specific activity 19.9 Ci/mmol) for 8, 24, 48, and 72 h. The medium and cells were then collected and steroids were extracted with 2×2 volumes of chloroform. After evaporation of the chloroform, the extracts were dissolved in acetonitrile (ACN) and filtered through a C_{18} Sep-Pak Vac cartridge (Waters Corp., Milford, MA) conjugated with a Teflon filter (0.22 mm, Micron Separation, Inc., Westboro, MA). Solvents were evaporated from the filtrates and analyzed by HPLC, as described previously [21]. Briefly, after evaporation of CAN, the samples were resuspended in 80 μl of ACN:methanol (50:50), mixed with 80 μl of the HPLC initial mobile phase of water:ACN:methanol (60:30:10), and injected into the HPLC system. HPLC was performed with a Nova-Pak C_{18} reverse phase column. A number of [^{3}H]-labeled steroids were used as internal standards for identification of the chromatographic peaks caused by the metabolites of P_5.

2.4. Pregnenolone binding studies

To facilitate cell attachment, the 24-well plates were coated with poly-L-lysine (0.05 mg/ml) for 30 min and dried. To determine the kinetics of [7-^{3}H]P_5 binding, LNCaP, PC-3, and COS-1 cells (1×10^5) were plated into the precoated 24-well plates and allowed to attach. The following day, the medium was replaced with serum-free, steroid-free IMEM supplemented with 0.1% bovine serum albumin and containing [7-^{3}H]P_5 (0.01–27 nM) in the presence or absence of a 200-fold excess of cold P_5 to determine non-specific binding. Following a 2-h incubation period at 37°C, the cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline (DPBS) and solubilized in DPBS containing 0.5% SDS and 20% glycerol. Extracts were removed and the cell-associated radioactivity was counted in a scintillation counter.

Competitive binding studies with [7-^{3}H]P_5 were performed essentially, as described by Wong et al. [22] and Yarbrough et al. [23]. When the concentration of [7-^{3}H]P_5 required to almost saturate the steroid-binding site was established (10 nM), the abilities of the steroids, progesterone, DHT, estrogen, and Dex (1–500

