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Inhibition of Spontaneous and Androgen-Induced Prostate Growth by a Nonhypercalcemic Calcitriol Analog

C. CRESCIOLI, P. FERRUZZI, A. CAPORALI, R. MANCINA, A. COMERCI, M. MURATORI, M. SCALTRITI, G. B. VANNELLI, S. SMIROLDO, R. MARIANI, D. VILLARI, S. BETTUZZI, M. SERIO, L. ADORINI, AND M. MAGGI

Departments of Clinical Physiopathology (C.C., P.F., R.M., A.C., M.M., M.S., M.M.), Anatomy, Histology, and Forensic Medicine (G.B.V.), and Urology (D.V.), University of Florence, Florence 50100, Italy; Department of Experimental Medicine, University of Parma (A.C., S.B.), Parma 43100, Italy; BioXell (S.S., R.M., L.A.), Milan 20132, Italy; and Department of Biomedical Science, University of Modena and Reggio Emilia (M.S.), Modena 41100, Italy

We have recently found that analog V (BXL-353, a calcitriol analog) inhibits growth factor (GF)-stimulated human benign prostate hyperplasia (BPH) cell proliferation by disrupting signal transduction, reducing Bcl-2 expression, and inducing apoptosis. We now report that BXL-353 blocks *in vitro* and *in vivo* testosterone (T) activity. BPH cells responded to T and dihydrotestosterone (DHT) with dose-dependent growth and reduced apoptosis. Exposure of BPH cells to BXL-353 significantly antagonized both T- and DHT-induced proliferation and induced apoptosis, even in the presence of T. To verify whether BXL-353 reduced prostate growth *in vivo*, we administered it orally to either intact or castrated rats, supplemented with T enanthate. Nonhypercalcemic doses of BXL-353 time- and dose-dependently reduced the androgen effect on ventral prostate weight, similarly to finasteride. Comparable results were obtained after chronic administration of BXL-353 to intact rats. Clusterin (an atrophy marker) gene and protein were up-regulated by BXL-353 in rat prostate, and nuclear fragmentation was widely present. The antiandrogenic properties of BXL-353 did not interfere with pituitary and testis function, as assessed by serum determination of rat LH and T. BXL-353 did not compete for androgen binding to BPH homogenates and failed to inhibit 5α-reductase type 1 and type 2 activities. In conclusion, BXL-353 blocks *in vitro* and *in vivo* androgen-stimulated prostate cell growth, probably acting downstream from the androgen receptor, without affecting calcemia or sex hormone secretion. BXL-353 and other vitamin D_3 analogs might thus represent an interesting class of compounds for treating patients with BPH. (*Endocrinology* 144: 3046–3057, 2003)

THE PROSTATE gland retains the plasticity to respond to androgenic signaling throughout the entire life span, reaching maximal volume in the aging man (1). This is essentially the physiological basis for the most common urological problem of the aging male, benign prostate hyperplasia (BPH). Approximately 40–50% of men over 60 yr of age suffer from symptoms related to BPH, and 25–30% of them require surgery (2). Androgens and age are considered the main determinants of prostate enlargement, and blocking androgen receptor (AR) activity is a straightforward therapeutic approach for BPH. Agents that inhibit androgen secretion or activity, such as GnRH agonist or AR antagonists, are indeed very active in reducing prostate growth (3), but the hypogonadal symptoms they induce strongly limit their clinical applicability. Even finasteride (F), a drug that selectively inhibits the 5α-reductase type 2 (5αR-2)-mediated conversion of testosterone (T) in dihydrotestosterone (DHT), shows consistent sexual side-effects, reducing its popularity in aging men, especially in those with borderline erectile function (4). In addition, although F decreases the rate of significant BPH-related events, such as acute urinary retention, and the need for surgery, its effect on prostate growth is relatively small, resulting in a 15–25% decrease from baseline (5). Hence, novel therapeutic approaches for age-related prostate enlargement, not directly related to a block of AR signaling, have a high medical need.

Since the pioneering experiments of Cunha and colleagues (6, 7), prostate growth and differentiation have been presumed to be stimulated by androgens and to be strongly influenced by intraprostatic stromal growth factors (GFs). Indeed, in murine models of AR deficiency, prostatic epithelium development from urogenital sinus depends upon androgen responsiveness of the stroma, and not the reverse (7). Interestingly, in organ cultures of neonatal rat ventral prostates, exogenous administration of keratinocyte GF (KGF), a stromal derived intraprostatic GF, completely replaced the requirement of T for prostate growth and branching morphogenesis (8). Recent studies indicate that KGF might also be important for human prostate cell growth (9–11). IGFs have also been implicated in BPH pathogenesis (12). Patients with the highest circulating levels of IGF-I have an elevated risk of BPH (13), and type 1 IGF receptor (IGFR1) as well as IGF-II are more concentrated in the periurethral zone, where BPH originates in humans, than in other regions

Abbreviations: AR, Androgen receptor; BPH, benign prostate hyperplasia; DHT, dihydrotestosterone; F, finasteride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GF, growth factor; hfPSMC, human fetal penile smooth muscle cells; IGFR1, type 1 IGF receptor; ISEL, *in situ* end labeling; KGF, keratinocyte GF; KGFR, KGF receptor; 5αR-2, 5α-reductase type 2; rLH, rat LH; T, testosterone; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling; VDR, vitamin D receptor.

of the prostate gland (14). Interestingly, in this study IGF-II gene and protein expression in the periurethral zone of the prostate was strongly related to androgen levels.

Because the prostate gland is one of the few tissues retaining sensitivity to androgen-stimulated growth in the aging male, targeting intraprostatic stromal GFs might represent an attractive option for the medical treatment of BPH. In previous studies we found that in human stromal BPH cell cultures, a less hypercalcemic analog of calcitriol, analog V or BXL-353, completely antagonized KGF receptor (KGFR)- and IGFR1-mediated mitogenic signaling (11, 15). In the case of KGFR, we also demonstrated that the effect of BXL-353 was at least partially mediated by a disruption of KGF-induced receptor autophosphorylation (11, 16). In the present study, we investigated whether BXL-353 interferes *in vitro* and *in vivo* with androgen-induced prostate growth.

Materials and Methods

Materials

MEM, DMEM/Ham's F-12 medium (1:1), Ham's F-12 medium, PBS, BSA (fraction V), glutamine, antibiotics, collagenase type IV, T, DHT, cyproterone acetate, NADPH, dithiothreitol, phenylmethylsulfonylfluoride, and a kit for measuring calcemia were purchased from Sigma-Aldrich Corp. (St. Louis, MO). The protein measurement kit was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). Fetal bovine serum was purchased from Unipath (Bedford, UK). The unlabeled synthetic androgen R1881, [^{3}H]R1881, and [^{3}H]testosterone were purchased from NEN Life Science Products (Boston, MA). Anti-IGFR1 monoclonal antibody (clone αIR-3) and rabbit normal immunoglobulin G (IgG) were obtained from Oncogene Research Products (Boston, MA). Anti-KGFR polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antirat clusterin antibody (mouse monoclonal IgG) specific for β-chain was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The Apop-Tag kit for *in situ* end labeling (ISEL) was obtained from Oncor (Gaithersburg, MD). Bicalutamide was a gift from AstraZeneca (Milan, Italy). CHO 1827 and CHO 1829 were provided by Serono International (Geneva, Switzerland). Instagel Plus was purchased from Packard (St. Louis, MO). Finasteride (pure substance; 17β-(*N*,*t*-butyl)carbamoyl-4-aza-5α-androst-1-en-3-one) was a gift from Merck Sharp & Dohme Research Laboratories (Rahway, NJ). The analog 1,25-dihydroxy-16-ene-23-yne D_3 (BXL-353) was provided by BioXell (Milan, Italy). Forward and reverse primers specific for AR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from MWG (Biotech, Florence, Italy). Forward and reverse primers for 5αR-1 and 5αR-2, In Situ Cell Death Detection Kit POD for terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling (TUNEL), and reagents for Western analysis were obtained from Roche (Indianapolis, IN). Plasticware for cell cultures was purchased from Falcon (Oxnard, CA). Disposable filtration units for preparation of growth medium were purchased from PBI International (Milan, Italy). Thin layer chromatography silica plates were obtained from Merck & Co., Inc. (Darmstadt, Germany). T enanthate was purchased from Geymonat (Anagni, Italy). The Coat-A-Count Total Testosterone detection kit was purchased from Medical Systems (Genova Struppa, Italy). The rat LH (rLH) ^{125}I assay system (RIA) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

BPH cell cultures

After obtaining the informed consent of the patients and the approval of the local ethical committee, BPH cells were obtained from prostate tissues derived from five patients who underwent suprapubic adenomectomy for BPH. Patients did not receive any pharmacological treatment in the 3 months preceding surgery. Cell cultures were prepared and maintained as previously described (11). Cells were used within the fifth passage.

