Quinazoline-derived α1-Adrenoceptor Antagonists Induce Prostate Cancer Cell Apoptosis Via an α1-Adrenoceptor-independent Action[1]

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ABSTRACT

Recent evidence suggests that the quinazoline-based α1-adrenoceptor antagonists, doxazosin and terazosin, exhibit a potent apoptotic effect against prostate tumor epithelial cells, whereas tamsulosin, a sulfonamide-based α1-adrenoceptor antagonist, was ineffective in inducing a similar apoptotic effect against prostate cells (Cancer Res., *60:* 4550–4555, 2000). In this study, to identify the precise molecular mechanism underlying this apoptosis induction, we examined whether doxazosin and terazosin (both piperazinyl quinazolines) affect prostate growth via an α1-adrenoceptor-independent action. Transfection-mediated overexpression of α1-adrenoceptor in human prostate cancer cells, DU-145 (that lack α1-adrenoceptor), did not alter the ability of prostate cancer cells to undergo apoptosis in response to quinazolines. Significantly enough, there was no modification of the apoptotic threshold of the androgen-sensitive prostate cancer cells, LNCaP, to either quinazoline-based α1-agonist by androgens. Furthermore, human normal prostate epithelial cells exhibited a very low sensitivity to the apoptotic effects of doxazosin compared with that observed for the malignant prostate cells. These findings provide the first evidence that the apoptotic activity of the quinazoline-based α1-adrenoceptor antagonists (doxazosin and terazosin) against prostate cancer cells is independent of: (*a*) their capacity to antagonize α1-adrenoceptors; and (*b*) the hormone sensitivity status of the cells. This may have potential therapeutic significance in the use of quinazoline-based α1-adrenoceptor antagonists (already in clinical use for the treatment of hypertension and benign prostate hyperplasia) for the treatment of androgen-independent human prostate cancer.

INTRODUCTION

Prostate cancer has become the most prominent contributor to cancer mortality among American males, resulting in >40,000 deaths every year (1). Prostate cancer mortality results from metastasis to the bone and lymph nodes and progression from androgen-dependent to androgen-independent prostatic growth (2). Common treatment regimes for patients with localized disease involves surgical interventions (radical prostatectomy) or nonsurgical approaches (hormone ablation or radiotherapy). A large percentage of prostate cancer patients, however, present an advanced stage disease with noncurative options available. Androgen ablation, the major therapeutic modality for advanced prostate cancer, is rarely curative because it is exclusively targeted against the androgen-dependent prostate cancer cells, whereas the androgen-independent cells survive (3). Attempts at combination regimes of hormonal ablation and chemotherapy failed to provide clinically convincing evidence of significant therapeutic success (4), primarily attributable to the slow rate of cell proliferation that characterizes prostatic tumors (5, 6).

Within the normal prostate gland, homeostasis is maintained by a unique balance between the rates of proliferation and apoptosis, such that neither overgrowth nor involution of the gland occurs (7). Compelling evidence suggests that the tumorigenic growth of the prostate depends on the evasion of normal homeostatic control mechanisms, because of an increase in cell proliferation and a decrease in apoptotic death (5, 6). Thus, enhancing the apoptotic process emerges as a significant therapeutic target for the effective elimination of androgen-dependent and androgen-independent prostate cancer cells (3). Characteristically, because increased bcl-2 expression correlates with development of androgen-independent prostate cancer, an apoptotic defect that renders prostatic tumors resistant to therapy (8, 9), recent efforts have targeted key apoptosis regulators to enhance the therapeutic response with minimal toxicity (3). Modulation of molecular pathways of apoptosis execution will have significant effects on prostate tumor vascularity and sensitivity to other therapeutic modalities (such as radiotherapy).

With continued intensified search for the identification of apoptosis modifiers as therapeutic targets for prostate cancer treatment, attention has recently been directed toward a group of existing drugs: α1-adrenoceptor antagonists that are members of the quinazoline chemical class. Doxazosin and terazosin are long-acting selective α1-adrenoceptor antagonists that are clinically used to provide acute relief of the obstructive symptoms associated with BPH[3] (10–12) and for the treatment of hypertension via reduction of total peripheral resistance by selective postsynaptic α1-blockade (13). Tamsulosin, a recently developed member of this class of α1-adrenoceptor antagonists, is selective for the predominant α1a-adrenoceptor subtype but has a different sulfonamide-related structure, and its potent therapeutic efficacy has been demonstrated in clinical trials (14). The therapeutic benefit of α1-adrenoceptor antagonists in the treatment of BPH has been historically attributed to a change in the periurethral tone of the prostate (15) via a direct action on α1-adrenoceptors present in the prostate smooth muscle (16).

