

EXPERIMENTAL STUDY

Inhibition of prostate cell growth by BXL-628, a calcitriol analogue selected for a phase II clinical trial in patients with benign prostate hyperplasia

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Abstract

Objective: Calcitriol analogues might represent an interesting new therapy for benign prostate hyperplasia (BPH). We here report the preclinical characterization of BXL-628, an analogue selected for an ongoing double-blind, randomized, placebo-controlled phase II trial in BPH.

Design: Experiments with BXL-628 were carried out in human BPH cells and in the ventral prostate of intact and castrated rats.

Methods: BPH cell and rat prostate growth were evaluated along with morphological and biochemical hallmarks of apoptosis.

Results: BXL-628 inhibited human BPH cell proliferation and induced apoptosis even in the presence of androgens or growth factors. It also decreased prostate growth to an extent similar to finasteride, inducing DNA fragmentation and apoptosis, both in intact and in testosterone-supplemented castrated rats. Accordingly, BXL-628, like finasteride, increased the expression of clusterin, a prostatic atrophy marker. However, BXL-628 did not inhibit 5 α -reductase 1 and 2, did not bind to the androgen receptor (AR) in BPH homogenates and did not affect AR-coupled luciferase activity. In addition, BXL-628 did not affect rat pituitary and testis activity or calcemia.

Conclusions: BXL-628 inhibited *in vitro* and *in vivo* prostate cell proliferation, and therefore might represent a novel, interesting option for the treatment of BPH.

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Introduction

The active form of vitamin D, calcitriol (1,25-(OH)₂D₃), is a secosteroid hormone originally identified as a key regulator of bone metabolism and calcium homeostasis. The biological actions of calcitriol have been subsequently shown to extend well beyond these classical functions to include regulation of immunity and angiogenesis, and growth, differentiation and apoptosis of many cell types, including malignant cells (1–5). Because human (6, 7) and rat (7) prostate cells express the vitamin D receptor (VDR) and respond to VDR agonists by decreasing their proliferation (6) we originally hypothesized (8) that VDR ligands could represent a novel option for the treatment of benign prostatic hyperplasia (BPH), a non-neoplastic enlargement of the prostate extremely common in the aging male. However, a problem with the therapeutic use of

calcitriol is its natural ability to induce hypercalcemia and hyperphosphatemia. Hence, analogues of calcitriol retaining biological activity but devoid of hypercalcemic side-effects have been developed and some of them approved for the treatment of secondary hyperparathyroidism (see 9 for review). We have recently reported that a calcitriol analogue, analogue V or BXL-353, not only reduced the growth of human BPH cells, but also induced a death program by decreasing the expression of the survival factor bcl-2 (8). In addition, we found that BXL-353 completely blocked intra-prostatic growth factor (GF) or androgen-induced BPH cell proliferation, most probably by preventing GF receptor phosphorylation and disrupting androgen–GF cross-talk (8, 10, 11). In a model of testosterone-supplemented, orchidectomized rats, this analogue was able to counteract the growth-promoting activity of testosterone by decreasing prostate volume

without affecting calcemia (11). The inhibitory effect of BXL-353 on prostate growth was comparable with that induced by finasteride, a 5α reductase inhibitor that reduces the formation of the active testosterone metabolite, dihydrotestosterone (DHT). Like finasteride, BXL-353 induced apoptosis in the prostate of treated rats and up-regulated a gene closely related to cell death and prostate atrophy, clusterin (11). However, at variance with finasteride or other anti-androgens, BXL-353 did not affect 5α reductase activity, did not bind to the androgen receptor (AR), and did not modify sex hormone production (11). Therefore, because calcitriol analogues did not share with finasteride any anti-androgenic properties, except the anti-proliferative activity on prostate cells, they appear to be free from the unwanted sexual side-effects limiting the popularity of 5α reductase inhibitors in the aging male (12). Hence, non-hypercalcemic calcitriol analogues might represent attractive potential therapeutic agents for BPH.

To test this hypothesis, a phase II clinical trial is ongoing in Italy to examine the capacity of a calcitriol analogue, BXL-628, to reduce prostate volume in patients with BPH. BXL-628 has been shown in pre-clinical and clinical studies to be significantly less hypercalcemic than calcitriol and other analogues. In a dose-ranging study, subjects have been treated with this compound and an abnormal elevation of serum calcium was never observed even at the highest dose, about twofold higher than that used in patients with BPH. We now report preclinical *in vitro* and *in vivo* studies on the capacity of BXL-628 to inhibit prostate cell growth and analyze the mechanisms involved.

Materials and methods

The trial is a multicenter, randomized, double blind, placebo controlled study involving 12 urologic centers across Italy.

Materials

Minimum essential medium (MEM), Dulbecco's MEM-F12 1:1 mixture, Ham's F12 medium, phosphate-buffered saline, bovine serum albumin (BSA) fraction V, glutamine, geneticine, collagenase type IV, vitamin D₃, testosterone, DHT, cyproterone acetate (Cyp), β -nicotinamide adenine dinucleotide 3'-phosphate reduced form, dithiothreitol, phenylmethylsulfonyl fluoride and a kit for measuring calcemia were purchased from Sigma (St Louis, MO, USA). The protein measurement kit was from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Fetal bovine serum was purchased from Unipath (Bedford, Beds, UK). Monoclonal anti-rat clusterin antibody (mouse monoclonal IgG) specific for β -chain was from UPSTATE Biotechnology (Lake Placid, NY, USA). Apop Tag kit for *in situ* end labeling (ISEL) was from Oncor (MD, USA).

CHO 1827 and CHO 1829 were provided by Serono International (Geneva, Switzerland). Instagel plus was purchased from Packard (St Louis, MO, USA). Finasteride (pure substance) (17 β -(N, t-butyl)carbamoyl-4-aza-5 α -androst-1-en-3-one) was a kind gift from Merck Sharp & Dohme Research Laboratories (Rahway, NJ, USA). Bicalutamide was a kind gift from AstraZeneca (Milan, Italy). Analogue 1- α -fluoro-25-hydroxy-16,23E-diene-26,27-bishomo-20-epi-cholecalciferol (BXL-628) was provided by BioXell (Milan, Italy). Keratinocyte growth factor (KGF) was from Pepto Tech EC (London, UK) and insulin-like growth factor-I, human (Des(1-3)IGF-I) was purchased from GroPep Limited (Adelaide, Australia). *In Situ* Cell Death Detection Kit Peroxidase for terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was from Roche Diagnostics Corporation (Indianapolis, IN, USA). Plastic ware for cell cultures was purchased from Falcon (Oxnard, CA, USA). Disposable filtration units for growth media preparation were purchased from PBI International (Milan, Italy). Lipofectamine 2000 and Opti-MEM I medium for luciferase transfection were from Invitrogen, Life Technologies (San Giuliano Milanese, Milan, Italy). Thin-layer chromatography silica plates were obtained from Merck (Darmstadt, Germany). Testosterone enanthate was from Geymonat (Anagni, Italy). The Coat-A-Count total testosterone detection kit was purchased from Medical System (Genova Struppa, Italy). Rat luteinizing hormone (rLH) [¹²⁵I] assay systems were from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Cells and tissues

Human BPH cells, assessed and maintained as previously described (8) were obtained from prostate tissues derived from seven patients who had undergone suprapubic adenectomy for BPH, after informed consent and approval by the Local Ethical Committee. Specific antibodies were used to characterize BPH cells. They showed positive staining for 11 α smooth-muscle actin, vimentin and desmin, suggesting fibromuscular morphological features. Conversely, they were negative for epithelial and endothelial markers such as cytokeratin and factor VIII (8).