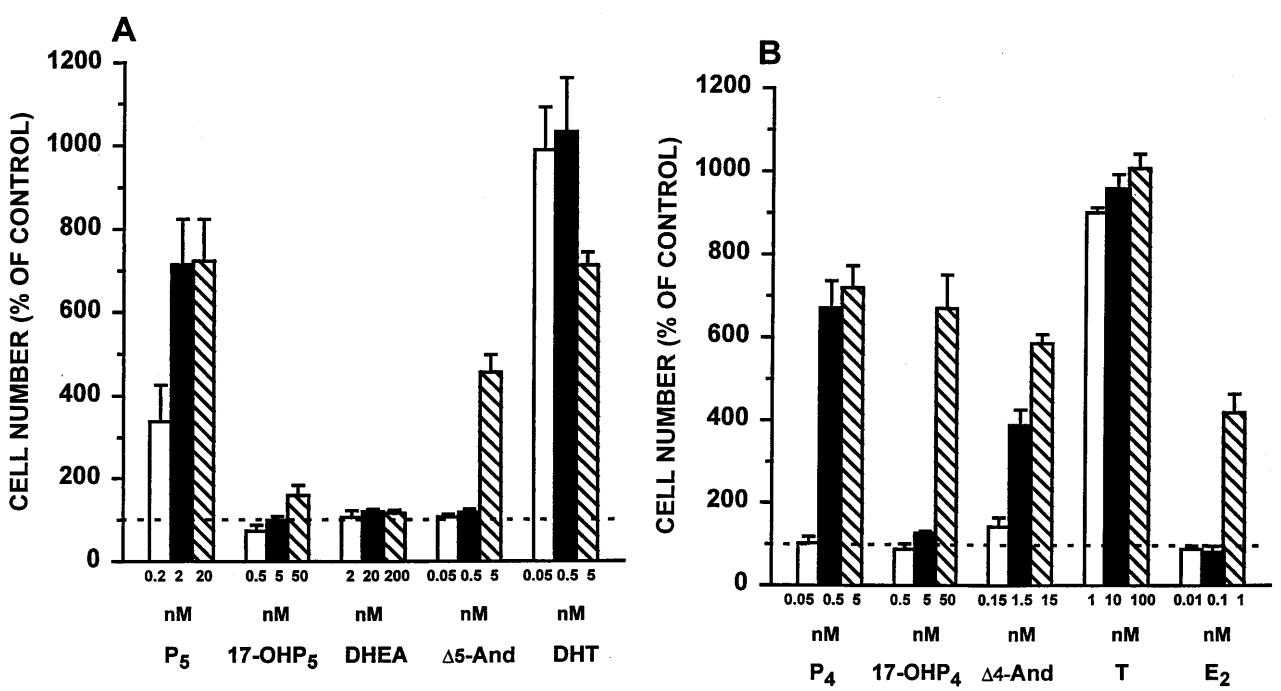

Fig. 1. The effects of different steroids on LNCaP cell proliferation. (A) The effects of different concentrations of Δ5-steroids and DHT on LNCaP-cell proliferation. P_5, pregnenolone; 17-OHP_5, 17α-hydroxypregnenolone; DHEA, dehidroepiandrosterone; Δ5-And, androstenediole; DHT, dihydrotestosterone; (B) the effects of different concentrations of Δ4-steroids, testosterone, and estrogen on LNCaP-cell proliferation. P_4, progesterone; 17-OHP_4, 17α-hydroxyprogesterone; Δ5-And, androstenedione; T, testosterone, E_2, estradiol. The effects of the steroids on cell proliferation was performed, as described in Section 2. The columns represent the mean of the cell numbers in triplicate wells from three separate experiments following a 9-day-treatment period. The cell number is expressed as a percentage of the mean number in the control wells. Values are expressed as mean ± S.E.M. Dotted line represents control (100%).

nM) to compete with [7-^{3}H]P_5 for binding to the cells were determined.

The cytosolic and nuclear fractions of LNCaP cells for [7-^{3}H]P_5-binding studies were prepared, as described by Sonnenschein et al. [8]. Briefly, cells were scraped from confluent T-75 flask in DPBS containing 1.5 mM EDTA, 10 mM DTT, 10 mM sodium molibdate, and 10% (v/v) glycerol, pH 7.4. Then, cells were homogenized with a Potter homogenizer and centrifuged at 100 000 × *g* for 1 h at 2°C. Receptor-binding studies were conducted with the cytosolic fraction (supernatant) and the membrane fraction (pellet), as described by Veldscholte et al. [24], using [7-^{3}H]P_5 and [7-^{3}H]R1881 as substrates. Maximum binding capacities (B_{max}) and affinities (K_d) were determined by Scatchard analysis, using the RADLIG 40 software (Biosoft, Ferguson, MO).

2.5. In situ photolabeling

In situ photolabeling studies were performed essentially, as described previously [3,25]. LNCaP cells were grown to confluency in 100-mm dishes and were exposed to 5-nM [^{3}H]-labeled R1881 or [7-^{3}H]P_5 for 2 h. After two washes with DPBS, the culture dishes were placed on a 300-nm UV-transilluminator and the cells were irradiated for 4 min. Then, cytosolic fraction was prepared, as described above, and proteins were separated by SDS-PAGE. Broad-range molecular weights markers (Bio-Rad) were used as references. The resultant gel was sliced and the slices dissolved in scintillation solution. The radioactivity associated with the slices was measured in a scintillation counter and plotted vs. protein molecular weights.

2.6. Transfection of LNCaP and CV1 cells with MAMneoLUC

LNCaP cells and CV1 cells were transfected with the pMAM*neo*LUC plasmid, as we have described previously [26]. Briefly, 2×10^5 cells in a 35 mm^2 dish were exposed to 3 ml of Opti-MEM (Gibco-BRL) containing LipofectAMINE (30 μl) and 6 μg of the pMAM*neo*LUC construct for 5 h at 37°C in a 5% CO_2 incubator. The medium was then changed to routine culture medium and the cells were incubated for 72 h. Cells were then grown in medium supplemented with 750 μg/ml geniticin (G418). The surviving colonies were selected and maintained in selective media. Stable selectants were tested twice a month for luciferase activity. Cells were treated with 5-μM dexamethasone for 24 h and luciferase activity was measured, as described in Section 2.7. After 3 months ($\sim$23 passages) the transfectants from LNCaP and CV-1 cells with the highest luciferase activity were selected and coined LNCaP-LUC and CV1-LUC, respectively.

2.7. Transfection of CV1-LUC cells with pCMV5-LNCaPAR and pCMV5-hAR

CV1-LUC cells were grown in steroid-free IMEM for 3 days and plated (4×10^4 cells/well) in 24-wells plate in antibiotic-free DMEM supplemented with 10% FBS. After 24 h, the cells were washed twice with DPBS and each well was incubated with 250 μl of phenol red-free IMEM containing 2-μl PLUS-regent, 4-μl LipofectAMINE and 0.5-μg pCMV5-hAR or pCMV5-LNCaPAR. After a 5-h incubation, 250 μl of routine medium were added and cells were incubated for additional 24 h. Then, the resultant CV1-LUC/pCMV5-hAR cells and CV1-LUC/pCMV5-LNCaPAR cells were assayed for luciferase activity as described in Section 2.8.