A provocative challenge for the existing concept on α1-blocker action, stemmed from evidence gathered in this laboratory, suggests that two quinazoline-derived α1-adrenoceptor antagonists, doxazosin and terazosin, have additional effects against cellular growth, which transcend smooth muscle relaxation: both doxazosin and terazosin at pharmacologically relevant doses exert an apoptotic activity in stroma smooth muscle and epithelial cell populations in prostate tissue from BPH patients without affecting their roliferative rate (17, 18). These clinical findings were in full accord with experimental studies using a mouse reconstitution model of prostate hyperplasia, in which doxazosin demonstrated a potent apoptotic effect against oncogene-induced prostate growth (19). Furthermore, more recent *in vitro* studies documented a potent antigrowth effect of the two quinazolines (doxazosin and terazosin), but not tamsulosin, the sulfonamide-based α1-adrenoceptor antagonist, against two highly aggressive, androgen-independent human prostate cancer cell lines, PC-3 and DU-145, via induction of apoptosis (20–22).

Considering the ability of the quinazoline-derived α1-adrenoceptor antagonists, but not the uroselective, high affinity (sulfonamide-based antagonist) tamsulosin, to induce prostate tumor cell apoptosis, in this study, we examined whether the apoptotic effect of terazosin and doxazosin is: (*a*) an α1-adrenoceptor-mediated mechanism; and (*b*) dependent on androgens. Our results demonstrate that the quinazoline-

[3] The abbreviations used are: BPH, benign prostatic hyperplasia; DHT, dihydrotestosterone; PSA, prostate-specific antigen; VEGF, vascular endothelial growth factor.

driven apoptosis is independent of α1-adrenoceptor action, and DHT does not affect the sensitivity of prostate cancer cells to the apoptotic effects of doxazosin and terazosin. Moreover, there was a minimal cell death effect (by quinazolines) in the normal prostate epithelial cells. The present findings may provide a rationale for advancing these long-acting, quinazoline-based α1-adrenoceptor antagonists toward the development of an effective therapeutic strategy for patients with androgen-independent prostate cancer.

MATERIALS AND METHODS

Cell Culture

The human prostate cancer cell lines, LNCaP and DU-145, were obtained from the American Type Tissue Culture Collection (Rockville, MD). Normal human prostate epithelial cells, PrEC, were purchased from Clonetics (Walkersville, MD). LNCaP cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% fetal bovine serum/2 mM L-glutamine (Collaborative Biomedical Products, Bedford, MA) and 100 units of penicillin/100 mg/ml streptomycin. DU-145 cells were maintained in RPMI (Life Technologies, Inc.), containing 10% fetal bovine serum (Collaborative Biomedical Products) and antibiotics. PrEC cells were maintained in PrEGM media from Clonetics.

Drugs

The three α1a-adrenoceptor antagonists used in this study were generously provided by the following pharmaceutical companies that manufacture them, respectively: Doxazosin (Cardura; Doxazosin Mesylate) was provided by Pfizer Pharmaceuticals (New York, NY); Terazosin (Hytrin; Terazosin Hydrochloride) was obtained from Abbott Laboratories (Abbott Park, IL); and tamsulosin (FLOMAX; Tamsulosin Hydrochloride) was provided by Yamanouchi Pharmaceuticals (Tokyo, Japan). DHT (5-androstan-17β-OL-3-one) was obtained from Sigma Chemical Co. (St. Louis, MO).

Transfection

DU-145 cells were seeded at 1×10^6 cells/100-mm tissue culture dish and after 24 h, were cotransfected with the pcDNA3 plasmid encoding the human α1a-adrenoceptor (a generous gift from Dr. P. Walden, New York University, New York, NY) and the neomycin resistance-encoding plasmid via the calcium phosphate precipitation procedure as described previously (23). Neomycin-resistant colonies were selected in the presence of G418 (500 μg/ml; Life Technologies, Inc.), cloned, and expanded into cell lines.

Cell Viability Assay

Subconfluent cultures of cells in six-well plates were exposed in duplicate to increasing concentrations of doxazosin, terazosin, or tamsulosin (1-50 μM). The number of viable cells was assessed 2 days posttreatment using the trypan blue exclusion assay. For the experiments involving treatment with DHT, before treatment with doxazosin, LNCaP cells were grown in charcoal-stripped serum-containing medium in the presence or absence of 1 nM DHT as described previously (24). Values are expressed as the percentage of mean cell viability relative to the untreated control cultures.