Prostatic tissues for binding assay were placed in liquid nitrogen immediately after surgery and stored at -80°C until processing. Patients had not received any pharmacological treatment in the 3 months preceding surgery.

CHO-1827 and CHO-1829 cells, transfected with 5α reductase type 1 ($5\alpha\text{R-1}$) or $5\alpha\text{R-2}$ respectively (13), were maintained in Ham's F12 medium supplemented with 5% fetal calf serum.

Human prostate adenocarcinoma PC3 cells were stably transfected with the plasmid p5HbhAR-A containing human AR and maintained as previously described (14).

Rat ventral prostate glands were collected, pooled and processed for either clusterin immunohistochemistry and apoptosis localization by TUNEL, or total RNA extraction as previously reported (11).

***In vitro* assays**

Proliferation assays and hemocytometer counts were designed and performed as previously described (11). Increasing concentrations (10^{-18} to 10^{-7} M) of calcitriol or BXL-628 with or without a fixed concentration of testosterone (10 nM), KGF or Des(1-3)IGF-I (10 ng/ml) were used. Growth assays were also carried out using a fixed concentration of androgens (10 nM) with or without BXL-628 (1 nM and 10 nM), the anti-androgens finasteride (1 nM) and Cyp (100 nM); using a fixed concentration of testosterone (10 nM) or GFs (10 ng/ml) with or without BXL-628 (10 nM). In the same experiment, each experimental point was repeated in triplicate or quadruplicate and experiments were performed three times. Results are expressed as % variation (means \pm S.E.M.) over control or over the maximal testosterone- or GF-induced stimulation, as appropriate.

The 5α reductase inhibition assay was performed using CHO 1827 cells transfected with 5α R-1 or CHO 1829 cells transfected with 5α R-2 as described (15). BXL-628 was added in a concentration range from 10^{-9} to 10^{-5} M, using finasteride as a control inhibitor in each experiment.

Binding assays on cytosol fractions of BPH fragments were carried out as previously reported (11), using a final protein concentration of 1.8 mg/ml. Protein content was determined by the method of Bradford (16), using BSA as a standard.

For luciferase assay, AR-transfected PC3 cells were transfected with luciferase gene as previously reported (17) and according to the manufacturer's instructions. After 48 h, the cells were incubated with DHT (10^{-12} to 10^{-6} M) or bicalutamide (10^{-9} to 10^{-5} M) in the presence of 3 nM DHT, and with equimolar concentrations of BXL-628 for 18 h. Steroids and BXL-628 analogue were dissolved in ethanol. Transfected cells incubated with only ethanol served as controls.

The luciferase assay was performed with a Berthold luminometer according to the manufacturer's instructions (Luciferase Assay System; Promega, Milan, Italy). Total protein measurement was performed by the Bradford method (16). At least three independent assays were done in duplicate. Results (means of three transfection experiments) are expressed as percentage of bioluminescence activity normalized per μ g total proteins.

Animal protocols

Male Sprague–Dawley rats (28 days old) were purchased from Charles River Laboratories (Calco, Lecco, Italy). All animal experimentation described

was conducted in accord with accepted standards of animal care. Castration procedure and treatment of castrated or intact animals were carried out following the 2-week protocol and the 1-month protocol respectively, as previously described (11). Briefly, castrated rats were given vehicle (miglyol 812), BXL-628 (10, 30, 100 and 300 μ g/kg) or finasteride (10 and 40 mg/kg) for 2 weeks and killed 1 day later. Intact, adult male rats (weight 250 g) were dosed orally with vehicle (miglyol 812), BXL-628 (10, 30, 100 and 300 μ g/kg) or finasteride (10 and 40 mg/kg) for 1 month, unless otherwise specified, and killed thereafter. Blood for calcium and hormone measurements was obtained at the end of each experimental protocol.

Gene and protein assays

To evaluate apoptosis onto BPH after the incubation with testosterone (10 nM), KGF (10 ng/ml) or Des(1-3)IGF-I (10 ng/ml) with or without BXL-628 (10 nM), ISEL was performed using Apop Tag *in situ* apoptosis detection kit peroxidase following the manufacturer's instructions, and the percentage of apoptotic cells was calculated as previously described (8). Results are expressed as means \pm S.E.M. from three separate experiments.

Total RNA for Northern hybridization analysis was extracted from rat tissues using RNAFast from Molecular Systems (San Diego, CA, USA). Blotting, labeling, hybridization conditions, probes (rat clusterin 1.5 kb full-length cDNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1.2 kb full-length cDNA) and quantitation of the autoradiograms were performed according to the reported procedures (11, 18, 19).

For immunohistochemistry, all the cryostatic sections obtained from controls and treated rats were processed and analysed in parallel following previously reported procedures (11, 20).

DNA fragmentation in rat prostate cryostatic sections, assessed by TUNEL, was performed using the *In Situ* Cell Death Detection Kit POD as recommended by the manufacturer and analysis performed as already described (11).

Serum measurements

Serum calcium levels were measured with a commercially available colorimetric assay according to the manufacturer's instructions. Serum levels of testosterone and rLH hormones were determined by commercially available radioimmunoassay kits, according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed by one-way ANOVA and paired or unpaired Student's *t*-tests, when appropriate. Binding data were analysed using the computerized program LIGAND (21).

The computer program ALLFIT (22) was used for the analysis of sigmoid dose–response curves to obtain estimates of half-maximal inhibition values (IC_{50}) and half-maximal stimulatory values (EC_{50}) as well as maximal inhibitory (I_{max}) and stimulatory effects. Data are expressed as means \pm S.E.M.

Results

Incubation of BPH cells with increasing concentrations of calcitriol or BXL-628 inhibits cell growth (Fig. 1A). Both compounds inhibited cell proliferation

dose-dependently. ALLFIT (22) analysis indicated that, although maximal inhibition of calcitriol and BXL-628 was not statistically significant different ($I_{max} = 43 \pm 1\%$), their relative potency was, BXL-628 being several log units more effective than calcitriol ($-\log IC_{50}$ BXL-628 = 15.8 ± 0.3 vs $-\log IC_{50}$ calcitriol = 10.2 ± 0.6 , $P < 0.005$).