CHO-1827 and CHO-1829 cells

CHO-1827 (transfected with 5αR-1) and CHO-1829 cells (transfected with 5αR-2) (17) were maintained in Ham's F-12 supplemented with 5% fetal bovine serum in a fully humidified incubator (95% air/5% CO_2) at 37 C.

Tissue specimens

Rat ventral prostate glands were rapidly excised out, weighed, and quickly frozen in dry ice. Sections of 14 μm were excised out by means of a cryostat from each specimen. Immunohistochemistry experiments were performed in contiguous cryosections for direct comparison of tissue morphology, clusterin expression, and apoptosis localization by TUNEL. For total RNA extraction and Western blot analysis, rat ventral prostates were pooled from four to six animals.

Animal protocols

Male Sprague Dawley rats (28 d old) were purchased from Charles River Laboratories, Inc. (Calco, Lecco, Italy). All animal experimentation was conducted in accord with accepted standards of human animal care. Castration was performed via the scrotal route under ketamine/xylazine anesthesia. Three days after castration, rats (five to eight animals per group) were treated, or not, with T enanthate (30 mg/kg) in one or two separate weekly sc injections and thereafter according to the different protocols. In the 1-wk protocol they were treated orally with vehicle (miglyol 812) or BXL-353 at increasing concentrations (1, 3, 5, 10, and 30 μg/kg) for four consecutive administrations. Finasteride (10 mg/kg) was also administered orally to a subset of T-treated castrated rats according to the same protocol. In the 2-wk protocol, castrated rats supplemented with T were treated for 5 d the first week and 4 d the second week with vehicle (miglyol 812), BXL-353 (3, 5, 10, and 30 μg/kg), or F (3, 10, and 30 mg/kg) for a total of nine administrations and killed 1 d later.

In the 1-month protocol, intact adult male Sprague Dawley rats (250 g) were treated orally with vehicle (miglyol 812), BXL-353 (3, 10, and 30 μg/kg), or F (10 and 40 mg/kg) 5 d/wk for 5 consecutive wk and for an additional 2 d the sixth week (total of 27 administrations), unless otherwise specified. Blood for calcium and hormone measurements was obtained at the end of each experimental protocol.

Binding assay

Cytosol fractions of BPH fragments were prepared as previously reported (18, 19). For BPH cell homogenate, BPH cells grown in DMEM/Ham's F-12 (1:1; without phenol red) supplemented with 10% stripped fetal bovine serum were harvested and homogenized as reported by Granchi and colleagues (19, 20). The binding assay for BPH cell or fragment preparations was essentially performed as previously reported (19), (final protein concentrations, 1 and 3.6 mg/ml, respectively). Incubations of cytosolic fractions or cell homogenates were carried out with increasing concentration (0.125, 0.25, 0.5, and 1 nM) of [^{3}H]R1881 (specific activity, 83.5 Ci/mmol) in the absence or presence ([^{3}H]R1881, 1 nM) of increasing concentrations of cold R1881 (10^{-10}–10^{-6} M), DHT (10^{-10}–10^{-6} M), T (10^{-10}–10^{-6} M), bicalutamide (10^{-10}–10^{-4} M), and BXL-353 (10^{-10}–10^{-4} M), when required by the experimental design. To prevent R1881 binding to progesterone receptor, 1 μM triamcinolone acetonide was added to each tube. Separation of bound and unbound ligand was achieved as previously described (19, 20). The protein content was determined by the method of Bradford (21), using BSA as standard.

RT-PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA) from BPH cells, BPH tissue, human fetal penile smooth muscle cells (hfPSMC; positive controls for AR), and CHO 1827 and CHO 1829 (positive controls for 5αR-1 or 5αR-2). RNA concentrations were determined by spectrophotometric analysis at 260 nm. The quality of total RNA was assessed by performing additional RT-PCR for the GAPDH gene. The conditions for total RNA (500 ng) RT and amplification, and specific primers for AR, 5αR-1, 5αR-2, and GAPDH were reported previously (19, 20). The contamination of genomic DNA was excluded by performing 35 cycles of amplification without RT.

BPH cell proliferation assay

All proliferation tests were performed after 24 h of cell starvation in phenol red- and serum-free medium containing 0.1% BSA. After starvation, cells were incubated in the same medium as before, with or without specific stimuli. Thereafter, cells were trypsinized, and each experimental point was derived from counting in the hemocytometer and then averaging at least six different fields for each well as previously reported (11). For time-course assays, 2×10^4 cells were seeded onto 12-well plates in growth medium and treated with increasing concentrations (1–100 nM) of T for various times (24–96 h). For cell proliferation assays, 4×10^4 cells were seeded as before and incubated with increasing concentrations of steroids (10^{-12}–10^{-7} M) for 48 h. Experiments were also performed using a fixed concentration (10 nM) of steroids 1) with or without anti-KGFR, anti-IGFR1 antibodies, and IgG (1 μg/ml); 2) with or without fixed (1 or 10 nM) or increasing (10^{-20}–10^{-7} M) concentrations of BXL-353; and 3) with or without antiandrogens (F, 1 nM; cyproterone acetate, 100 nM), for 48 h. In the same experiment each experimental point was repeated in triplicate or quadruplicate and experiments were performed at least three times. Cell growth results are expressed as the number (±SE) of cells or as the percentage (±SE) of growth compared with their relative controls.

ISEL

To evaluate the apoptotic index, ISEL on BPH cells was performed using Apop-Tag *in situ* apoptosis detection kit peroxidase following the manufacturer's instructions (11). Cells were incubated with T (10 nM), BXL-353 (10 nM), or an equimolar (10 nM) combination of both. The percentage of apoptotic cells (the number of stained cells divided by the total number of cells) was calculated in at least five separate fields per slide in five different slides. Results are expressed as the mean ± SEM in three separate experiments.

5αR inhibition test

The 5αR inhibition assay was performed with CHO cells transfected with 5αR-1 (CHO 1827) or 5αR-2 (CHO 1829) according to reported procedures (22). BXL-353 was added in a concentration range from 10^{-9}–10^{-5} M, using F as a control inhibitor in each experiment.

Northern hybridization analysis

Total RNA was extracted using RNAFast from Molecular Systems (San Diego, CA). Blotting, labeling, hybridization conditions, and probes (rat clusterin, 1.5-kb full-length cDNA; GAPDH, 1.2-kb full-length cDNA) were performed according to previously reported procedures (23, 24). Quantitation of the autoradiograms was obtained by densitometric scanning using an Ultrascan XL densitometer (LKB, Rockville, MD).

Western blot analysis

Prostates were quickly removed, and tissue was homogenized in TTX buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.2% Triton X-100, 50 μg/ml deoxyribonuclease, and 50 μg/ml ribonuclease] with Complete protease inhibitor according to the manufacturer's instructions (Roche). Fifty micrograms of total protein extract were run on 10% polyacrylamide gel. After protein transfer onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ), the filters were blocked for 1 h at room temperature in 1% blocking agent and incubated using monoclonal antihuman clusterin antibodies in 0.5% blocking agent. After several washes in Tris-buffered saline/0.05% Tween, membranes were incubated with horseradish peroxidase-conjugated antimouse IgG antibody for 1 h at room temperature. Clusterin-immunoreactive bands were detected with the Lumi-Light Plus Western blotting substrate (POD, Roche).

Immunohistochemistry

The entire experimental procedure was performed at room temperature as previously described (25). All cryosections obtained from controls and treated animals were processed in parallel under identical reaction conditions. For every experimental condition, three alternate sections from three different rat prostates were examined. Negative controls, made by excluding the specific antibody from the reaction, showed no specific staining. Counterstaining was performed with hematoxylin, and coverslips were mounted with Eukitt (O. Kindler GmbH & Co., Germany). Digital high magnification color images were acquired by means of a CCD camera (Diagnostic Instruments, Chicago, IL) through the microscope.

TUNEL

DNA fragmentation in prostate cryosections, assessed by means of TUNEL, was performed using the In Situ Cell Death Detection Kit (POD, Roche) as recommended by the manufacturer. TUNEL-positive apoptotic nuclei were documented by digital high magnification color images acquired by CCD camera through the microscope. Counterstaining was performed with eosin, and coverslips were mounted with Eukitt (O. Kindler GmbH, Freiburg, Germany).