Cell Proliferation Assays

Rate of DNA Synthesis. The effect of α1a-adrenoceptor antagonists on the rate of DNA synthesis in normal and malignant human prostate epithelial cells was evaluated using the [^{3}H]thymidine uptake assay, as described previously (20).

Cell Cycle Analysis. Subconfluent cultures of the androgen-sensitive prostate cancer cells LNCaP were treated with doxazosin or terazosin (25 μM) for 48 h. Cells were subsequently harvested and washed with PBS (pH 7.4), and pellets were resuspended with PBS and 70% ethanol. They were stored at $-20°$C overnight and resuspended (10^6 cells/ml), at which time 1 mg/ml DNase-free RNase was added. The cell suspension was incubated at 37°C (30 min) and stained overnight at 4°C with 50 μg/ml propidium iodide (Sigma Chemical Co.). Cells were filtered through a 35 μM nylon filter and analyzed using Beckman-Coulter fluorescence-activated cell sorter analyzer. G_1, S, and

G_2-M populations were quantitated using the Multicycle software program (Phoenix Flow Systems). Results are expressed as a percentage of cells in each stage of cell cycle.

Apoptosis Evaluation

The morphological appearance of apoptosis was assessed using the Hoechst staining as described previously (24). Briefly, cells were seeded in six-well plates (in triplicate) at 10^5 cells/well, and at subconfluency, they were treated with one of the three antagonists, doxazosin, terazosin, or tamsulosin (25 μM), for 48 h. After treatment, cells were fixed in 4% paraformaldehyde/PBS and stained with 10 μg/ml Hoechst 33342 dye (Sigma Chemical Co.)/0.1% Triton X-100. Fixed cells were incubated overnight at 4°C and visualized under a 365 nM UV light microscope (Zeiss Axiovert 10). Quantitative analysis was performed by counting the green fluorescent (apoptosis positive) cells under $\times$400 magnification from three independent fields. Values are expressed as the percentage of apoptotic cells relative to the total number of cells per field (the average number of cells that were positive for apoptosis varied from 2–40/field, whereas the total number of cells counted per field was $\sim$50–100).

Protein Analysis

Western Blot Analysis. LNCaP cells were treated with either doxazosin or terazosin (25 μM) for 1, 2, or 3 days. Cells from treated and control untreated cultures were lysed in 150 mM NaCl, 50 mM Tris (pH 8.0), 0.5% deoxycholic acid, and 1% NP40 with 1 mM phenylmethyl sulfonyl fluoride. Total cell lysates were electrophoretically analyzed through a 15% (w/v) polyacrylamide gel, and proteins were subsequently transferred onto a Hybond-P membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Western blot analysis was performed using the rabbit polyclonal antibody against VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) and the rabbit polyclonal antibody against PSA (DAKO, Carpenteria, CA). α-Actin expression was determined using the monoclonal-actin antibody (Oncogene Research, Cambridge, MA) as a loading and normalizing control. Protein detection was achieved using the Enhanced Chemilluminesence System (Amersham International, Arlington Heights, IL). Values for specific protein expression are expressed as fold-change, relative to α-actin expression (loading control) using the SCION Image analysis.

ELISA Assay for PSA. LNCaP cells were treated with doxazosin, terazosin, or tamsulosin (at 25 μM) for 24 h as described above. Supernatants from treated and nontreated LNCaP cells were assayed for PSA expression using an ELISA assay. PSA monoclonal antibody (Fitzgerald Industries, Concord, MA) was used as the capture antibody (5 μg/ml). Supernatants were then added after blocking in 1% BSA/PBS (1 h at room temperature). The antirabbit PSA (1 μg/ml; Fitzgerald Industries) and the Goat F(ab$'$)$_2$ antirabbit IgG-biotin conjugate (1 μg/ml; Biosource International, Camarillo, CA) were subsequently applied (1 h). After washing, ExtrAvidin Alkaline Phosphatase Conjugate 1:1000 (Sigma Chemical Co.) was applied, and PSA protein was detected using p-nitrophenyl phosphate (Sigma Chemical Co.) according to the manufacturer's instructions. Values represent the percentage of PSA expression relative to the untreated control samples.