As previously reported (8, 10, 11), BPH cell proliferation was significantly increased ($P < 0.01$) by testosterone ($156 \pm 8\%$), and GFs, such as Des(1-3)IGF-I ($194 \pm 6\%$) or KGF ($183 \pm 5\%$). When cell growth was stimulated for 48 h with testosterone or GFs (Fig. 1B)

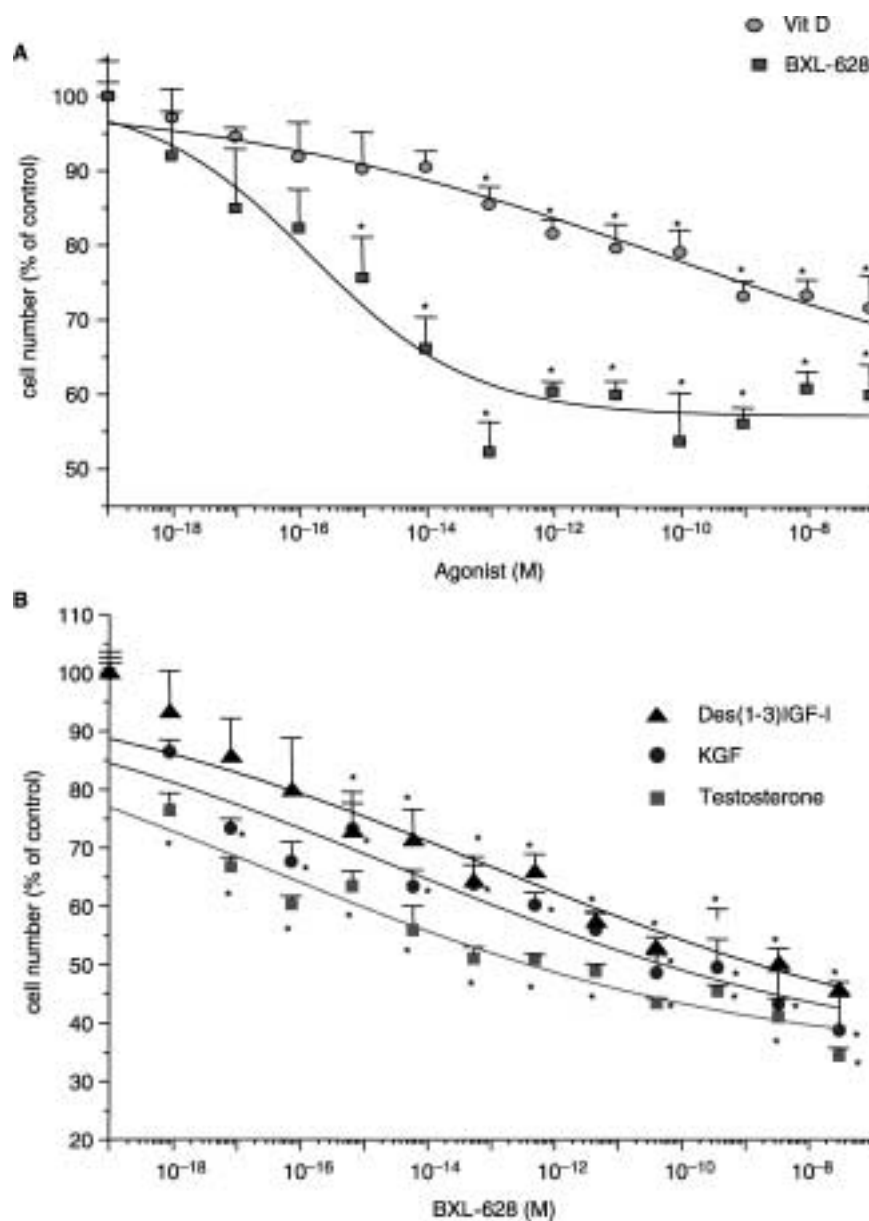


Figure 1 Inhibition of BPH cell proliferation by calcitriol and BXL-628. (A) Incubation for 48 h with increasing concentrations of calcitriol (Vit D; circles) or BXL-628 (squares). * $P < 0.01$ vs control. (B) Effect of increasing concentrations of BXL-628 on BPH cell proliferation stimulated by testosterone (10 nM; squares), KGF (10 ng/ml; circles) or Des(1-3)IGF-I (10 ng/ml; triangles). * $P < 0.01$ vs testosterone- or GF-treated cells. Values are means \pm S.E.M.

the inhibitory effect of BXL-628 was even more pronounced ($I_{\max} = 66.6 \pm 7.3\%$). Mathematical modelling (22) of inhibition curves indicated that BXL-628 was more potent in BPH cells stimulated with testosterone ($-\log IC_{50}$ values = 16.4 ± 0.6) than with the other two growth factors ($-\log IC_{50}$ values = 12.7 ± 0.6 , and $-\log IC_{50} = 14.2 \pm 0.6$ for Des(1-3)IGF-I and KGF respectively; $P < 0.0001$).

BXL-628 (1 nM) antagonized not only testosterone- but also DHT-stimulated BPH cell proliferation to an extent similar to the AR antagonist Cyp (100 nM; Fig. 2). Conversely, the 5α reductase inhibitor finasteride (1 nM) antagonized only testosterone-induced cell growth (Fig. 2A). In addition, BXL-628 reduced growth even in androgen-unstimulated cells (Fig. 2A).

To evaluate the potential anti-androgenic properties of BXL-628, in addition to BPH cell growth inhibition,

we investigated its interaction with the AR. First, we ruled out the possibility that BXL-628 binds to the AR by performing competition studies in human BPH homogenates, using the synthetic androgen [3 H]R1881 as labeled ligand. LIGAND analysis (21) of the data indicated that unlabeled R1881, DHT, testosterone and the AR antagonist bicalutamide completely displaced [3 H]R1881 binding (Table 1). Conversely, BXL-628 did not compete for [3 H]R1881 binding at any concentration tested (Table 1). These results were confirmed and extended using a luciferase reporter gene assay. In PC3 cells expressing the full-length AR coupled to a luciferase report gene, DHT stimulated a dose-dependent increase in luciferase activity ($EC_{50} = 2 \pm 1.3$ nM; Fig. 3A), while bicalutamide inhibited DHT-stimulated activity ($IC_{50} = 194 \pm 80$ nM; Fig. 3B). In this system, increasing concentration of