T and rLH measurements

Serum levels of T and rLH hormones were determined using commercially available RIA kits (specified in *Materials and Methods*), according to the manufacturer's instructions. To measure serum T in rats, samples were first added to 4 vol diethyl ether, mixed by gentle inversion for 15 min, and then centrifuged for 5 min at 2000 rpm. The aqueous phase was frozen in dry ice, and the organic phase was recovered and evaporated to dryness under a nitrogen stream. The dried extract was reconstituted in the assay buffer as follows: 1:1 (vol/vol) in intact rats and 4:1 (vol/vol) in castrated rats.

Calcium measurements

Serum calcium levels were measured with a commercially available colorimetric assay (Sigma-Aldrich Corp.), according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed by one-way ANOVA and paired or unpaired *t* tests where appropriate. Binding data were analyzed using the computerized program LIGAND (26).

The computer program ALLFIT (27) was used for analysis of sigmoid dose-response curves to obtain estimates of IC_{50} and EC_{50} as well as maximal inhibitory (I_{max}) and stimulatory (E_{max}) effects. Data were expressed as the mean (±SE).

Results

In vitro effects of androgens, antiandrogens, and BXL-353 on BPH cells

BPH cells express all genes and proteins related to full androgen sensitivity. In particular, they express specific transcripts for the AR gene and bind with high affinity ($K_d = 72 \pm 34$ pM; n = 3), and low capacity (2.64 ± 0.5 fM; n = 3) to the synthetic androgen R1881. The receptor density in BPH cells is similar to that we previously observed in hfPSMC (19), a classic androgen target. BPH cells also express transcripts for both subtypes of 5αR enzymes, of the same sizes as in hfPSMC and in CHO cells transfected with type 1 or type 2 5αR genes (not shown).

Figure 1A shows that different concentrations of T (1–100 nM) induced a time- and dose-dependent stimulation of BPH cell growth (n = 3). After a 48-h stimulation, the effect of T was comparable to that induced by its 5α-reduced metabolite, DHT (Fig. 1A, *inset*, n = 3). This was confirmed by the simultaneous fitting, using the program ALLFIT (27), of the growth curves induced by 48-h stimulation with different concentrations of the two androgens that showed no statistically significant difference in either 50% effective concen-

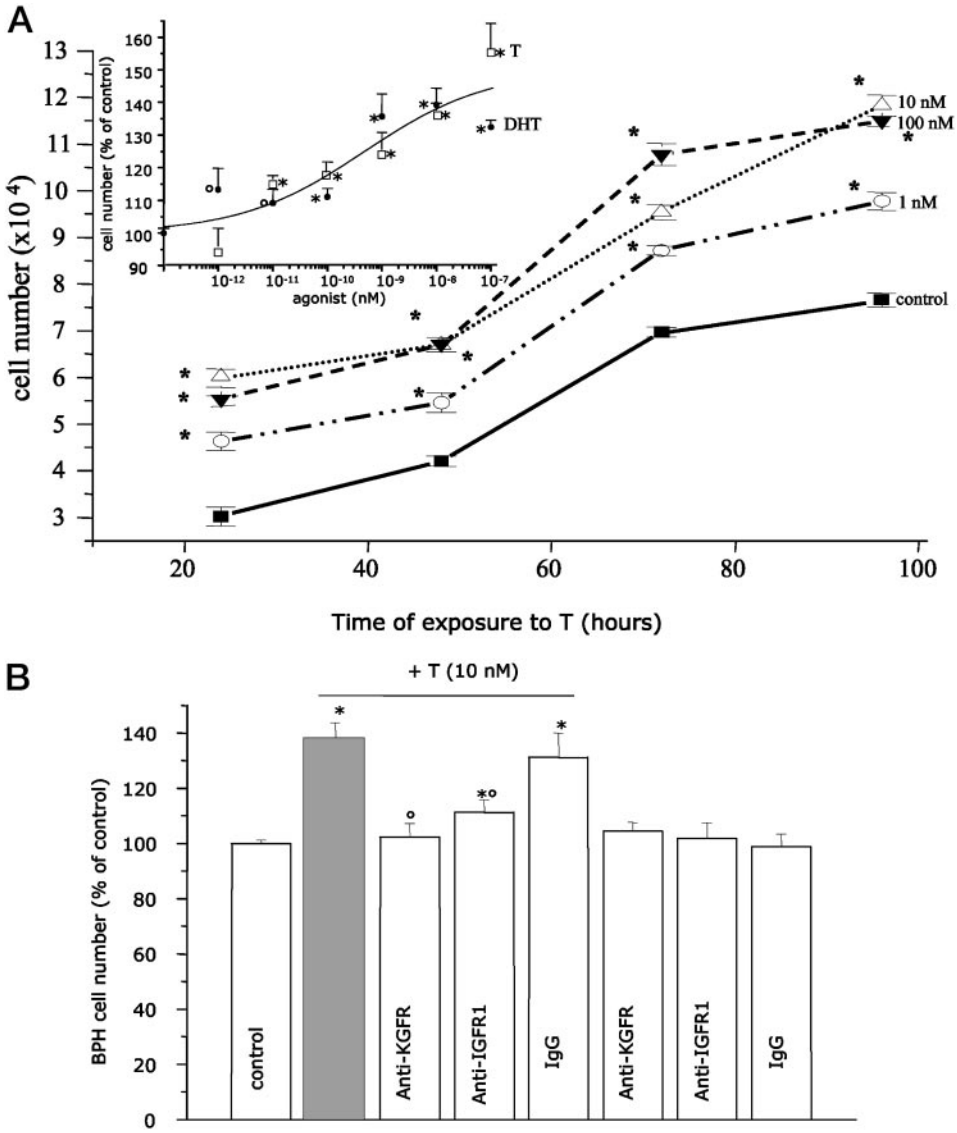

FIG. 1. Effect of androgens on BPH cell growth. A, Time course (24–96 h) of BPH cell proliferation in the absence (control; ■) or presence of different doses of T (○, 1 nM; △, 10 nM; ▼, 100 nM). Results are the mean ± SEM of three separate experiments performed in quadruplicate (*, $P < 0.01$ *vs.* control). *Inset*, Effect of 48-h incubation with increasing concentrations (10^{-12}–10^{-7} nM) of T (□) or DHT (●) on BPH cell proliferation. Results are expressed as the percent increase (mean ± SEM) over their relative controls in three different experiments performed in triplicate. °, $P < 0.05$; *, $P < 0.01$ (*vs.* control). After ALLFIT interpolation, T and DHT exert a similar mitogenic effect in BPH cells, with a 50% effective concentration of 0.38 ± 0.2 nM and a maximum effective concentration of 150 ± 13.8%. B, Effects of anti-KGFR antibody (KGFR Ab; 1 μg/ml) and anti-IGFR1 antibody (IGFR1 Ab; 1 μg/ml) or unrelated immunoglobulins (IgG, 1 μg/ml) on T-stimulated (10 nM) BPH cell growth. After 24-h starvation, BPH cells were stimulated with T and antibodies as indicated for an additional 48 h. Results are expressed as the percent increase (mean ± SEM) over their relative controls in four different experiments performed in quadruplicate. *, $P < 0.01$ *vs.* control; °, $P < 0.01$ vs. T-treated cells.

tration (0.38 ± 0.2 nM) or maximum effective concentration (150 ± 13.8%). T-induced proliferation is at least partially mediated by autocrine growth factors (Fig. 1B). We previously observed that the specific blocking antibodies anti-KGFR and anti-IGFR1 abolished the KGF and des(1–3)-IGF-I (IGF-I analog that does not bind to IGF-binding proteins) mitogenic signaling in BPH cells (15). The same anti-KGFR and anti-IGFR1 antibodies, but not unrelated Igs, significantly blunted T-induced cell proliferation, suggesting the involvement of GFs in mediating T-dependent growth (Fig. 1B; n = 4).

The vitamin D_3 analog BXL-353 also blocked T-induced BPH cell proliferation (Fig. 2). BXL-353, at subfemtomolar concentrations [50% inhibitory concentration (IC_{50}), 0.22 ± 0.14 fM] abrogated the growth stimulatory effect of T and at higher concentrations induced a significant decrease in proliferation (maximum inhibitory concentration, 65.8 ± 4.4%) even when compared with untreated control cells (Fig. 2A; n = 3). This effect was at least in part due to activation of a death program in BPH cells (Fig. 2B; n = 3). The percentage of apoptotic nuclei detected by ISEL dramatically increased after a 48-h exposure to 10 nM BXL-353, affecting more than half of the total cell population. Conversely, treatment with T significantly reduced the number of BPH cells committed to death. The simultaneous incubation of T (10 nM) and BXL-353 (10 nM) partially decreased BXL-353-induced apoptosis; however, more than 40% of cell nuclei still showed fragmentation (n = 3). Thus, BXL-353 blocked the mitogenic and antiapoptotic properties of T in BPH cells. Figure 3 shows a comparison between the antiandrogenic activity of BXL-353 (1 nM) and that of well-characterized antiandrogen compounds, such as the 5αR inhibitor F (1 nM) or the AR antagonist cyproterone acetate (100 nM), on T- and DHT-stimulated (10 nM) BPH cell growth. As expected, although cyproterone acetate completely counteracted the proliferative activity of both T and DHT, F was effective only against T. In contrast to F, BXL-353 completely blocked both T- and DHT-stimulated BPH cell growth and again significantly reduced cell proliferation even when compared with untreated cells (n = 3).