Statistical Analysis

Statistical analyses of the numerical data were performed using ANOVA in the Graph Pad Prism program. All values are represented as averages $\pm$SE. Values were considered statistically significant at $P < 0.05$.

RESULTS

To elucidate whether the mechanism underlying the apoptotic effect of doxazosin and terazosin in prostate cancer cells is dependent on their ability to antagonize the α1a-adrenoceptor, human prostate cancer cells DU-145, which totally lack α1a-adrenoceptor expression (20), were engineered to overexpress α1a-adrenoceptor, and cloned transfectants were evaluated *in vitro*. Fig. 1 represents the Western blot analysis of the representative α1a-adrenoceptor transfectant clones. Clone-32 was selected for additional experiments as it expressed a band at M_r 30,000, indicative of α1a-AR reactivity (Fig. 1; Ref. 16). The binding affinity of the transfected α1a-adrenoceptor to
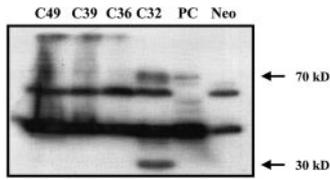
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Fig. 1. Expression of α1a-adrenoceptor in DU-145 prostate cancer cells. Transfection of DU-145 cells with α1a-adrenoceptor-encoding plasmid expression vector, as well as the neomycin expression vector, was performed as described in "Materials and Methods." Western blot analysis of selected cloned G418-resistant transfectants and neomycin control transfectant cells was performed using an antibody from Santa Cruz Biotechnology. C-49, C-39, C-36, and C-32 represent individual α1a-adrenoceptor/neomycin transfectant clones. *PC,* the positive control (rat brain); *Neo,* the neomycin-only control tranfectant. The size (M.W.) of the exogenously introduced α1a-adrenoceptor subtypes is shown on the *right*.
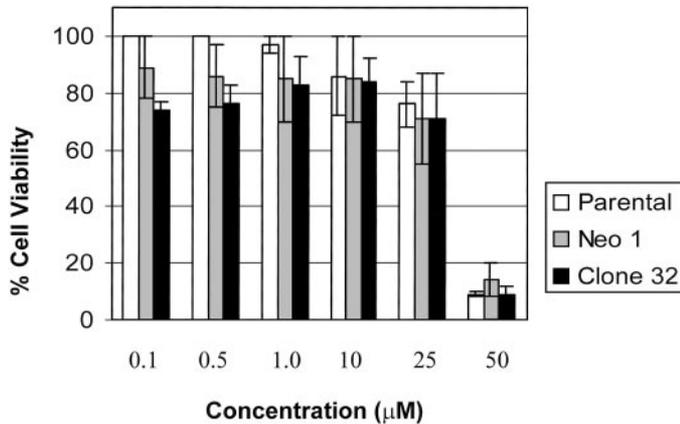

Fig. 2. Effect of α1-adrenoceptor overexpression on the apoptotic response of DU-145 prostate cancer cells to quinazoline-based α1-adrenoceptor antagonist doxazosin. Parental prostate cancer cells DU-145, neo control transfectants, and clone-32 α1a-adrenoceptor transfectants were treated with increasing concentrations of doxazosin as shown (1–50 μM). Cell viability was evaluated after 48 h of treatment on the basis of trypan blue exclusion assay. Results represent the average of three experiments performed in triplicate (mean $\pm$SE).

exogenous norepinephrine was documented by comparatively analyzing it with that of prostate smooth muscle cell line SMC-1 (20) in the presence or absence of phenoxybenzamine (1 μM; an irreversible inhibitor of α1a-adrenoceptor binding). We found that the DU-145 Clone-32 α1a-AR-transfectant cell line exhibited similar biding capacity to norepinephrine as the smooth muscle cell line (data not shown).

A characteristic dose response of DU-145 Clone-32 transfectant cells to doxazosin is shown on Fig. 2. There was no significant difference in doxazosin-induced cell death in the DU-145 Clone-32 cells, compared with parental DU-145 and neo control transfectants. Evaluation of the morphological appearance of apoptosis in response to doxazosin revealed a similar level of apoptosis induction after 2 days of treatment for all three cell lines (data not shown).