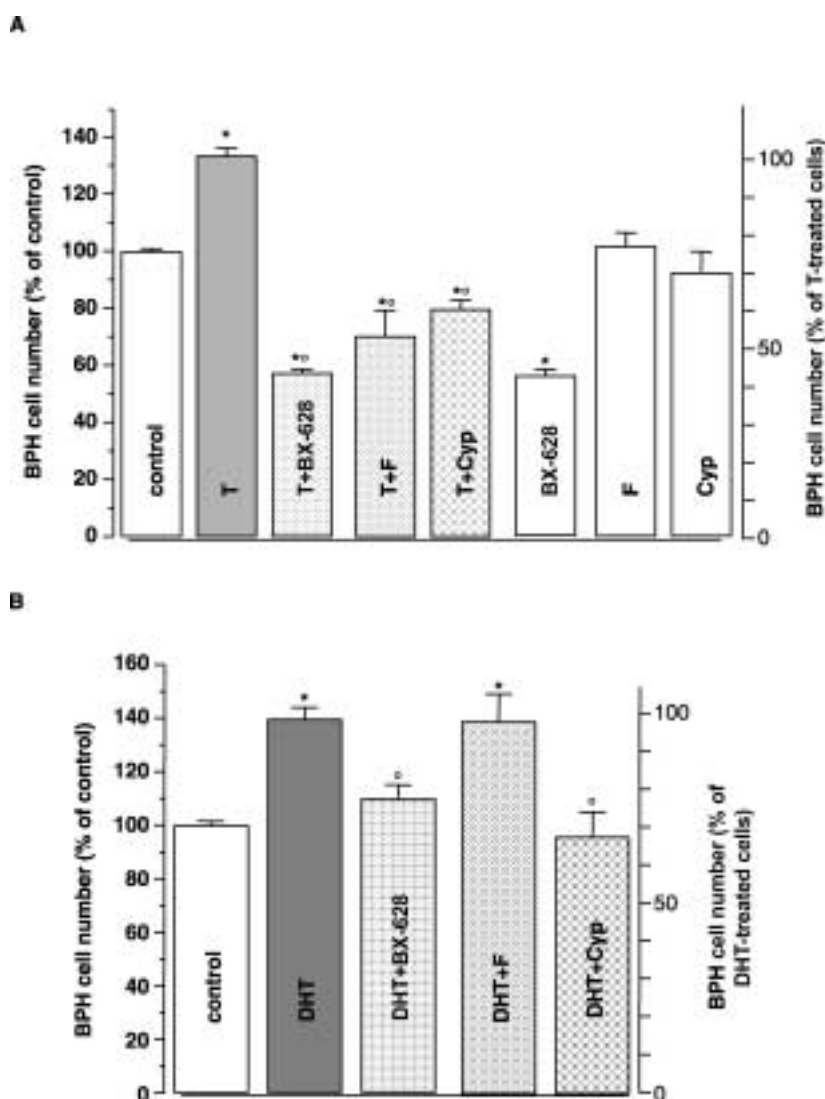


Figure 2 Effect of BXL-628, Cyp and finasteride on androgen-stimulated BPH cell growth. BPH cells were incubated for 48 h with BXL-628 (BX-628; 1 nM) or anti-androgens (finasteride (F), 1 nM; Cyp, 100 nM) in the presence of (A) testosterone (T; 10 nM) or (B) DHT (10 nM). * $P < 0.01$ vs control, ° $P < 0.01$ vs androgen-treated cells. Values are means \pm s.e.m.

Table 1 Affinity constants of androgen agonists (R1881, DHT, testosterone), antagonist (bicalutamide) and BXL-628 in human BPH homogenates as detected by [³H]R1881 binding. Values are means ± S.E.M.

AR ligand	Affinity constants (K_d nmol/l)
R1881	0.16 ± 0.06
DHT	0.07 ± 0.03
Testosterone	1.89 ± 0.9
Bicalutamide	159 ± 82
BXL-628	> 100 000

BXL-628 neither stimulated nor inhibited AR-mediated luciferase activity increase (Fig. 3).

Finally, to verify whether or not BXL-628 interacts with the formation of DHT, the active metabolite of

testosterone, we performed experiments in CHO cells transfected with 5 α R-1 and 5 α R-2 (11). Results were compared with those obtained with finasteride. While finasteride inhibited testosterone conversion into DHT with the expected IC₅₀ values (IC₅₀ for 5 α R-1 = 659 ± 100 nM and IC₅₀ for 5 α R-2 = 53.7 ± 11 nM, *n* = 3), BXL-628 did not interfere with either isoenzyme up to the micromolar range (not shown).

The effect of BXL-628 in BPH cells was, at least in part, due to activation of programmed cell death as detected by ISEL (*n* = 3; Table 2). The percentage of apoptotic nuclei dramatically increased (270%) after a 48-h exposure to 10 nM BXL-628 (*P* < 0.01 vs control). Conversely, treatment with testosterone (10 nM) or GFs (10 ng/ml) significantly (*P* < 0.01) reduced

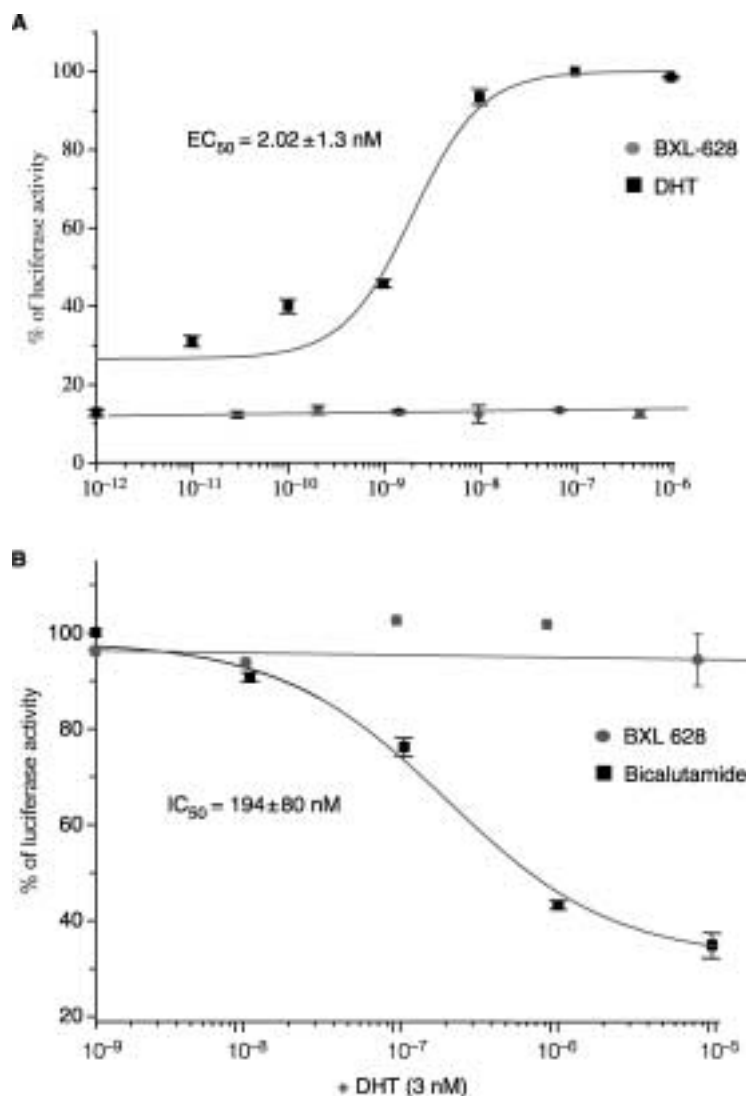


Figure 3 Lack of agonistic or antagonistic properties of BXL-628 on human AR. PC3 AR transfected cells were incubated with (A) increasing concentrations of DHT (squares), BXL-628 (circles) or (B) with a fixed concentration of DHT (3 nM) in the presence of bicalutamide (squares) or BXL-628 (circles) for 18 h. The effect of the different treatments was assessed using a luciferase receptor gene assay. Values are means ± S.E.M.