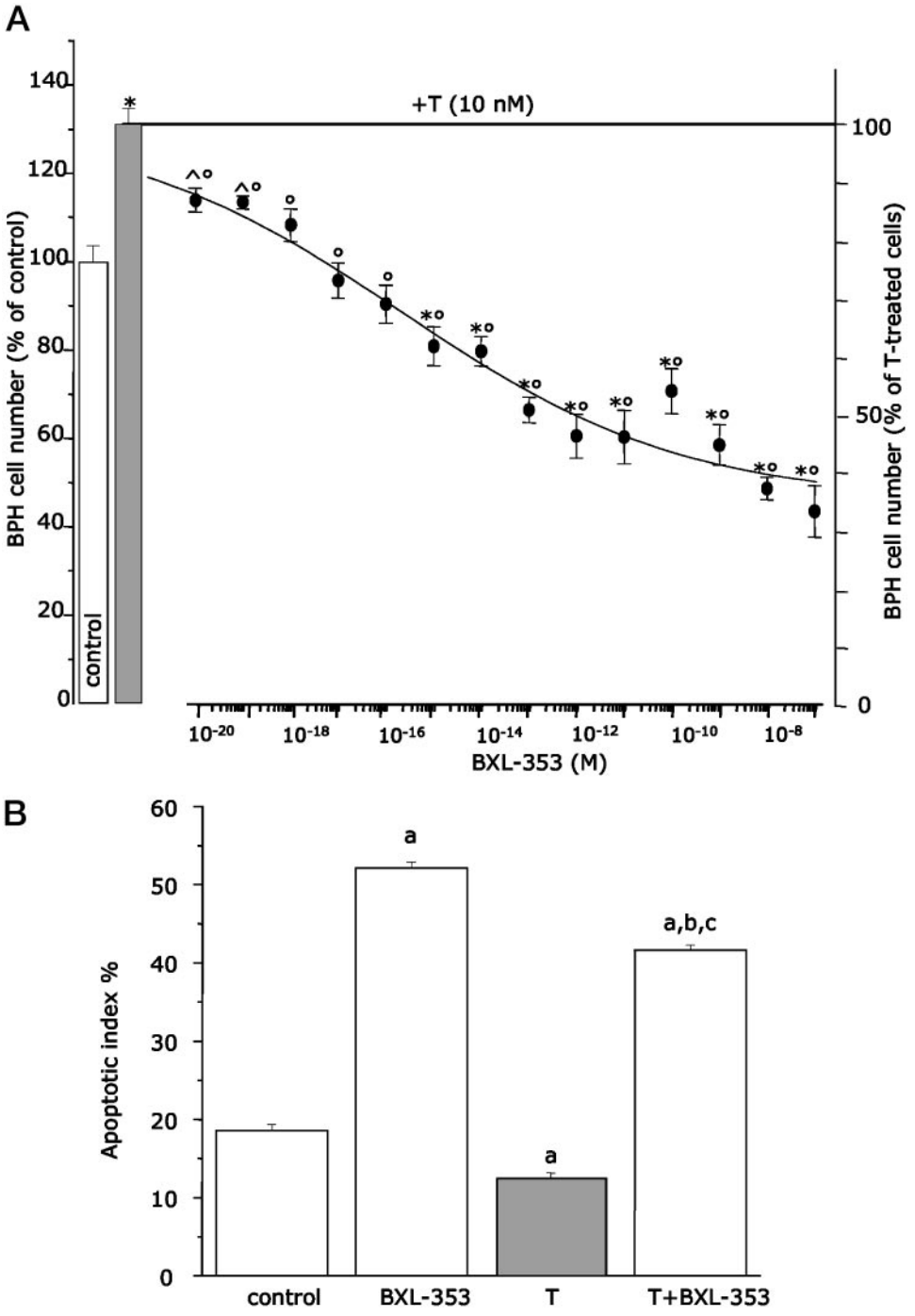

FIG. 2. Effect of BXL-353 on BPH cell proliferation and apoptosis. A, Effect of 48-h incubation with increasing concentrations (10^{-20}–10^{-7} M) of BXL-353 on T-stimulated (10 nM) BPH cell proliferation (IC_{50} = 0.22 ± 0.14 fM; maximum inhibitory concentration, 65.8 ± 4.4%). Results are expressed as the percent variation (mean ± SEM) over their relative controls (□; see *left* ordinate axis) or over the maximal T stimulation [10 nM (■); see *right* ordinate axis] in three different experiments performed in triplicate. ^, $P < 0.05$; *, $P < 0.01$ (*vs.* control). °, $P < 0.01$ (*vs.* T-treated cells). B, Effects of BXL-353 (10 nM), T (10 nM), and equimolar concentrations (10 nM) of T and BXL-353 on DNA fragmentation on BPH cells as detected by ISEL. The apoptotic index (percentage) represents the number of stained nuclei over BPH cells in each of at least five separate fields per slide. Results are expressed as the mean ± SEM in three separate experiments. a, $P < 0.01$ (*vs.* control). b, $P < 0.01$ (*vs.* BXL-353-treated cells). c, $P < 0.01$ (*vs.* T-treated cells).

In vivo effects of androgens, antiandrogens, and BXL-353 on prostate growth

To verify whether BXL-353 could counteract the T growth stimulatory effect in *in vivo* models, we first orally treated T-replaced castrated rats with increasing concentrations of this compound or F for different time periods (Fig. 4). As shown in Fig. 4, A and B, castration dramatically reduced ventral prostate weight, whereas T enanthate administration (30 mg/kg) restored it partially (after 1 wk) or completely (after 2 wk). After 1 wk of T administration (Fig. 4A), BXL-353 dose-dependently reduced T-induced prostate growth, with a maximal effect (44% inhibition) at 5 μg/kg. In this model, F (10 mg/kg) was ineffective in abrogating androgen stimulation. After 2 wk of T treatment (Fig. 4B), BXL-353 was still able to significantly reduce T-stimulated rat ventral prostate growth, with a maximal effect at 30 μg/kg (35% inhibition). This inhibition was comparable to that induced by 3 mg/kg F, but was lower than the inhibition obtained with higher doses of F (10 and 40 mg/kg). In intact adult rats (Fig. 4C), chronic administration (1 month) of both BXL-353 and F significantly reduced prostate weight. Maximal inhibition (38%) was obtained with the highest dose of BXL-353 (30

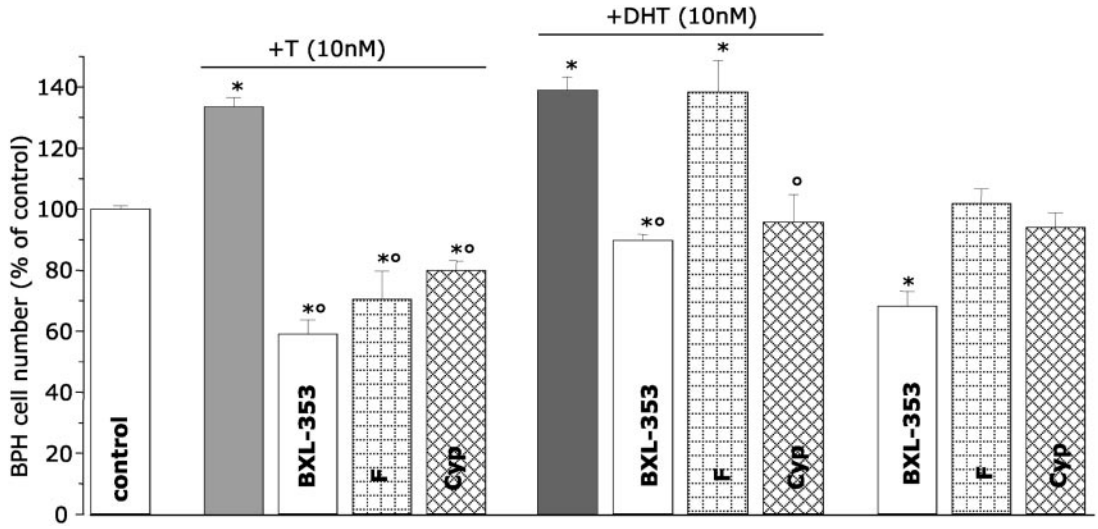

FIG. 3. Effects of antiandrogens and BXL-353 on androgen-stimulated BPH cell growth. After 24 h starvation, BPH cells were incubated for 48 h with BXL-353 (1 nM) or antiandrogens [F, 1 nM; cyproterone acetate (Cyp), 100 nM] in the presence or absence of T (10 nM) or DHT (10 nM). Results are expressed as the percent variation (mean ± SEM) over their relative controls in four different experiments performed in quadruplicate. *, $P < 0.01$ (*vs.* control). °, $P < 0.01$ (*vs.* androgen-treated cells).