We have demonstrated previously that the androgen-independent prostate cancer cells PC-3 and DU-145 undergo apoptosis in response to quinazoline-derived α1-blockers but not the sulfonamide-derived tamsulosin (20, 21). In this experiment, the effect of the three drugs (doxazosin, terazosin, and tamsulosin) on the cell viability and rate of DNA synthesis was examined in the androgen-sensitive human prostate cancer cells LNCaP (Fig. 3). Fig. 3*A* demonstrates that 2-day treatment with doxazosin (at 25 μM) had a dramatic effect on cell viability resulting in ~80% cell death. In a similar pattern, terazosin treatment also resulted in a significant loss of cell viability (40%), whereas tamsulosin had virtually no effect on cell death in LNCaP cells. Fig. 3*B* reveals a characteristic appearance of the apoptotic

LNCaP cells in control untreated cultures and after treatment with doxazosin, terazosin, or tamsulosin (for 48 h). The results from the apoptosis detection (using the Hoechst staining) are summarized on Table 1. The quinazolines doxazosin and terazosin resulted in a significant increase in the number of apoptotic cells (14 and 13%, respectively), whereas tamsulosin treatment had no apoptotic effect against prostate cancer cells (1%) compared with the basal apoptosis levels in untreated controls (Table 1).

To determine whether androgens have the ability to modify the apoptotic response of prostate cancer cells to the quinazoline-based α1-antagonists, the effect of and DHT on doxazosin-induced cell death in LNCaP cells was examined. Fig. 4*A* indicates the LNCaP cell viability cells after 48 h of doxazosin treatment, in the presence or absence of DHT (1 nM). At midrange doxazosin dose, (10 μM), there appeared to be a modest "protective" effect by DHT on cell viability compared with doxazosin-treated cells alone. For the higher doxazo-
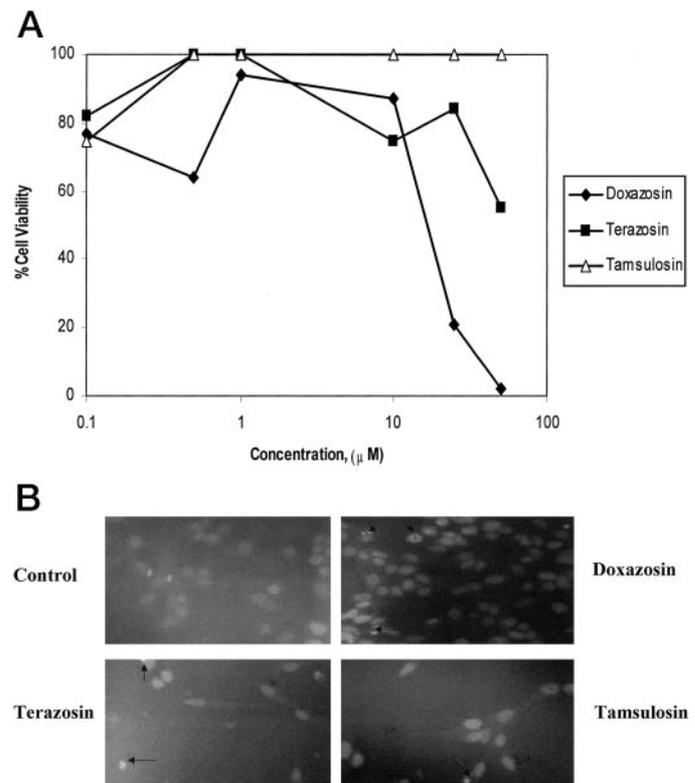

Fig. 3. Effect of doxazosin, terazosin, and tamsulosin on apoptosis in LNCaP prostate cancer cells. *A,* cell viability dose response of the three α1-adrenoceptor antagonists after 48 h of treatment. *B,* characteristic results of the Hoechst staining for the detection of LNCaP cells with the apoptotic morphology after the various treatments. LNCaP cells were treated with doxazosin, terazosin, or tamsulosin for 48 h, and cells were subsequently fixed and stained with Hoechst 33342. The fragmented nuclei of apoptotic cells were observed using a UV filter and quantitated (summarized on Table 1). *Arrows,* cells that are undergoing apoptosis.