2.8. Luciferase assay

Cells were transferred into steroid-free medium 3 days prior to the start of the luciferase experiments. CV1-LUC cells were plated into 24-well culture plates and cotransfected with pCMV5-hAR or pCMV5-LNCaPAR plasmids, as described above. LNCaP-LUC cells were plated at 10^5 cells/well in steroid-free medium. Cells were then treated with vehicle (0.02% ethanol) or selected steroids (5.0 μM) in triplicate wells. After a 24-h treatment period, the cells were washed twice with ice-cold DPBS and assayed using the luciferase kit (Promega) accordingly to manufacturer's protocol. Briefly, cells were lysed with 200-μl luciferase-lysing buffer (Promega), collected in microcentrifuge tubes and pelleted by centrifugation. Supernatants (100 μl) were transferred to the corresponding wells of white 96-well plates (Polyfiltronics, Inc., Boston, MA) and 50 μl of luciferin was added to each well. The light produced during the luciferase reaction was measured in a Victor 1420 Multilabel Counter (Wallac Inc., Gaithersburg, MD). The effects of steroid-receptor antagonists on P_5-activated transcription were determined using the same protocol.

2.9. Animal studies

Male SCID mice (4–6 weeks of age) were provided by NCI, Fredrick, MD. The animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water ad libitum. LNCaP tumors were grown s.c. in the mice essentially, as we have previously described [26]. Briefly, wild-type LNCaP cells were grown to 80% confluency in T-150 flasks. Cells were then scraped into DPBS, counted, and suspended in Matrigel (3×10^7 cells per ml). Male SCID mice were injected s.c. with 100 μl of the cell suspension at one site on each flank. LNCaP tumors were allowed to grow for 4–5 weeks following cell inoculation. The mice were then grouped (six mice

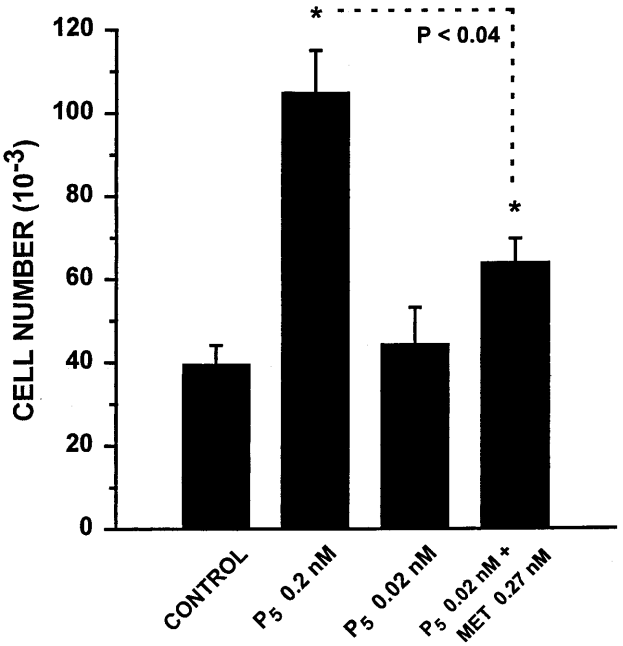

Fig. 2. Effects of pregnenolone metabolites on LNCaP cell growth. The abilities of pregnenolone and its metabolites to induce LNCaP-cell proliferation were determined, as described in Section 2. The columns represent the mean of the cell numbers in triplicate wells from three different experiments following a 9-day-treatment period. Cell number is expressed as a percentage of the mean number in the control wells. Values are expressed as mean ± S.E.M. (P_5, pregnenolone; MET, metabolites). *$P < 0.04$.

per group) for castration and/or treatment with vehicle (0.3% HPC) or pregnenolone (50 mg/kg per day). Tumors were measured weekly for the 4 weeks of treatment and tumor volumes were calculated using the formula $0.5236 \times r_1^2 \times r_2 (r_1 < r_2)$. At the end of the treatment period, the animals were sacrificed by decapitation and tumors were excised, weighed and stored in liquid nitrogen.

3. Results

3.1. Effects of different steroids on cell growth

The effects of physiological, subphysiological, and pharmacological concentrations of different steroids on the growth of LNCaP cells were evaluated (Fig. 1). At their physiological concentrations, both testosterone (10 nM) and DHT (0.5 nM) markedly increased cell number by 9–10-fold (Fig. 1A Fig. 1B). P_5 (2 nM; Fig. 1A) and progesterone (0.5 nM; Fig. 1B) were the only steroids that stimulated LNCaP-cell growth to a level comparable with that of testosterone and DHT, at their physiological concentrations. Proliferation of LNCaP cells was stimulated 7–8-fold by physiological (2 nM) and 3–4-fold by subphysiological concentrations (0.2 nM) of P_5. Subphysiological concentrations of progesterone (0.05 nM) had no effect on LNCaP-cell proliferation. However, its physiological concentration (0.5 nM) stimulated LNCaP-cell growth 6–7-fold.