μg/kg), an effect comparable to that exerted by high doses of F. Importantly, in all of the experimental protocols, oral administration of different doses of BXL-353 did not cause hypercalcemia (Table 1) or any other discernible toxicity.

BXL-353 up-regulates clusterin gene and protein expression in rat ventral prostate

Clusterin is a glycoprotein overexpressed in rat ventral prostate undergoing organ involution and cell atrophy after different protocols of androgen ablation (23), including F administration (25). Accordingly, as shown in Fig. 5A, castration dramatically up-regulated clusterin mRNA abundance in the rat ventral prostate. The effect of orchidectomy was completely reversed by a 2-wk administration of T. However, simultaneous treatment with both BXL-353 (30 μg/kg) and F (40 mg/kg) partially blunted the effect of T. In intact rats (Fig. 5B), chronic administration (1 month) of both BXL-353 (30 μg/kg) and F (40 mg/kg) induced a similar increase in clusterin gene expression. As shown in Fig. 5C, the effect of BXL-353 on clusterin mRNA abundance in the ventral prostate of intact rats was time and dose dependent. It was apparent after the first week of treatment with the highest dose of BXL-353 (30 μg/kg) and was evident after 2 wk with both doses tested (3 and 30 μg/kg). In intact rats, clusterin protein also increased after either 1 or 2 wk of treatment with 30 μg/kg BXL-353 (Fig. 5D).

BXL-353 induces atrophy and apoptosis in rat ventral prostate

Next we analyzed the effects of BXL-353 on the morphology of the ventral prostate from intact and castrated rats (Fig. 6). Clusterin expression, as revealed by immunohistochemistry, is reported in the *upper panels* (Fig. 6, A–F), and nuclear fragmentation, as detected by TUNEL, is shown in the *bottom panels* (Fig. 6, G and H). Figure 6A shows a control section; in the prostate of intact rats, clusterin labeling was virtually absent. Orchidectomy induced, after only 4 d, marked atrophy of the ventral prostate, characterized by a homogeneous and intense positivity for clusterin in the flattened epithelial cells of the gland (Fig. 6B). Figure 6C shows the effect of 2-wk administration of the lowest dose of BXL-353 (3 μg/kg) to intact rats. Clusterin was expressed in the majority of the epithelial cells, even in the absence of evident morphological hallmarks of atrophy. Conversely, after the same length of exposure, the highest dose of BXL-353 (30 μg/kg) induced diffuse epithelial atrophy and clusterin expression (Fig. 6D). Figure 6E emphasizes, at a higher magnification, the clusterin labeling of the cuboidal atrophic cells of the glandular epithelium. Figure 6F shows that in T-replaced castrated rats, BXL-353 administration for 2 wk induced the appearance of clusterin staining only in the scanty atrophic epithelial cells. It is interesting to note that apoptosis was clearly evident in all preparations studied after treatment with BXL-353, as shown in Fig. 6G (intact rats) or Fig. 6H (T-replaced castrated rats). Evident nuclear fragmentation was also detected in stromal cells (Fig. 6G).

BXL-353 does not affect 5αR or AR activity

Because we found that BXL-353 and F exerted similar antiandrogenic effects both *in vitro* and *in vivo*, we examined the possibility that BXL-353 might also effect 5αR activity. Therefore, we tested the ability of BXL-353 and F to inhibit 5αR activity in CHO cells transfected with type 1 (Fig. 7A) and type 2 (Fig. 7B) 5αR isoenzymes. Although F inhibited T conversion into DHT with the expected IC_{50}, BXL-353 did not affect the activity of both subtypes of 5αR up to the micromolar range (n = 3). In addition, to rule out the possibility that BXL-353 might directly interact with the AR, we performed binding studies in human BPH homogenates using [^{3}H]R1881. Figure 7C shows displacement curves obtained in BPH prostate homogenates (n = 3). LIGAND analysis (26) of results from three separate experiments indicated

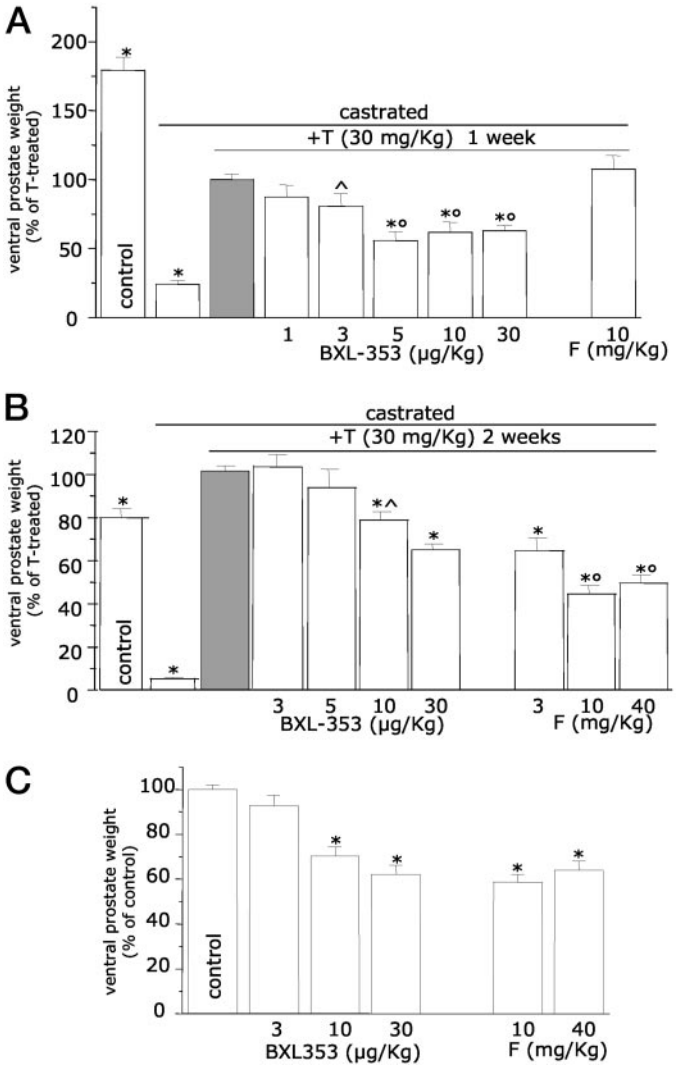

FIG. 4. Effect of BXL-353 or F on rat ventral prostate weight. A, Castrated rats were supplemented with a single injection of T enanthate (30 mg/kg·wk) and treated orally for 4 d with vehicle or increasing concentrations of BXL-353 (1–30 μg/kg) or F (10 mg/kg). Ventral prostate weight is expressed as the percent variation (mean ± SEM) in weight of T-replaced castrated rats in two separate experiments. Ventral prostate weight in vehicle-treated intact (control) or castrated rats is also reported. ^, $P < 0.05$; *, $P < 0.01$ (*vs.* T-supplemented vehicle-treated rats). °, $P < 0.01$ (*vs.* T-supplemented F-treated rats). *B*, Castrated rats were injected with T enanthate (15 mg/kg·wk) and treated orally for 5 d/wk for 2 consecutive wk with vehicle or increasing concentrations of BXL-353 (3–30 μg/kg) or F (3–40 mg/kg). Ventral prostate weight is expressed as the percent variation (mean ± SEM) in weight of T-replaced castrated rats (n = 4). Ventral prostate weight in vehicle-treated intact (control) or castrated rats is also reported. *, $P < 0.01$ (*vs.* T-supplemented vehicle-treated rats). °, $P < 0.01$; ^, $P < 0.05$ [*vs.* T-supplemented BXL-353 (30 μg/kg)-treated rats]. C, Intact adult rats were treated orally for more than 1 month (5 times/wk for a total of 27 administrations) with vehicle (control) or increasing concentrations of BXL-353 (3–30 μg/kg) or F (10 and 40 mg/kg). Ventral prostate weight is expressed as the percent variation (mean ± SEM) in weight of control rats (n = 4). *, $P < 0.01$ (*vs.* control rats).

that R1881 ($K_d = 0.18 \pm 0.06$ nM), DHT ($K_d = 0.17 \pm 0.08$ nM), T ($K_d = 1.6 \pm 0.86$ nM), and the AR antagonist bicalutamide ($K_d = 178 \pm 84$ nM) completely displaced [^{3}H]R1881 binding.