Table 2 Effect of BXL-628 (10 nM), GFs (10 ng/ml) or testosterone (10 nM) on DNA fragmentation in BPH cells. Apoptotic index (%) represents the number of stained nuclei, as detected by ISEL, over BPH cells in each of at least five separate fields per slide. Values are means ± S.E.M.

	Apoptotic index (%)	
	Control	BXL-628
Control	18.55 ± 0.8	68.44 ± 1.26 ^a
Des(1–3)IGF-I	10.69 ± 0.6 ^a	45.85 ± 0.66 ^{a,b,c}
KGF	8.5 ± 0.42 ^a	44.46 ± 0.57 ^{a,b,c}
Testosterone	13.56 ± 0.72 ^a	49.06 ± 1.87 ^{a,b,c}

^a*P* < 0.01 vs control; ^b*P* < 0.01 vs BXL-628-treated cells; ^c*P* < 0.01 vs GF- or testosterone-treated cells.

the number of apoptotic BPH cells as compared with untreated cells (Des(1-3)IGF-I = -42%; KGF = -54%; testosterone = 27%). However, even in the presence of GFs or testosterone, BXL-628 induced a sustained (more than 250%) and significant (*P* < 0.01) increase in the number of ISEL-positive BPH cells.

To test the anti-proliferative properties of BXL-628 in *in vivo* models of prostate growth, we treated castrated and intact rats orally with increasing concentrations of BXL-628 (10–300 µg/kg) or finasteride (10 and 40 mg/kg). As shown in Fig. 4A, castration dramatically reduced ventral prostate weight, while a 2-week treatment with testosterone enanthate (30 mg/kg) not

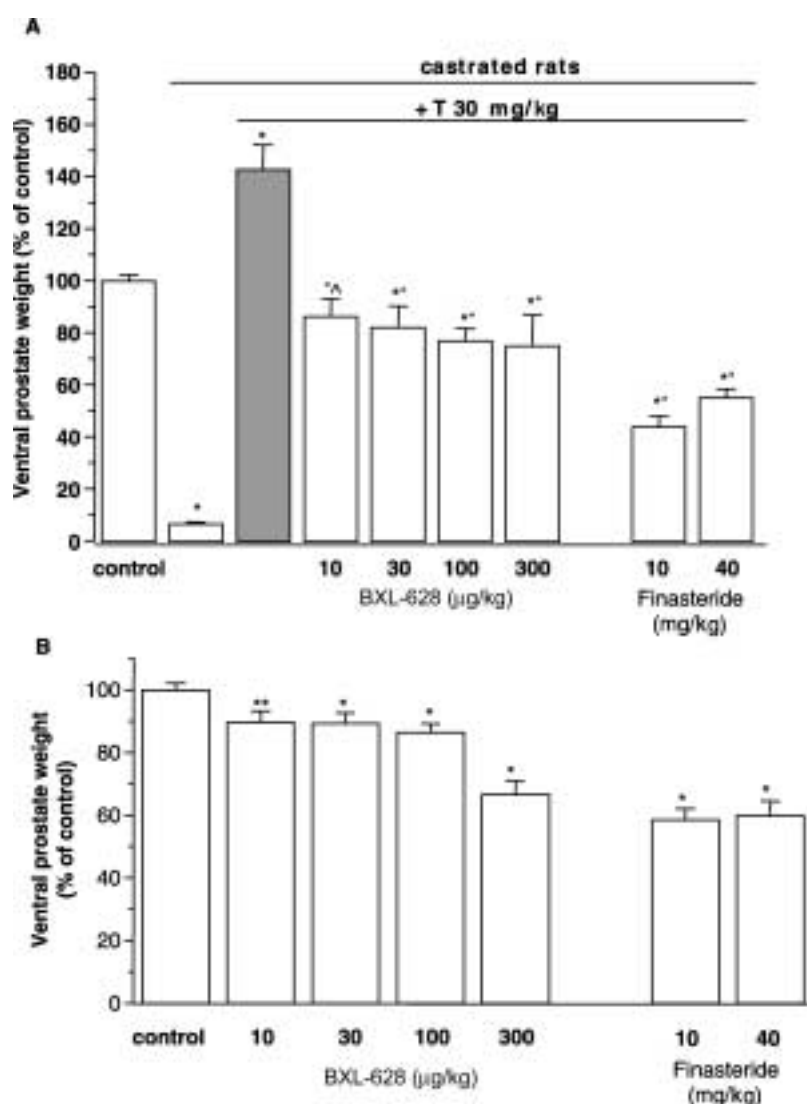


Figure 4 Inhibition of rat ventral prostate growth by BXL-628 or finasteride. (A) Castrated rats, injected with testosterone enanthate (T) were treated for 2 weeks with vehicle or increasing doses of BXL-628 or finasteride. Ventral prostate weight is expressed as % variation (means ± S.E.M.) of the weight of intact, vehicle-treated (control) rats. [^]*P* < 0.05, ^{*}*P* < 0.01 vs control rats, [°]*P* < 0.01 vs testosterone-supplemented rats. (B) Intact adult rats were treated orally for 1 month with vehicle (control) or increasing concentrations of BXL-628 or finasteride. Ventral prostate weight is expressed as % variation (means ± S.E.M.) of the weight of control, vehicle-treated rats. ^{*}*P* < 0.01 vs control rats.

only completely restored, but further stimulated its growth. BXL-628, at any dose tested, completely blunted testosterone-stimulated prostate over-growth, reducing ventral prostate weight below that of untreated rats. Similar results were obtained with finasteride (10 and 40 mg/kg). A 1-month treatment of intact adult rats with BXL-628 significantly decreased ventral prostate weight, with a maximal reduction (30%) at the highest dose tested (300 µg/kg). At this dose, the inhibitory effect of BXL-628 on prostate growth was comparable with that induced by 10 or 40 mg/kg finasteride (Fig. 4B). In all the experimental protocols, oral administration of different doses of BXL-628 caused a very modest hypercalcemia only at the highest dose tested (300 µg/kg) (Table 3). No other discernible side-effects were observed.

To better understand the molecular mechanisms underlying BXL-628-induced prostate weight reduction, we evaluated the expression of clusterin gene and protein and the morphological hallmarks of apoptosis by TUNEL. Clusterin is an ubiquitous product gene, strictly related to cell cycle arrest and atrophy (18, 23–25), the expression of which is down-regulated by androgens (18, 26, 27). Figure 5A shows the prostatic expression of clusterin mRNA, as detected by Northern analysis, in orchidectomized rats supplemented or not with testosterone. Castration dramatically up-regulated clusterin mRNA abundance, while this effect was completely reversed by a 2-week administration of testosterone. The simultaneous treatment with different concentration of BXL-628 (300 and 100 µg/kg) or finasteride (40 mg/kg) partially blunted the testosterone-induced down-regulation of clusterin gene expression. In intact rats (Fig. 5B), a 1-month administration of different concentrations of BXL-628 (30 and 100 µg/kg) induced a sustained increase in clusterin gene expression in the prostate, comparable to, or even higher than that induced by 40 mg/kg finasteride.