In order to determine whether the growth stimulatory effects of P_5 were specific to LNCaP cells, the effect of P_5 on PC-3 and MCF-7 cell growth was determined. The androgen receptor negative PC-3 prostate cancer-cell line, and the hormone-dependent MCF-7 breast cancer-cell line were treated with P_5 for 9 days and its effect on cell proliferation was compared with the effects of DHT and estradiol, respectively. Neither DHT nor P_5 stimulated the proliferation of PC-3 cells. Similarly, P_5 had no effect on the growth of MCF-7 breast-cancer cells, which were highly responsive to estrogen stimulation (data not shown).

3.2. Role of pregnenolone metabolites in LNCaP-cell proliferation

The ability of LNCaP cells to metabolize radiolabeled P_5 ([7-^{3}H]P_5) was determined by HPLC. LNCaP cells (5×10^4) were treated with [7-^{3}H]P_5, and following an 8-h incubation, one minor P_5 metabolite was detected with a 1.5-min retention time (*r.t.*). The amount of this metabolite (M_1) was increased following a 24- and 48-h culture period. By 24 h, a second metabolite (M_2) was also detected, at a low concentration, with an *r.t.* of 6 min. By 48 h, the levels of the two metabolites were 14% (M_1) and 9% (M_2). By 72 h, the metabolites were 18% (M_1) and 30% (M_2) of the starting amount of P_5 added. No progesterone was detected at any incubation time point.

To explore whether the growth stimulatory effects of P_5 were due to the metabolites in the medium, cells were treated with P_5 that had been pre-exposed to a high density of LNCaP cells so that it contained an increased proportion of the metabolites (P_5:metabolites = 0.02:0.27 nM, as determined by HPLC; Fig. 2). Medium containing 0.02-nM P_5 and 0.27-nM metabolites increased LNCaP-cell growth (1.5-fold vs. vehicle). However, this was significantly less ($P < 0.04$) than the growth effect of P_5 (0.2 nM) alone (3-fold vs. vehicle). Further studies were also performed with the conjugated pregnenolone, P_5-S, which is unable to cross the cell membrane. P_5-S (0.2–20 nM) had no effect on the growth of LNCaP prostate cancer cells (data not shown). These results suggested that the growth-stimulatory effects of P_5 on LNCaP cells were being mediated directly by the steroid itself and not by the metabolites (Fig. 2), and that, in order to illicit its effects, P_5 has to enter the cell.

3.3. Receptor-binding assays

In order to determine whether P_5 was binding to a specific steroid receptor in the LNCaP cells, receptor

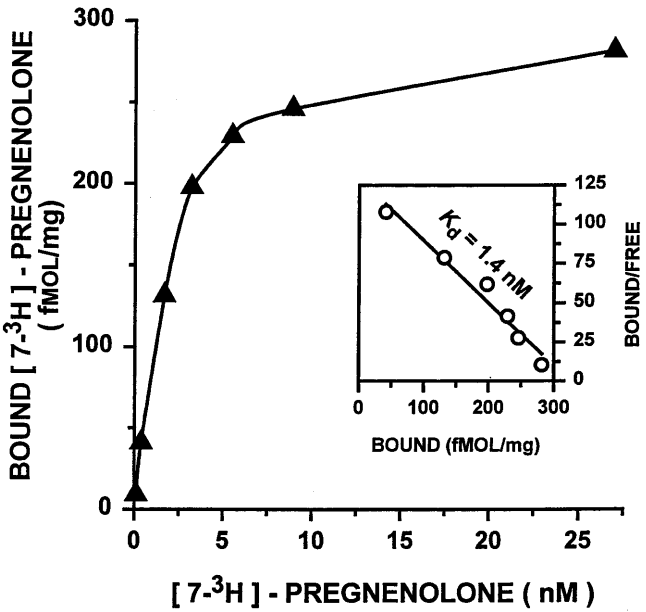

Fig. 3. Kinetics of pregnenolone binding to LNCaP cells. LNCaP cells were incubated with 0.1–30 nM of [^{3}H]pregnenolone for 2 h at 37°C in serum-free medium, as described in Section 2. Following washing and lysing, cell-associated radioactivity was determined by liquid scintillation counting.