TABLE 1. Calcemia (milligrams per deciliter) in T-replaced castrated rats after 1 wk of treatment with different doses of BXL-353

Castrated rats (1 week)	Serum calcium
Control	9.72 ± 0.18
BXL-353 (1 μg/kg)	9.02 ± 0.27
BXL-353 (3 μg/kg)	8.96 ± 0.27
BXL-353 (5 μg/kg)	9.76 ± 0.31
BXL-353 (10 μg/kg)	8.94 ± 0.26
BXL-353 (30 μg/kg)	10.28 ± 0.23

One-week treatment with different doses (1, 3, 5, 10, and 30 μg/kg) of BXL-353 did not change calcium serum levels in castrated rats replaced with T enanthate (30 mg/kg·wk) compared with controls. Results are the mean ± SEM of rats per group [five to eight animals per group (as specified in *Materials and Methods*)]. Similar results were obtained at the other time points or in in intact animals (not shown).

Conversely, BXL-353 did not compete for [^{3}H]R1881 binding at any of the concentrations tested (up to 100 μM; n = 3).

BXL-353 does not change rLH and T serum levels

To investigate whether BXL-353 reduced prostate growth by interfering with pituitary or testis function, we measured rLH and T serum levels in castrated or intact rats after treatment with different doses of BXL-353 (3, 10, and 30 μg/kg) or F (10 mg/kg). As shown in Table 2B, castration significantly reduced T and increased rLH serum levels. A 2-wk administration of T enanthate (30 mg/kg) significantly reduced rLH concentrations and restored serum T levels to those observed in control rats. BXL-353 at any dose tested failed to significantly affect either rLH or T serum levels. Similarly, chronic administration (1 month) of different doses of BXL-353 (3, 10, and 30 μg/kg) did not modify rLH and T serum levels in intact rats (Table 2A).

Discussion

Our results show, for the first time, that a vitamin D analog, BXL-353, reduces *in vitro* and *in vivo* prostate growth by decreasing cell proliferation and inducing apoptosis, even in the presence of T, the most important trophic factor for the prostate gland.

Vitamin D_3 and its active metabolite, calcitriol, are secosteroid hormones modulating calcium homeostasis through actions on kidney, bone, and the intestinal tract. Increasing evidence accumulated during the last decade indicates that the prostate gland is also a target of secosteroids. The human prostate expresses the vitamin D receptor (VDR) (28), and calcitriol inhibits the *in vitro* growth of both epithelial (29) and stromal (11) BPH cells. In addition, calcitriol exhibits antiproliferative and prodifferentiating activities in malignant prostate cell lines (30–32) and in some *in vivo* models of prostate cancer (33, 34). It is presumed that these effects are essentially due to a double action of calcitriol: a G_o/G_1 arrest in the cell cycle progression and an induction of apoptosis, by increasing, respectively, cyclin-dependent kinase inhibitors and the ratio of survival factors such as Bax/Bcl-2 (see Refs. 35 and 36 for reviews). In addition, recent evidence indicates that calcitriol is able to inhibit angiogenic signaling between endothelial and cancer cells (37). Based on these observations, clinical trials of calcitriol as a

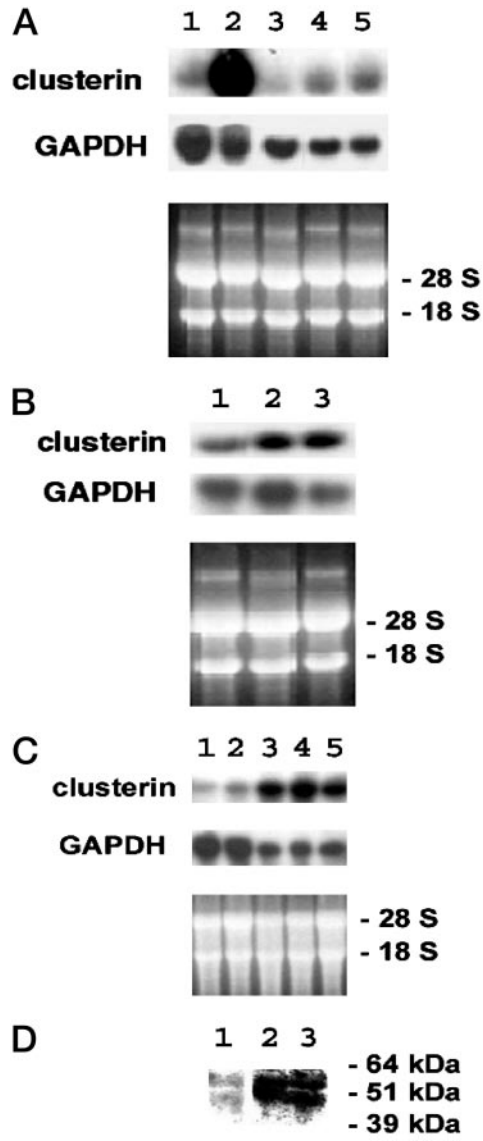

FIG. 5. Effect of BXL-353 and F on clusterin gene and protein expression in the rat ventral prostate. A, Northern analysis of clusterin mRNA expression in the ventral prostate of vehicle-treated intact (lane 1) or castrated (lane 2) rats. Lanes 3–5, Clusterin gene expression in orchidectomized rats supplemented for 2 wk with T enanthate (30 mg/kg) and orally treated with vehicle (lane 3), BXL-353 (30 μg/kg; lane 4), or F (40 mg/kg; lane 5). Every lane was loaded with 10 μg total RNA. The corresponding GAPDH expression and ethidium bromide staining of the gel are shown *below* the blot. The blot is representative of two separate experiments. B, Northern analysis of clusterin mRNA expression in the ventral prostate of adult intact rats treated orally for more than 1 month (5 times/wk, 27 administrations) with vehicle (lane 1), BXL-353 (30 μg/kg; lane 2), or F (40 mg/kg; lane 3). Every lane was loaded with 10 μg total RNA. The corresponding GAPDH expression and the ethidium bromide staining of the gel are shown *below* the blot. The blot is representative of two separate experiments. C, Northern analysis of clusterin mRNA expression in the ventral prostate of intact adult rats orally treated for 1 wk (4 administrations) with vehicle (lane 1) or different doses of BXL-353 (3 and 30 μg/kg; lanes 2 and 4, respectively). Effect of 2-wk oral treatment (9 administrations) of different doses of BXL-353 (lane 3, 3 μg/kg; lane 5, 30 μg/kg) is also shown. Every lane was loaded with 10 μg total RNA. The corresponding GAPDH expression and the ethidium bromide staining of the gel are shown *below* the blot. The blot is representative of two separate experiments. D, Western blot detection of clusterin protein in the ventral prostate of adult intact rats treated for 1 wk with vehicle (lane 1) or BXL-353 (30 μg/kg; lane 2). Results after 2-wk treatment (9 administration) with BXL-353 (30 μg/kg) are shown in lane 3. All lysates were obtained as described in *Materials and Methods*, and 30 μg proteins were separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and probed for clusterin expression with antirat clusterin polyclonal antibody (1:100). Molecular mass markers (kilodaltons) are indicated to the *right* of the blot. The blot is representative of two separate experiments.

treatment for human prostate cancer have been started (38, 39). Although preliminary results were promising, a major drawback of calcitriol is the dose-limiting effect of hypercalcemia and hypercalciuria, which prevents the administration of pharmacological doses of the hormone. Therefore, analogs of calcitriol that retain antiproliferative activity without exhibiting side-effects on calcium metabolism have been developed. Among these analogs, BXL-353 is one of the most effective in inhibiting growth and promoting differentiation in several *in vivo* (40, 41) and *in vitro* (16, 42) experimental models of prostate cancer. In particular, BXL-353 reduced the proliferation of prostate cancer cell lines and induced apoptosis even in the presence of potent growth factors, such as KGF (16). BXL-353 was able to reduced KGF-induced Bcl-2 overexpression and cell survival by directly interfering with KGFR autophosphorylation (16). Hence, we hypothesized a novel mechanism to explain the antiproliferative effect of vitamin D analogs in prostate cells: disruption of prostate GF signaling.

We recently observed that GF signaling was blocked by BXL-353 not only in malignant prostate cancer cells (16) but also in nonmalignant prostate cells derived from BPH patients (11, 15). Therefore, we asked whether BXL-353 might also antagonize the growth stimulatory properties of the major GF for normal prostate cells, T. If this is the case, BXL-353 could be considered as a treatment for human BPH. For *in vitro* studies we employed BPH cells, which are not only positive for the AR gene and protein, but are also responsive to androgen stimulation with a time- and dose-dependent growth increase. Moreover, BPH cells express both subtypes of 5αR and are sensitive to F treatment.