Table 1 *Quantitative analysis of apoptosis induction in normal and malignant prostate epithelial cells in response to α1-adrenoceptor antagonists*[a]

Treatment	LNCaP (% apoptotic cells)	Normal prostate cells (PrEC) (% apoptotic cells)
Control	$1.1 \pm 0.2\%$	$2 \pm 0.7\%$
Doxazosin (25 μM)	$14 \pm 0.8\%$	$5 \pm 1.1\%$
Terazosin (25 μM)	$13 \pm 1.2\%$	$1 \pm 0.3\%$
Tamsulosin (25 μM)	$1.2 \pm 0.3\%$	ND

[a] Prostate cancer cells LNCaP and normal prostate epithelial cells (PrEC) were treated with doxazosin, terazosin, or tamsulosin (25 μM) for 2 days, and cells exhibiting the apoptotic morphology were assessed using the Hoechst staining as described in the legend for Fig. 3B. Values represent the average percentage of apoptotic cells of three independent experiments performed in duplicate (mean $\pm$SE).
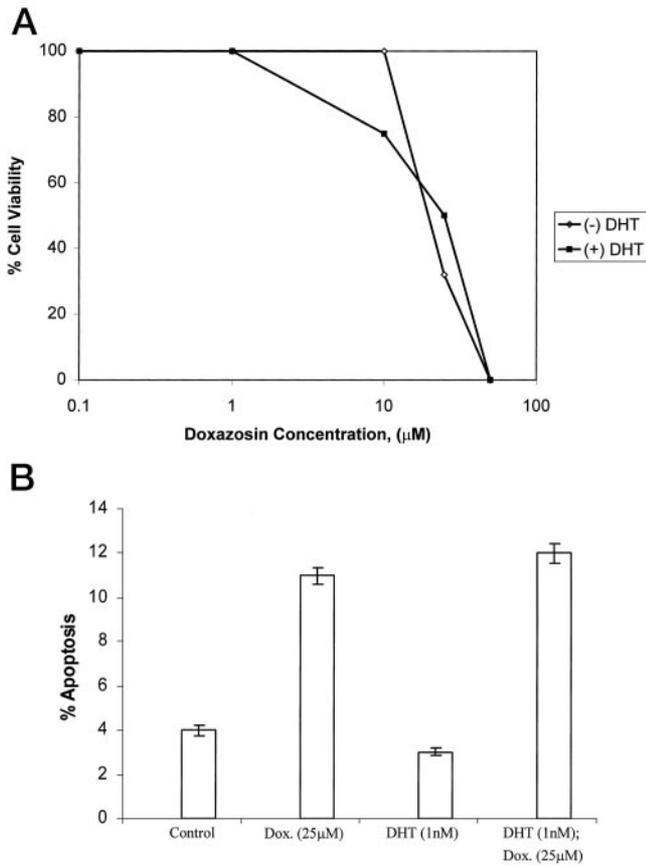
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Fig. 4. Effect of androgens on sensitivity of LNCaP cells to doxazosin-induced apoptosis. *A,* the results of cell viability dose response to increasing concentrations of doxazosin in the presence or absence of DHT (1 nM). *B,* the quantitative evaluation of apoptosis of LNCaP prostate cancer cells after doxazosin treatment (48 h). Cells exhibiting the apoptotic morphology were detected using the Hoechst staining and counted under fluorescence microscopy as described in "Materials and Methods." The values shown represent results from three independent experiments performed in duplicate (mean values ±SE).

prostatic epithelial cells was evaluated *in vitro* in the normal cell line, PrEC. Treatment with doxazosin (2 days) resulted in a notable loss of cell viability at higher concentrations, whereas terazosin had no significant effect against normal prostate cells (Fig. 5*A*). Comparative analysis of the effect of doxazosin on the rate of DNA synthesis in the normal and cancer epithelial cell lines, PrEC and LNCaP, demonstrated that whereas there was no significant effect in the normal prostate cells, a moderate dose-dependent decrease in the rate of DNA synthesis was detected in the LNCaP cells (data not shown). Induction of apoptosis in normal prostatic epithelial cells, PrEC, was evaluated by Hoechst staining. The results from the quantitative evaluation of apoptosis induction in response to all three α1-adrenoceptor antagonists in normal prostate epithelial cells are shown on Table 1. After 2 days of treatment with

Table 2 *Effect of α1-adrenoceptor antagonists on cell cycle progression in LNCaP prostate cancer cells[a]*

Treatment	Control	Day-1 post-treatment	Day-2 post-treatment
Doxazosin (25 μM)	74.5% (G$_1$)	70.6% (G$_1$)	67.5% (G$_1$)
	17.8% (S)	21.8% (S)	22.2% (S)
	7.7% (G$_2$)	7.7% (G$_2$)	10.3% (G$_2$)
Terazosin (25 μM)	74.4% (G$_1$)	70.7% (G$_1$)	66.0% (G$_1$)
	17.8% (S)	19.7% (S)	23.0% (S)
	7.7% (G$_2$)	9.6% (G$_2$)	11.0% (G$_2$)