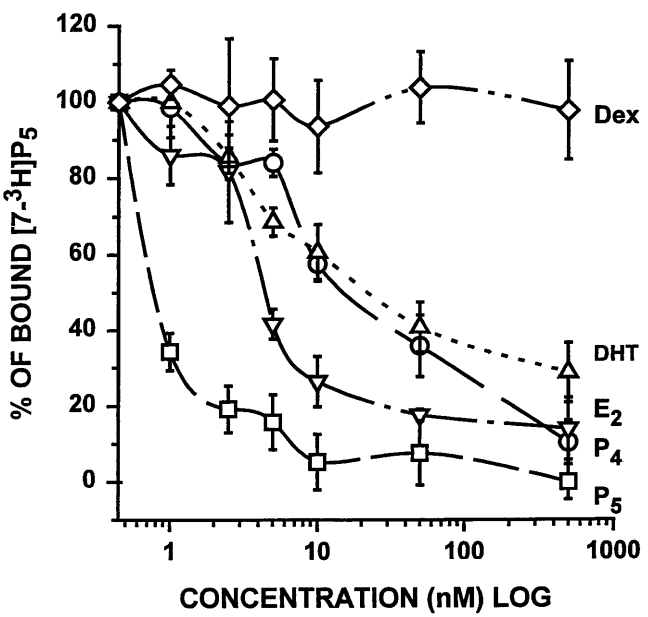

Fig. 4. Competition studies with 10-nM pregnenolone and different steroids. LNCaP cells were incubated in steroid-free media containing 10 nM of [^{3}H]pregnenolone with or without 1–1000 nM of cold pregnenolone and other steroids. After a 2-h incubation period the medium was removed and cells were washed with DPBS. Cells were collected and the amount of [^{3}H]pregnenolone bound to the cells was determined, as described in Section 2. The results show the per cent of [^{3}H]pregnenolone (pmol per cell) bound to the cells and are expressed as a percentage of total bound [^{3}H]pregnenolone. Values from three different experiments are expressed as mean ± S.E.M. (◇Dex, dexamethasone; △DHT, dihydrotestosterone; ▽E_2, estradiol; □P_5, pregnenolone; ○P_4, progesterone).

binding analyzes were performed in LNCaP, PC-3, and COS-1 cells, and competitive binding studies were performed with LNCaP cells. Kinetic analysis of P_5 binding in intact LNCaP cells revealed a single class of high-affinity binding sites for this steroid with $K_d = 1.4$ nM and $B_{max} = 0.3$ pmol/mg of protein (Fig. 3). There was no specific binding of P_5 to PC-3 and COS-1 cells (data not shown). To obtain direct evidence for the cellular localization of the P_5-specific binding sites, ligand binding studies were conducted in the membrane and cytosolic fractions of LNCaP cells. Fractionation was accomplished by centrifugation of the cellular lysate, as described previously [8]. Both fractions were assayed with P_5 and the synthetic androgen, R1881 which was used as a positive control. Kinetic analysis revealed high-affinity binding sites for both ligands in the cytosolic, but not membrane fraction of LNCaP cells. Scatchard analysis showed a single class of binding sites for R1881, which is the LNCaP androgen receptor, with $K_d = 0.78$ nM and $B_{max} = 0.07$ pmol/mg, and a single, and, as yet undefined, binding site for P_5 with $K_d = 2.2$ nM and $B_{max} = 0.063$ pmol/mg.

In LNCaP cells, there was almost saturation of the binding sites for P_5 at 10-nM concentration of the ligand (Fig. 3). Therefore, this concentration of P_5 was used in the competitive binding studies. Concentrations of 1 nM of cold P_5, progesterone, estradiol, DHT, and dexamethasone displaced [^{3}H]P_5 (10 nM) from its binding site by 63, 2, 13, 0 and 0%, respectively (Fig. 4). Concentrations of 10 nM of cold P_5, progesterone, estradiol, DHT, and dexamethasone displaced [^{3}H]P_5 from its binding site by 96, 42, 76, 39 and 0%, respectively (Fig. 4). These results showed that the stimulatory effects of P_5 on the growth of LNCaP prostate cancer cells is likely to be mediated via a specific high-affinity binding site for this steroid.

In situ photolabeling was performed to determine the size of the P_5 cellular-binding site. Using this method, a major peak at approximately 110 kDa (size of AR [25]) was recorded for R1881 and P_5 (data not shown). This suggests that P_5 may be binding to the same site as R1881, that is the LNCaPAR.

3.4. Effects of antagonist of different steroid receptors on pregnenolone-stimulated LNCaP-cell proliferation

LNCaP cells were treated with physiological concentrations of P_5, progesterone, estrogen, and DHT either alone or in the presence of their corresponding receptor antagonists. The antagonists were, TRA which is an antagonist of the PR, ICI 182,780 which is an antagonist of the ER, casodex, an antagonist of the AR, and VN/63-1, a novel inhibitor of androgen synthesis, as previously reported by us and which is also a potent AR antagonist [26]. LNCaP-cell proliferation was stimulated by the corresponding steroid or P_5, and the