In this study, we report that BXL-353, even at subpicomolar concentrations, completely antagonized T-stimulated BPH cell growth and, in a rat model, reduced T-induced ventral prostate growth to an extent comparable to that produced by F. In addition, like F, BXL-353 blunted the effect of T on castration-induced clusterin mRNA up-regulation in rat ventral prostate and induced a time- and dose-dependent increase in clusterin gene and protein expression in intact rats, with a concomitant decrease in prostate size. Clusterin is a ubiquitous sex steroid-down-regulated protein (23, 43, 44) with as yet unknown functions. In the prostate, clusterin expression is clearly inversely related to progression in the cell cycle (45, 46) and directly related to gland atrophy (25, 47). Transient overexpression of clusterin induced cell cycle arrest and decreased DNA synthesis in human prostate epithelial cells (45). As previously described (47), immunopositivity for clusterin was virtually absent in the normal rat ventral prostate, whereas a dramatic increase in labeling was observed soon after orchidectomy or F administration (25). Similarly, BXL-353 administration strongly increased the

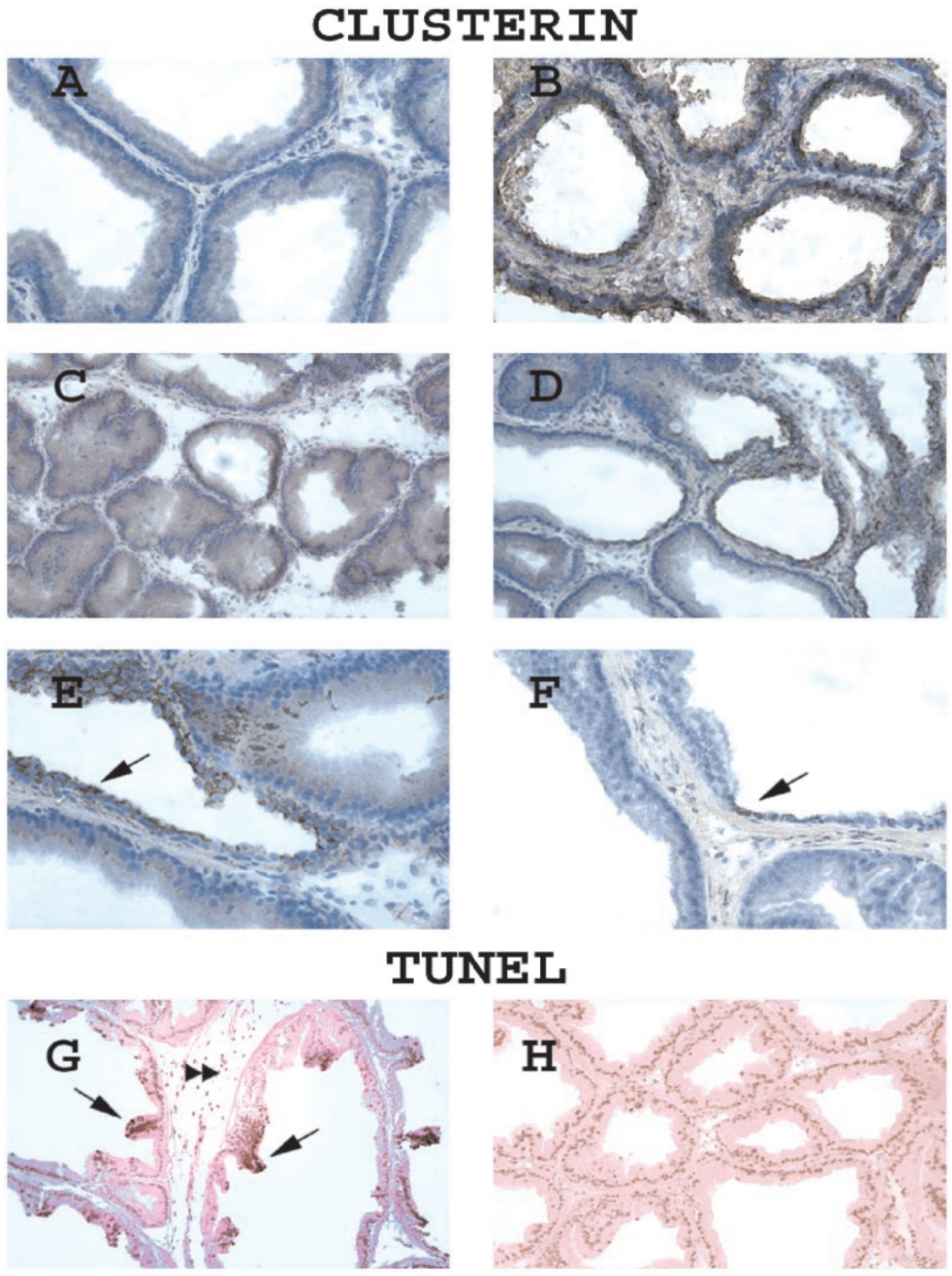

FIG. 6. Morphological effects of BXL-353 on rat ventral prostate. A–F, Representative fields obtained from cross-sections (14 μm) of whole prostate glands immunostained with a monoclonal antibody against rat clusterin and counterstained with hematoxylin. In vehicle-treated intact adult rats, clusterin labeling is barely detectable in the cytoplasm of a few epithelial cells (A; magnification, ×10). After orchidectomy (B; magnification, ×10), almost all of the cuboidal atrophic epithelial cells were intensively stained. Some stromal cells were also labeled. After 2-wk (nine administrations) treatment of intact rats with the lowest dose of BXL-353 (3 μg/kg), almost all of the columnar epithelial cells of the glands were positive for clusterin (C; magnification, ×10), without evident morphological hallmarks of atrophy. Conversely, the highest dose of BXL-353 (30 μg/kg) induced both epithelial gland atrophy and clusterin expression (D; magnification, ×10). E, Higher magnification (×20) of results obtained under the same experimental conditions as those in D. The *arrow* indicates typical atrophic cuboidal cells of the glandular epithelium with an evident clusterin staining. F, Typical results obtained in the ventral prostate of castrated and T-supplemented rats after 2-wk treatment (nine administrations) with BXL-353 (30 μg/kg). In T-treated glands, BXL-353 administration induced scanty foci of atrophy and clusterin staining (*arrow*). G and H, DNA fragmentation in rat prostate cryosections as assessed by TdT-mediated deoxy-UTP nick end-labeling (TUNEL). Two-week treatment (nine administrations) of intact (G; magnification, ×10) or T-treated castrated rats (H; magnification, ×10) with BXL-353 (30 μg/kg)-induced apoptosis in the majority of epithelial and stromal cells.