The local expression of clusterin in the prostate of orchidectomized rats is shown in Fig. 6. Castration induced a marked and widespread atrophy in the prostate gland, and nearly all the cuboidal epithelial cells facing the gland lumen were clusterin positive

Table 3 Calcemia (mg/dl) in testosterone-replaced castrated rats after different doses (10, 30, 100 and 300 µg/kg) of BXL-628. BXL-628 did not change calcium serum levels in castrated rats replaced with testosterone enanthate (30 mg/kg per week) as compared with controls. Similar results were obtained in intact rats (not shown). Results represent the means ± S.E.M. of rats/group.

	Calcemia
Control	10.2 ± 0.16
BXL-628 (10 µg/kg)	10.16 ± 0.24
BXL-628 (30 µg/kg)	9.87 ± 0.15
BXL-628 (100 µg/kg)	10.55 ± 0.18
BXL-628 (300 µg/kg)	10.85 ± 0.1

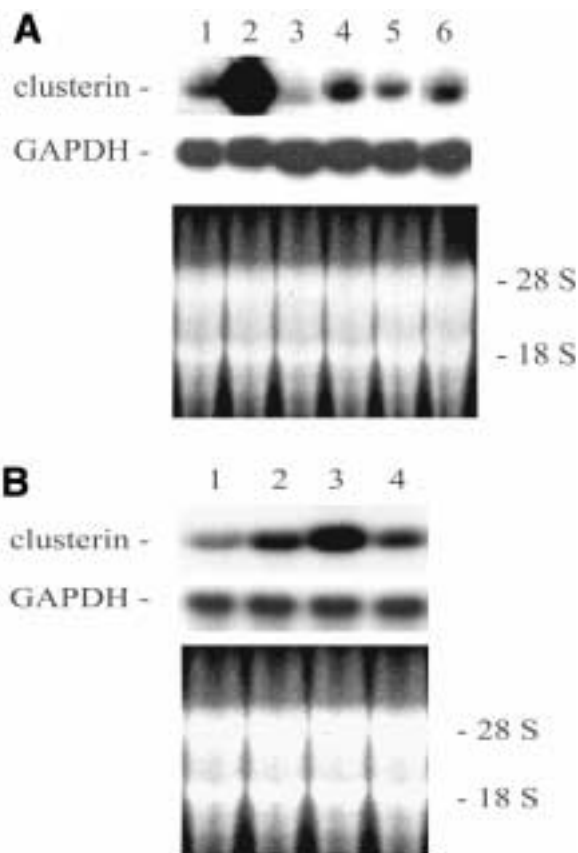


Figure 5 Effect of BXL-628 and finasteride on clusterin gene expression in the rat ventral prostate. (A) Northern analysis of clusterin mRNA expression in the ventral prostate of vehicle-treated intact (lane 1) or orchidectomized (lane 2) rats. Lanes 3–6 show clusterin gene expression in orchidectomized rats supplemented for 2 weeks with testosterone enanthate and treated with vehicle (lane 3), BXL-628 (300 µg/kg, lane 4 and 100 µg/kg, lane 5) or finasteride (40 mg/kg, lane 6). (B) Northern analysis of clusterin mRNA expression in the ventral prostate of adult intact rats treated with vehicle (lane 1), increasing concentrations of BXL-628 (30 µg/kg, lane 2 and 100 µg/kg, lane 3) or finasteride (40 mg/kg, lane 4). Every lane was loaded with 10 µg total RNA. GAPDH expression and the ethidium bromide staining of the gels are shown below the blots. Each blot is representative of two separate experiments.

(Fig. 6A). Testosterone replacement (Fig. 6B) reversed the morphological hallmarks of atrophy and consistently reduced clusterin staining. Such an effect was prevented by the simultaneous administration of BXL-628 (Fig. 6C and E). Figure 6D and F show TUNEL results in sections adjacent to those shown in Fig. 6C and E. BXL-628 treatment (Fig. 6D; 100 µg/kg and Fig. 6F; 300 µg/kg) induced an evident nuclear fragmentation in epithelial and stromal cells, and apoptosis was detectable in both clusterin-positive and -negative cells. The first two panels of Fig. 7 show the morphology of the prostate gland of an intact rat processed for clusterin detection, with (Fig. 7A) or without (Fig. 7B) the omission of the primary antibody. Note that clusterin labeling is almost absent in the

prostate of untreated adult rats (Fig. 7B), as is nuclear fragmentation (TUNEL; Fig. 7C). Conversely, treatment with different doses of BXL-628 induced clusterin expression (Fig. 7D–F) and apoptosis (Fig. 7C, G and I). Figure 7F shows, for comparison, the effect of finasteride (40 mg/kg) on clusterin positivity in the prostate gland.

To rule out the possibility that BXL-628 reduced *in vivo* prostate growth by interfering with pituitary or testis function, we measured rLH and testosterone serum levels in castrated and intact rats. As expected (Table 4A), castration significantly reduced testosterone while it increased rLH serum levels. Testosterone enanthate (30 mg/kg) administration (2 weeks) completely reversed the effect of orchidectomy. Oral treatment with BXL-628 (100 and 300 µg/kg) of testosterone-replaced castrated rats did not significantly affect rLH or testosterone serum levels. Similar results were obtained in intact rats (Table 4B). In fact, chronic administration (1 month) of BXL-628 (10, 30 and 100 µg/kg) or finasteride (40 mg/kg) to intact rats did not modify rLH and testosterone serum levels.

Discussion

Pharmacological intervention to treat BPH is an important medical need due to the high incidence of this disease and the associated deterioration in the patients' quality of life. In particular, it is desirable to prevent irritative and obstructive lower urinary tract symptoms (LUTS), acute urinary retention (AUR) and the associated need for surgical intervention. BPH has essentially two major components: a static component, closely related to an increased prostate volume, and a dynamic component, mostly reflecting an increased smooth muscle tone in prostate, prostatic urethra and bladder neck. At present, two different classes of agents are available for BPH treatment: α -adrenergic receptor blockers and 5α reductase inhibitors. While α 1 blockers are very effective in reducing symptoms related to the BPH dynamic component (LUTS), they

are ineffective in reducing the prostate volume and therefore in preventing BPH-related surgery (28). Conversely, 5α reductase inhibitors such as finasteride and dutasteride, by decreasing DHT formation, reduce prostate size and the need for surgery (28, 29). In addition, recent results from the 7-year Prostate Cancer Prevention Trial, involving more than 18 000 healthy aged man, confirmed that finasteride is able to decrease LUTS, AUR and the need for surgery but it has also indicated that it can prevent or delay the appearance of prostate cancer (30). However, as expected (see 31), finasteride was not free from anti-androgenic adverse effects on sexual function, such as decreased sexual potency, sexual desire and gynecostasia (30), factors that substantially lessen its attractiveness as a cancer-preventing agent (32). In addition, finasteride treatment was associated with an increased detection of high-grade prostate cancer, probably because the finasteride-induced low-androgen milieu selected the most aggressive, androgen-insensitive malignant growing cells (32).