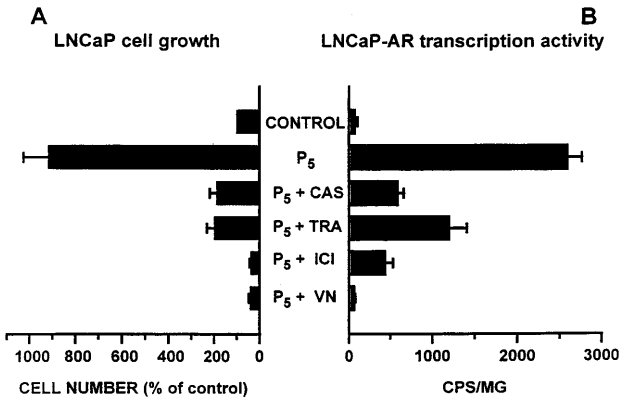

Fig. 5. The effects of DHT and pregnenolone on wild-type (A) and LNCaP (B) androgen receptor-mediated transcription. CV-1-LUC/pCMV5-hAR (A) and CV-1-LUC/pCMV5- LNCaPAR (B) cells were transferred into steroid-free medium and plated into 24-wells plate 3 days prior to the start of the experiment. Cells were treated with the medium containing vehicle (0.02% ethanol), DHT (5 nM), and P_5 (5 nM) for 24 h. Cells were then assayed for luciferase activity, as described in Section 2. The columns represent the mean of the light units (counts per second (CPS) per mg of protein) in triplicate wells from three different experiments. Values are expressed as mean ± S.E.M.

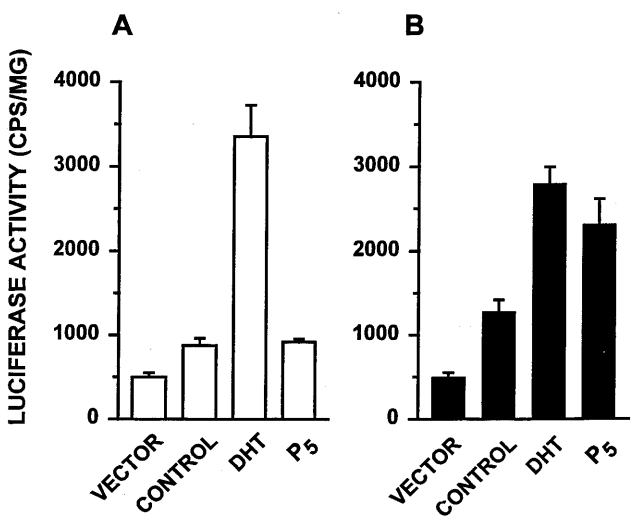

Fig. 6. The effects of receptor antagonists on the pregnenolone stimulated growth (A) and pregnenolone-stimulated transcription activity (B) in LNCaP prostate cancer cells. (A) The effects of antagonists of the AR (casodex), PR (triamcinolone acetate), ER (ICI 182,780), and the inhibitor of androgen synthesis with potent antiandrogenic activity (VN/63-1) (all at 5 μM) on the growth of LNCaP cells induced by P_5 (2 nM) were determined, as described in Section 2. The columns represent the mean of the cell numbers in triplicate wells from three different experiments after a 9-day incubation period. Cell number is expressed as a percentage of the mean number in the control wells. Values are expressed as mean ± S.E.M.; (B) the ability of the steroid-receptor antagonists to inhibit the stimulatory effects of 2-nM pregnenolone on transcriptional activity in LNCaP-LUC cells was tested, as described in Section 2. The columns represent the mean of the light units (counts per second (CPS) per mg of protein) in triplicate wells from three different experiments. Values are expressed as mean ± S.E.M. (P_5, pregnenolone; CAS, casodex; TRA, triamcinolone acetonide; ICI, ICI 182,780; VN, VN/63-1).

ability of the receptor antagonists to inhibit this proliferation was determined. Casodex inhibited P_5-stimulated cell growth by 79.5, TRA by 78, ICI 182,780 by 96, and VN/63-1 by 95.5%, respectively, (Fig. 5A) and casodex, TRA, and ICI 182,780 inhibited cell growth stimulated by DHT, progesterone, and estrogen, respectively (data not shown).

3.5. Effects of pregnenolone on LNCaP and wild-type androgen receptor-mediated transcription

CV1-LUC cells were transfected with LNCaP and wild-type AR and the resultant CV1-LUC/pCMV5-LNCaPAR cells and CV1-LUC/pCMV5-hAR were used for transcriptional activity studies. Cells were treated with DHT or P_5 and the effects of these steroids on luciferase production were determined. DHT (5 nM) stimulated luciferase production mediated by the wild-type androgen receptor by 4-fold compared with the control, whereas P_5 (5 nM) did not have any effect on luciferase production in CV1-LUC/p5CMV-hAR cells (Fig. 6A). In cells transfected with the mutated LNCaPAR, both DHT and P_5-stimulated luciferase production by 2.2-fold and 1.8-fold vs. control, respectively (Fig. 6B). These results suggest that the growth stimulatory effects of P_5 on LNCaP cells are being mediated by the mutated AR.