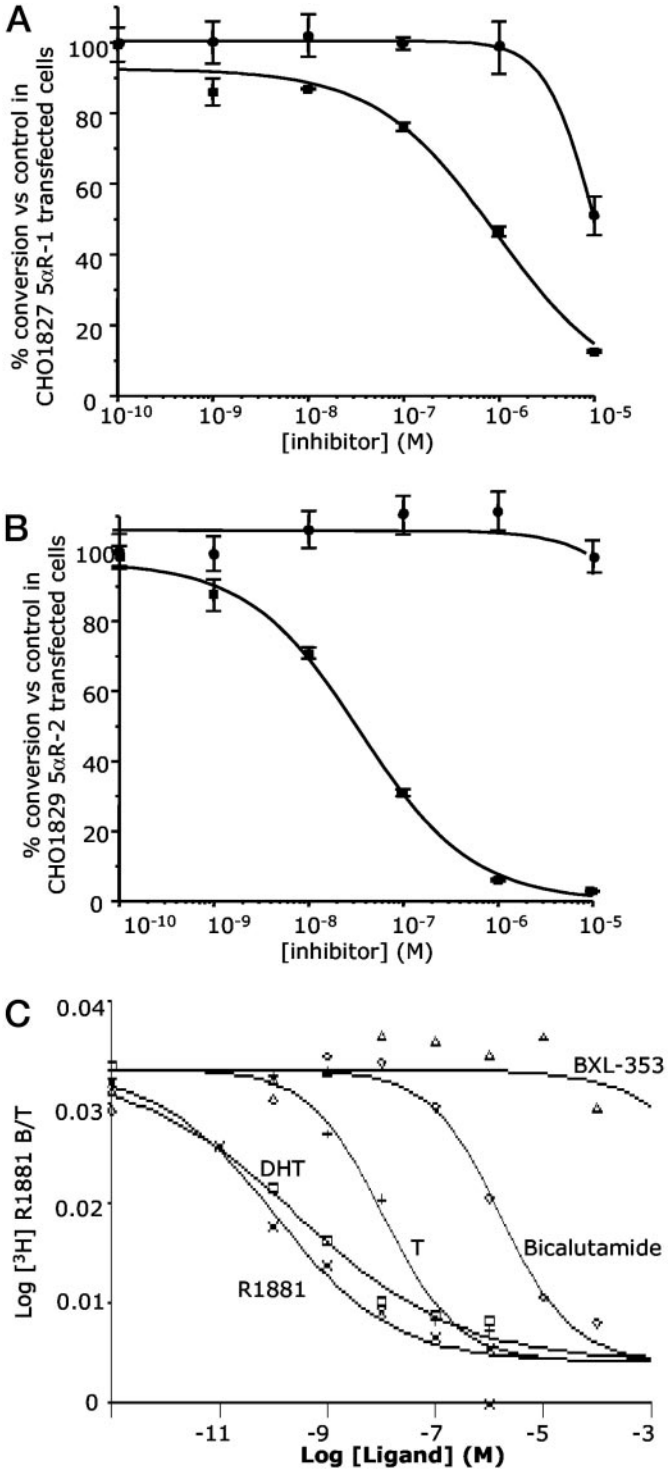

FIG. 7. Lack of antiandrogenic properties by BXL-353. A and B, Inhibition curves toward 5αR activity in CHO 1827 and CHO 1829, stably transfected with type 1 (A) or type 2 (B) isoenzymes, respectively, were measured in the presence of increasing concentration (10^{-10}–10^{-5} M) of BXL-353 (●) or F (■). While F inhibits 5αR activity with the expected IC_{50} (type 1 isoenzyme: $IC_{50} = 0.9 \pm 0.2$ μM; type 2 isoenzyme: $IC_{50} = 35 \pm 5$ nM), BXL-353 did not interfere with the conversion up to the micromolar range (type 1 isoenzyme: $IC_{50} = 10.2 \pm 0.29$ μM; type 2 isoenzyme: $IC_{50} > 10$ μM). Similar results were obtained in two other separate experiments. C, Effects of increasing concentrations of BXL-353 and several AR ligands on [^{3}H]R1881 binding to human prostate homogenates. Cytosol preparations (n = 3) of human prostate (obtained at surgery from patients affected by BPH) were incubated in the presence of [^{3}H]R1881 and increasing concentrations of the corresponding unlabeled ligand (X), DHT (□), T (+), bicalutamide (◇), and BXL-353 (△). Although all of the AR ligands displaced [^{3}H]R1881 binding as expected (R1881: $K_d = 0.18 \pm 0.06$ nM; DHT: $K_d = 0.17 \pm 0.08$ nM; T: $K_d = 1.6 \pm 0.86$ nM; bicalutamide: $K_d = 178 \pm 84$ nM), BXL-353 did not compete at any of the concentrations tested (up to 100 μM).

TABLE 2. rLH (nanograms per milliliter) and T (nanomolar concentrations) serum levels in intact or T-replaced castrated rats after treatment with different doses of BXL-353

	rLH	T
A. Intact rats		
Control	3.16 ± 0.51	7.20 ± 1.95
F	2.85 ± 0.30	8.55 ± 1.26
BXL-353 (3 μg/kg)	3.46 ± 0.27	8.18 ± 1.82
BXL-353 (10 μg/kg)	4.2 ± 0.13	7.67 ± 1.77
BXL-353 (30 μg/kg)	2.92 ± 0.2	6.92 ± 1.67
B. Castrated rats		
Control	3.74 ± 0.61	4.4 ± 0.54
Castrated	25.18 ± 3.76[a]	1.65 ± 0.12[a]
T-replaced	7.2 ± 2.23	3.02 ± 0.56
F	4.44 ± 1.14	4.61 ± 0.74
BXL-353 (3 μg/kg)	5.52 ± 1.86	5.38 ± 1.33
BXL-353 (10 μg/kg)	6.28 ± 1.40	2.98 ± 0.47
BXL-353 (30 μg/kg)	5.88 ± 2.11	3.92 ± 0.30

A, Chronic administration (1 month) of F (40 mg/kg) or BXL-353 (3, 10, and 30 μg/kg) did not change rLH and T serum levels in intact rats. B, In castrated rats serum T is significantly reduced, whereas serum rLH significantly increased ([a], $P < 0.01$ *vs.* control). After 2-wk treatment with T enanthate (30 mg/kg·wk) rLH concentrations were significantly reduced, and T serum levels were restored as in control rats. BXL-353 at all doses tested did not significantly affect either rLH or T serum levels.

number of clusterin-positive cells, in parallel with enhanced morphological evidence of glandular atrophy. BXL-353 was also able to increase the number of prostate cell doomed to die. Indeed, in both BPH cells and rat prostate, BXL-353 induced a massive increase in DNA fragmentation, as detected by ISEL and TUNEL, respectively. Similar results were previously reported in rat ventral prostate after treatment with F (48). However, at variance with F, BXL-353 did not affect 5αR-1 or 5αR-2 activity in CHO cells transfected with either 5αR isoenzyme subtype. Furthermore, the antiproliferative effect of BXL-353 in BPH cells was evident not only in T-stimulated, but also in DHT-stimulated, cells, as observed with the AR antagonist cyproterone acetate. However, BXL-353 does not bind to the human AR. Hence, although BXL-353 behaves as an antiandrogen, it does not interfere directly with the AR. Interestingly, BXL-353 administration to either castrated or intact rats does not affect pituitary and gonadal release of rLH and T. In addition, previous studies have indicated that VDR ligation upregulates, rather than represses, the AR gene and protein expression (49, 50). It is therefore possible that BXL-353 counteracts the mitogenic action of androgens in the prostate by acting downstream from the AR. One possible target for BXL-353 is androgen-dependent intraprostatic GF signaling. We have previously demonstrated that both KGFR and IGFR1 activities are involved in BPH cell proliferation (9, 11, 15). Antibodies against both types of receptors not only com-

pletely abrogated the mitogenic effect of their specific agonist (15), but also blunted the proliferative activity of T, as shown in the present study. Hence, it is possible that BXL-353 also interferes with the androgen activity on prostate growth by disrupting intraprostatic GF signaling (11, 15, 16). An explanation for the present and previous findings is that the VDR, via an extranuclear/nongenomic effect, is functionally coupled to one or more phosphatase activities, and that VDR interaction with its ligand promotes a series of protein kinase dephosphorylations. Indeed, calcitriol and its analogs induced a prompt decrease not only in phosphorylated KGFR (11, 16), but also in phospho-Erk and phospho-Akt (51, 52). Consistent with this hypothesis, recent evidence (51) indicates that the VDR, upon ligand binding, physically interacts with the catalytic subunit of some protein phosphatases, such as PP1 and PP2Ac, promoting their enzymatic activities. This implies, for instance, the consequent inactivation of $p70^{S6k}$, a kinase essential in the G_0/G_1 transition (53). The activated AR is also a multiple phosphorylated protein, and some of its phosphorylation sites (such as Ser^{650}) are required for full transcriptional activity (see Ref. 54 for review). Hence, it is possible that calcitriol (and some of its analogs) might activate the catalytic subunit of distinct families of phosphatases, exerting antiproliferative effects on AR-GF signaling or transcriptional activities. This hypothesis might explain why BXL-353, but not the other antiandrogens tested, also reduced the growth of untreated BPH cells and induced apoptosis (and Bcl-2 down-regulation) even in the absence of GF (11, 15, 16) or T itself (present study).

In conclusion, BXL-353, at nonhypercalcemic doses, counteracts in normal prostate cells AR-induced proliferation and under several experimental conditions is comparable to F. Hence, its clinical use to reduce prostate size might be postulated. Because BXL-353 does not bind to the AR nor does it interfere with DHT formation, it should not share with other antiandrogens sexual side-effects, such as decreased libido, altered sexual potency, or ejaculatory dysfunctions (55), that may limit the patient compliance for 5αR-2 inhibitors (4) as well as dual 5αR inhibitors, such as dutasteride (56). Because the prostate gland is one of the few AR targets retaining the plasticity to respond to T with a rather continued proliferation throughout the entire life span (1), the antiandrogenic activity of BXL-353 could be limited to the inhibition of prostate gland growth. The present results indicate that BXL-353 or other well-tolerated calcitriol analogs might therefore represent a new class of drugs beneficial for the most common urological problem of the aging male, BPH.

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Address all correspondence and requests for reprints to: Prof. Mario Maggi, Dipartimento di Fisiopatologia Clinica, Unità di Andrologia, Università di Firenze, Viale Pieraccini 6, 50139 Florence, Italy. E-mail: m.maggi@dfc.unifi.it.

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