Hence, an ideal new class of drugs for medical therapy of BPH should be able to prevent AUR, together with its related need for surgery, by decreasing androgen-induced prostate growth but without directly interfering with AR, and therefore without anti-androgenic prostatic and extra-prostatic adverse effects. This ideal medicament, by disrupting intra-prostatic GF signaling, might be useful not only for treating BPH but also for preventing prostate cancer, possibly without selecting AR-insensitive, malignant clones. The present study suggests that the non-hypercalcemic, well-tolerated calcitriol analogue BXL-628, by acting downstream from the AR and targeting GF-mediated prostate proliferation, represents a promising candidate for this new class of drugs.

In this preclinical study, we have demonstrated that BXL-628 reduces prostate size in intact rats to an extent similar to finasteride. In addition, like finasteride, BXL-628 abrogates the *in vitro* and *in vivo* proliferative activity of testosterone. However, at variance

Table 4 rLH (ng/ml) and testosterone (nM) serum levels in testosterone (T)-replaced castrated (A) or intact (B) rats after treatment with different doses of BXL-628. Values are means \pm S.E.M.

	rLH	Testosterone
(A)		
Control (intact rats)	2.36 \pm 0.46	11.5 \pm 2.44
Castrated	20.64 \pm 6*	0.9 \pm 0.32**
Castrated+T-replaced	2.08 \pm 0.36	21.25 \pm 4.12
Castrated+T-replaced + BXL-628 (100 µg/kg)	1.8 \pm 0.2	11.13 \pm 1.02
Castrated+T-replaced + BXL-628 (300 µg/kg)	3.15 \pm 0.65	15.73 \pm 2.75
(B)		
Control (intact rats)	2 \pm 0.16	11.98 \pm 2.87
Finasteride	2.2 \pm 0.4	18.11 \pm 3.23
BXL-628 (10 µg/kg)	2.22 \pm 0.25	19.13 \pm 3.83
BXL-628 (30 µg/kg)	2.32 \pm 0.36	9.39 \pm 2
BXL-628 (100 µg/kg)	1.96 \pm 0.13	11 \pm 2.14

* $P < 0.05$, ** $P < 0.01$ vs controls.

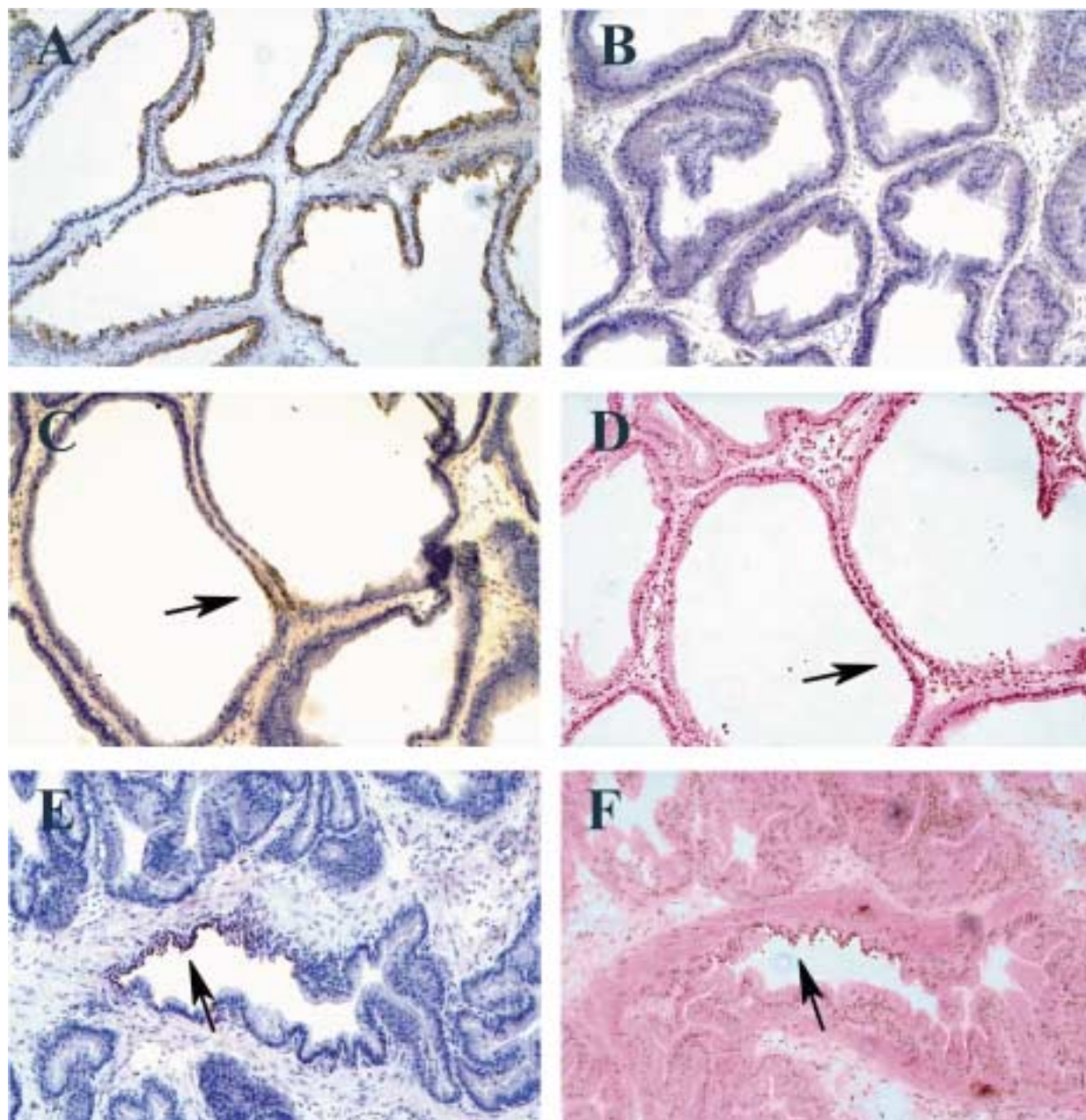


Figure 6 Morphological effects of BXL-628 on ventral prostate of castrated and testosterone-supplemented rats. (A–D) Representative fields obtained from cross-sections of whole prostate glands immunostained with a monoclonal antibody against rat clusterin and counterstained with hematoxylin. (A) In vehicle-treated rats castrated 4 days earlier, clusterin labeling is detectable in the cytoplasm of the atrophic cuboidal epithelial cells ($10\times$). (B) After 2 weeks of testosterone supplementation ($10\times$), almost all the clusterin labeling disappeared. Conversely, clusterin-positive cells were still present in rats treated with testosterone and different doses of BXL-628 ((C) $100\mu\text{g}/\text{kg}$ and (E) $300\mu\text{g}/\text{kg}$; black arrows). (D and F) show sections consecutive to those in (C) and (E), to highlight DNA fragmentation as assessed by TUNEL. Treatment for 2 weeks with BXL-628 ((D) $100\mu\text{g}/\text{kg}$ and (F) $300\mu\text{g}/\text{kg}$) induced massive apoptosis in the majority of epithelial and stromal cells. Note (black arrows) that all the clusterin-positive cells were undergoing apoptosis, while a consistent portion of clusterin unlabeled cells also show nuclear fragmentation.