[a] LNCaP cells were exposed to either doxazosin or terazosin for 1 and 2 days, and cell cycle analysis was performed after treatment as described in "Materials and Methods." Values represent the mean percentage of cells in each phase of the cell cycle (shown in parentheses).
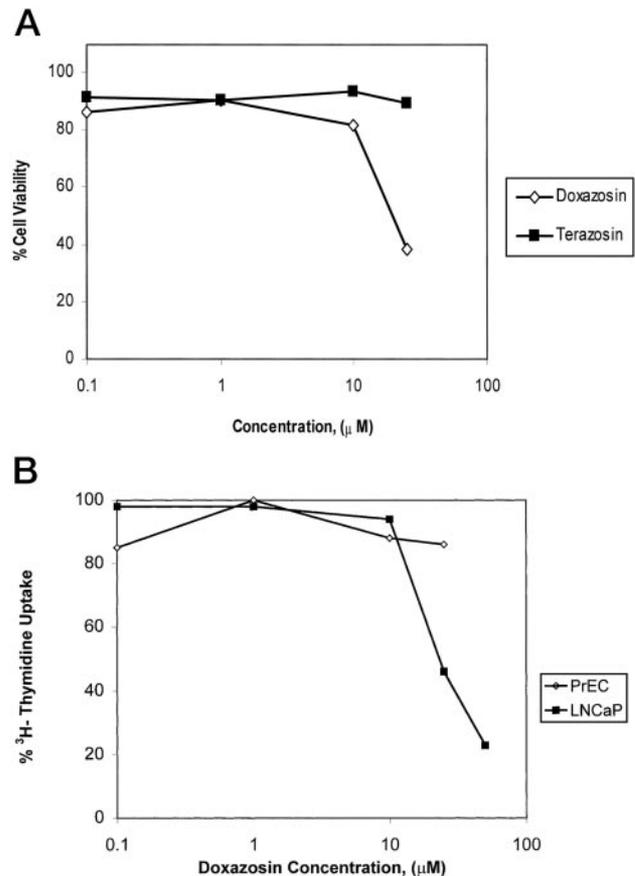
sin doses, DHT had no effect on the temporal profile of cell death (as determined by loss of cell viability; Fig. 4*A*). To determine whether DHT could modify the apoptotic response of LNCaP cells to the quinazoline-derived α1-adrenoceptor antagonists, cells were treated with doxazosin alone (25 μM), doxazosin in the presence of DHT, or DHT alone (1 nM). As shown in Fig. 4*B,* the level of doxazosin-mediated apoptosis induction was similar to that obtained in the presence of DHT, whereas DHT (alone) treatment resulted in basal apoptosis levels, comparable with the values observed for the control, untreated cultures. Thus, DHT (at physiologically relevant doses) did not exert a significant protective effect on doxazosin-mediated apoptosis induction in LNCaP prostate cancer cells (Fig. 4*B*).

Doxazosin treatment at high concentrations resulted in a decrease in the rate of DNA synthesis in LNCaP prostate cancer cells (data not shown). In contrast, terazosin and tamsulosin had no significant effect on the rate of DNA synthesis in this cell line at any of the doses tested. The effect of doxazosin and terazosin on LNCaP cell cycle progression was determined by flow cytometry. After 2 days of treatment with either drug at 25 μM, the percentage of relative distribution of the prostate cancer cells in each phase of the cell cycle remained very similar (Table 2). There was not a significant increase in the number of cells in the G$_2$-M phase, which supports the concept that the prostate antigrowth effect of the quinazolines results from targeting the apoptotic pathway without an antiproliferative activity (Table 2).

The effect of doxazosin and terazosin on the growth of normal

Fig. 5. Effect of quinazoline-derived α1-adrenoceptor antagonists on normal prostate epithelial cells. *A,* a representative cell viability dose response to doxazosin and terazosin in normal human prostate epithelial cells. *B,* a comparative analysis of the effect of doxazosin on the rate of DNA synthesis in LNCaP and normal epithelial cells, PrEC. The values shown are the results of two independent experiments performed in triplicate.