The effects of the steroid-receptor antagonists (1 μM) on P_5 (1 nM)-activated transcription were investigated in LNCaP-LUC cells. These studies revealed that our novel compound VN/63-1 was able to block completely the transcriptional activating effects of P_5 in LNCaP-LUC cells (Fig. 5B). While VN/63-1 has activity against 17α-hydroxylase/$C_{17,20}$-lyase [17], it is also a potent antiandrogen [26]. This inhibitor is analogue of P_5 and presumably competes with P_5 for the same binding site. All of the other steroid-receptor antagonists were also able to block increased transcriptional activity induced by P_5, but were less effective than VN/63-1. Surprisingly, the antiestrogen, ICI 182,780 was more effective than the antiandrogen casodex. The order of inhibitory potency of the receptor antagonists was, VN/63-1 > ICI 182,780 > Casodex > TRA, and activity was inhibited by 96, 85, 77, and 54%, respectively.

3.6. The effects of pregnenolone on LNCaP-tumor growth in male SCID mice

Intact and castrated male SCID mice bearing LNCaP-tumor xenografts were treated with P_5 (50 mg/kg per day) or with vehicle (HPC) alone for 4 weeks and the tumor volumes were measured weekly (Fig. 7). After 4 weeks of treatment, the total tumor volume in the intact mice treated with P_5 was 143% of that in control group. To ensure that this was not due to the testicular conversion of the exogenous P_5 to androgens,

P_5 was also administered to mice that had been castrated. The total tumor volume in the castrated mice treated with P_5 was 147% of that in the castrated group that were treated with vehicle only (Fig. 7). The tumor-weight values correlated well with the tumor volumes (Fig. 7). These results show that P_5 can stimulate the growth of LNCaP cell in vivo as well as in vitro.

4. Discussion

While evaluating the growth-inhibitory effects of some of our novel inhibitors of androgen synthesis, we found that LNCaP human prostate-cancer cells were growth stimulated by a low concentration (1 nM) of P_5. After this unexpected finding, the effects of other steroids on the growth of LNCaP cells were also evaluated (Fig. 1). Only progesterone and P_5, at their physiological concentrations, were able to stimulate LNCaP-cell growth at a level comparable with that of the androgens, DHT and testosterone. P_5, a common precursor of all steroid hormones, is present in the blood of normal adult men at a concentration of 1–3 nM [18–20]. We found that, at a 2-nM concentration, P_5-stimulated LNCaP-cell proliferation 7–8-fold. In addition, a subphysiological concentration of P_5 (0.2 nM) was also growth stimulatory to the cells (3–4-fold increase in cell number compared with vehicle-treated cells). These results suggested that P_5 may mediate the growth of hormone-dependent prostate tumors and could be important particularly during androgen deprivation therapy. However, as P_5 had no effect on the growth of PC-3 prostate cancer cells or on MCF-7 human breast cancer cells (data not shown), it appears that this phenomenon is most likely limited to LNCaP prostatic carcinoma cells.

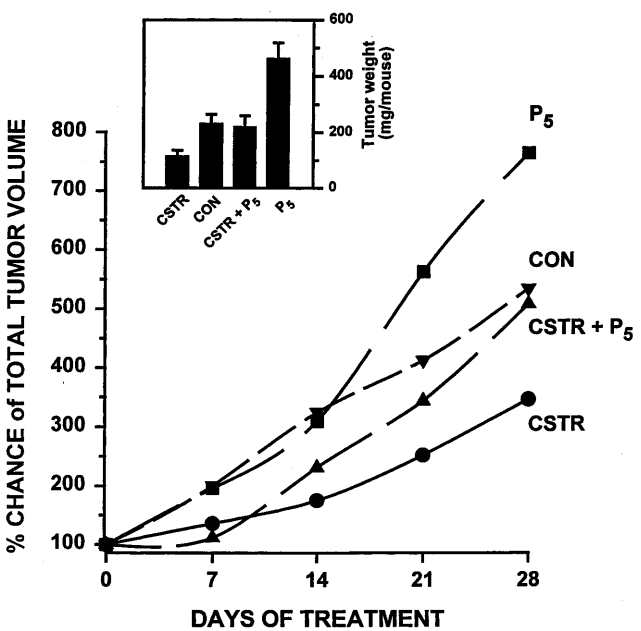

Fig. 7. Effect of pregnenolone on growth of LNCaP-tumor xenograft and tumor weights (insert) in male SCID mice. Groups of six mice, each with 2 LNCaP tumors, were treated by castration and/or with 50 mg/kg per day pregnenolone. Tumor volumes were measured weekly and the percentage change in tumor volume determined. Following 28 days of treatment, the mice were sacrificed and the tumors were removed and weighed. (P_5, pregnenolone; CON, control; CSTR, castrates).