with finasteride, BXL-628 does not inhibit $5\alpha\text{R}-1$ or $5\alpha\text{R}-2$ activity and can counteract not only testosterone- but even DHT-induced BPH cell growth. These anti-androgenic properties of BXL-628 are independent from interaction with the AR, as shown by the failure

of BXL-628 to bind to the AR, and to act as AR agonist or antagonist in AR-transfected PC3 cells. Furthermore, BXL-628 does not affect sex hormone secretion since, in the rat, gonadotropin and testosterone plasma levels were unchanged by daily administration

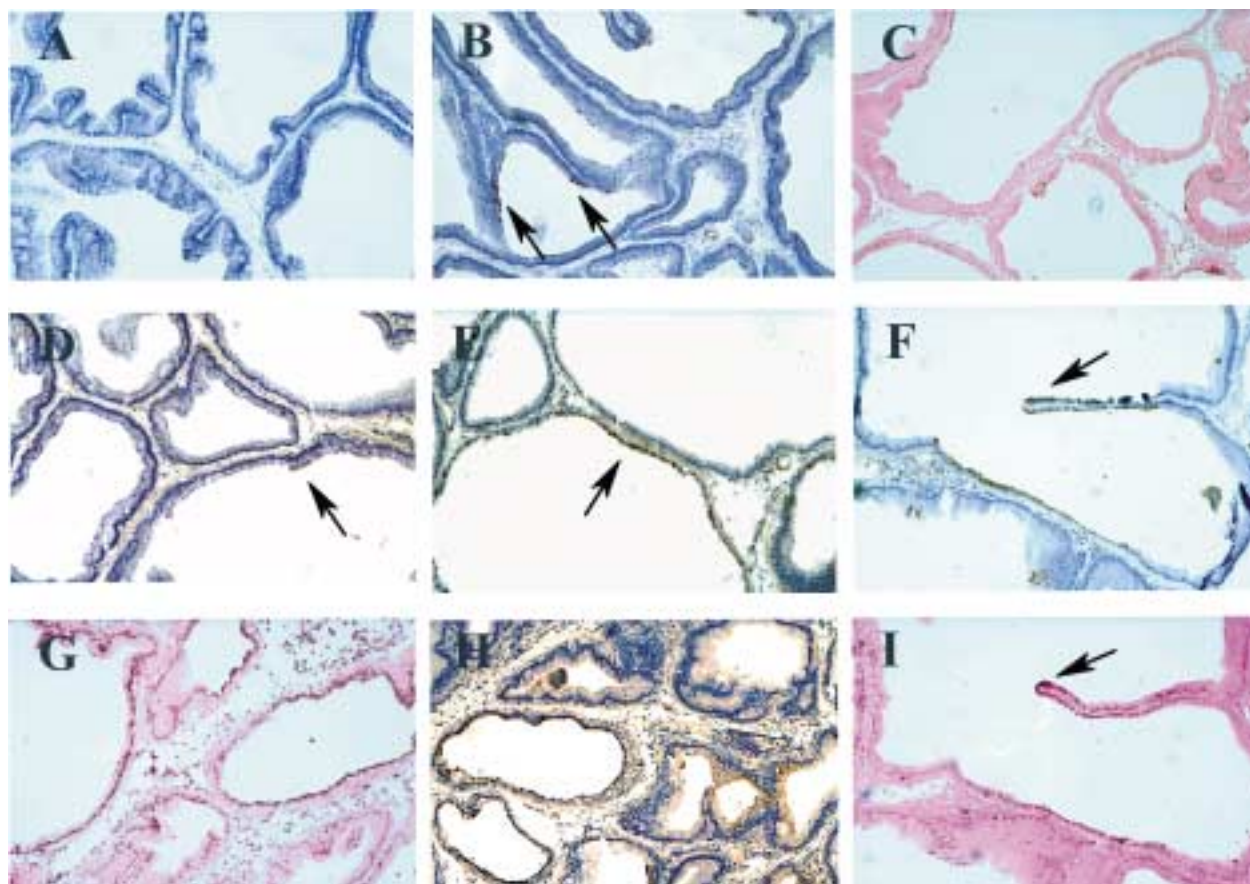


Figure 7 Morphological effects of BXL-628 and finasteride on the prostate of intact, adult rats. (A, B, D, E, F and H) Representative fields obtained from cross-sections of whole prostate glands immunostained with a monoclonal antibody against rat clusterin and counterstained with hematoxylin. In (A) (10 ×) the primary antibody was omitted. (B) (10 ×) shows that in untreated adult rats only a few, scanty epithelial cells were labeled in some glands (black arrows). Conversely, in prostate glands from rats treated with increasing concentrations of BXL-628, cuboidal epithelial cells showing the hallmark of atrophy were dose-dependently stained for clusterin (see black arrows, (D) 10 µg/kg, (E) 30 µg/kg and (F) 100 µg/kg; 10 ×). (H) Similar results were obtained with finasteride (40 mg/kg; 10 ×). (C, G and I) show serial, consecutive slices to those depicted in (B, D and F) respectively, to highlight DNA fragmentation as assessed by TUNEL. Note (black arrows) that all the clusterin-positive cells were undergoing apoptosis, while a consistent portion of clusterin unlabeled cells also show nuclear fragmentation.

of BXL-628 for up to 1 month. Hence, BXL-628 acts downstream of the AR–ligand interaction, most probably by disrupting the testosterone-growth factor cross-talk, as previously demonstrated with other VDR ligands (11). Very low concentrations of BXL-628 were able to completely antagonize not only testosterone-stimulated BPH cell proliferation, but also proliferation induced by the two most important intra-prostatic growth factors: IGF-I and KGF. In addition, even in the presence of testosterone or GFs, BXL-628 induced apoptosis in BPH cells. The BXL-628-induced death program was evident also in the prostate of both intact and testosterone-supplemented orchidectomized rats and was characterized by the diffuse appearance of DNA fragmentation with a concomitant increase in clusterin gene and protein expression. Clusterin is a protein tightly regulated in

the prostate by androgens (18). Although clusterin function is still not well understood, it is markedly up-regulated in conditions of gland atrophy (23, 25) and apoptosis (33). Thus, clusterin induction by BXL-628 treatment is consistent with the capacity of this compound to inhibit proliferation and induce apoptosis in prostate cells. The capacity to induce growth arrest and programmed death in prostate cells (8, 10, 11), even in malignant prostate cells (34), appears to be a common property of VDR ligands. These effects are unrelated to the presence of a functioning AR (34), but could depend on the ability of VDR ligands to interfere with GF receptor autophosphorylation (8). This mechanism is supported by the observation that calcitriol can inhibit ligand-dependent phosphorylation of epidermal growth factor receptor (EGFR) and extra-cellular signal regulated protein kinase (ERK1/2) in

human epidermoid cells A431, in which EGFR and transforming growth factor- β constitute the major autocrine signal (35).

In conclusion, the present study indicates that BXL-628, a compound previously known for its activity on the bone (36, 37), is effective in reducing prostate cell growth in different experimental models. The ongoing phase II clinical trial on BXL-628 in patients with BPH will indicate if this translates to an effective agent able to reduce prostate volume in BPH-affected individuals.

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