The HPLC studies of the metabolism of P_5 by LNCaP cells showed that P_5 cannot be converted by LNCaP cells to another active steroid progesterone and that unidentified metabolites of P_5 had low proliferative activity. These results suggested that the increase in LNCaP-cell proliferation by P_5 was being mediated by P_5 itself. Receptor-binding studies showed that LNCaP cells express high-affinity binding sites for P_5 with K_d = 1.4 nM and B_{max} = 0.3 pmol/mg of protein (Fig. 3). The [^{3}H]P_5 competitive binding studies showed that none of the steroids evaluated were able to displace P_5 with the same efficiency as P_5 itself (Fig. 4). These results confirmed the high specificity of the P_5-binding site for its ligand in LNCaP cells. It has been reported previously that P_5 and P_5-S are active neurosteroids [27] that can modulate cellular responses via a G-protein coupled mechanism in hippocampal CA1 neurons [28] and produce NMDA-mediated responses [29]. The failure of P_5-S to stimulate the LNCaP-cell growth ruled out this mechanism of action of P_5 in LNCaP cells. The inability of this conjugated steroid to cross the cellular membrane also suggested that the location of the receptor was intracellular and not membrane-associated. This was confirmed by the studies using fractionated LNCaP cells, which detected specific binding sites for P_5 in the cytosolic fraction of LNCaP cells, but not in the membrane fraction (data not shown). Moreover, the similar B_{max} values obtained with R1881 (0.07 pmol/mg protein) and P_5 (0.063 pmol/mg protein) suggest that both ligands bind to the same receptor. The in situ-photolabeling studies [3] support this suggestion by revealing a similar pattern of protein binding for the synthetic androgen, R1881, and for P_5 in LNCaP-cellular lysate, which confirmed that P_5 was binding to the mutated LNCaPAR.

It has been previously shown that progesterone and estrogen can stimulate LNCaP-cell growth through the mutated AR [3,7] and that antagonists of the PR and ER inhibit this effect [30]. We confirmed these results in LNCaP cells, and additionally found that antagonists of the AR, PR, and ER were effective against P_5-stimulated LNCaP-cell growth (Fig. 5A). The inhibitory potencies of these antagonists against their corresponding steroids were similar to their potencies against P_5 (data not shown). The antiestrogen ICI 182, 780 and our novel pregnenolone derivative, VN/63-1, were the most potent inhibitors of P_5-stimulated cell growth.

These results correlated with competitive binding data, which showed that estradiol competed with P_5 for LNCaPAR binding more efficiently than progesterone and DHT. This implied that all of the antagonists were acting through LNCaPAR, and further supported that the binding site for P_5 is the LNCaPAR.

In order to show that P_5 not only binds to the AR but also activates gene transcription, the luciferase reporter system was employed. This assay showed that P_5 activated transcription through the LNCaPAR (Fig. 6A) but not through the wild-type AR (Fig. 6B). This system also demonstrated that the inhibitory effects of the receptor antagonists on P_5 stimulation were also being mediated through the mutant AR (Fig. 5).

Studies in male SCID mice bearing LNCaP tumors demonstrated that the growth-stimulatory effects of P_5 on LNCaP cells occurred in vivo as well as in vitro (Fig. 7). Moreover, the studies confirmed that the growth stimulatory effects of P_5 on LNCaP tumor growth were being mediated directly by P_5 itself, and not by androgens or other metabolites that may be produced from this steroid. This was evident because LNCaP-xenograft growth was stimulated to the same extent in intact as well as in castrated animals (Fig. 7).

This is the first report describing the ability of physiological concentrations of P_5 to stimulate LNCaP-cell proliferation. We have shown that the effect of P_5 is being mediated by the steroid binding to the mutated LNCaPAR. Our investigations have demonstrated that the stimulation induced by P_5 can be inhibited by antagonists of the AR, PR, and the ER. However, the greatest inhibition was by our novel compound, VN/63-1. This compound is a Δ5-steroid which is a derivative of P_5. We have previously reported that this compound inhibits 17α-hydroxylase/$C_{17,20}$-lyase and is a potent antiandrogen [17,26]. Given that mutated forms of the AR are relatively common in prostate-cancer patients, these findings could have important implications for the treatment of prostate cancer. They suggest that some prostate-cancer patients who received androgen-ablation therapy and appear to become androgen independent, may have tumors that are growth stimulated by P_5 in the absence of androgens. Since P_5 is produced by the adrenals and is not inhibited by the current treatments, such as LHRH analogs, ketoconazole and flutamide, and incompletely by casodex, our results suggest that these patients could benefit from treatment with our novel inhibitor.

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