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Table 3 *Effect of α1a-adrenoceptor antagonists on PSA secretion by human prostate cancer cells, LNCaP[a]*

Dose (μM)	Doxazosin % PSA levels	Terazosin % PSA levels	Tamsulosin % PSA levels
0	100	100	100
0.1	86	82	100
0.5	59	80	100
1.0	63	64	100
10	52	55	75
25	14	27	75
50	ND	21	75

[a] LNCaP cells were treated with increasing concentrations of the α1-adrenoceptor antagonists (as shown), and PSA secretion in the culture medium was assessed after 2 days of treatment using an ELISA assay as described in "Materials and Methods." Values are expressed as the percentage of secreted PSA levels relative to the untreated controls. Data are representative of three independent experiments, performed in triplicate.

doxazosin (25 μM), there was a slight increase in the number of apoptotic cells (5%) that was not, however, statistically significant from the control cells (2%; $P > 0.05$). A comparative analysis shows that there was a considerable increase in apoptosis of LNCaP cells. Neither terazosin nor tamsulosin had a significant effect on apoptosis induction in normal prostate epithelial cells.

Because the androgen-sensitive human prostate cancer cells LNCaP produce and secrete PSA, we subsequently determined the PSA secretion in the growth medium of LNCaP cells after treatment with increasing concentrations of the three α1-adrenoceptor antagonists, doxazosin, terazosin, and tamsulosin. As shown in Table 3, treatment with the quinazolines (doxazosin and terazosin) resulted in a dramatic suppression of PSA levels at doses of 10 μM (52 and 55%, respectively), compared with the untreated controls. In contrast, there was no significant effect on PSA secretion levels by the sulfonamide α1-blocker, tamsulosin, at any of the treatment doses (Table 3).

In an attempt to gain a mechanistic insight into the apoptotic effect of quinazoline-derived α1-antagonists in androgen-sensitive prostate cancer cells, we subsequently evaluated the effect of doxazosin on the cellular expression of VEGF and PSA protein in LNCaP cells. Elevated VEGF has been associated with prostate cancer progression (25) and has become a target for antiangiogenesis therapy in androgen-responsive prostatic tumors (26). As shown in Fig. 6, Western blot analysis revealed that within 1 day of doxazosin treatment (25 μM), a moderate decrease in both the M_r 23,000 and 20,000 protein variant forms of VEGF was detected (Fig. 6). This down-regulation was maintained after 2 days of exposure to the quinazoline-based α1-adrenoceptor antagonist.

For the PSA expression profile, a significant decrease in the intracellular PSA protein levels in LNCaP cells was observed within the 1st day of treatment with doxazosin, and by 3 days of treatment, there was a 2-fold reduction in PSA levels compared with the values of the untreated controls. This decrease in PSA correlates with the suppression of PSA secretion (Table 3).

DISCUSSION

Current therapy for prostate cancer is limited by the propensity of the disease to progress from an androgen-dependent to an androgen-independent state. The present findings support the concept that the novel antigrowth activity of the quinazoline-based α1-adrenoceptor antagonists, doxazosin and terazosin, is selectively targeted at apoptosis in androgen-independent and androgen-sensitive prostate cancer cells. Interestingly enough, normal prostate epithelial cells seem to be relatively protected from this quinazoline-mediated apoptotic effect. Our data established that neither of the two quinazoline-derived α1-adrenoceptor antagonists evaluated exerted a significant effect on cell cycle progression of prostate cancer cells. These observations are

in accord with our previous clinically gathered data (17, 18, 27), as well as experimental studies by others (19), that doxazosin and terazosin inhibit benign and malignant prostate growth via induction of apoptosis (over the normal therapeutic dose range) without affecting the rate of cell proliferation. However, this is in contrast with a recent report demonstrating that doxazosin inhibits G_1-S transition in human coronary smooth muscle cells (28), lending support to the possibility that cell type specificity exists with regards to the cellular process targeting of the α1-adrenoceptor antagonist antigrowth function.

Of major mechanistic significance was the observation that this apoptotic effect against prostate cancer cells was independent of the ability of the two α1-adrenoceptor antagonists to antagonize the α1a-adrenoceptor binding in prostate cancer cells. Our results are consistent with previous studies in other cellular systems, demonstrating that doxazosin inhibits growth and migration of the human vascular smooth muscle cells (29) and human coronary smooth muscle cells (28), independent of an antagonistic effect on α1-adrenoceptor. Moreover, this action is in accord with our previous observations that documented that preincubation with phenoxybenzamine, an irreversible inhibitor of α1-adrenoceptor binding (that inactivates α1-adrenoceptors in vascular smooth muscle cells), did not affect the cellular response to doxazosin-induced apoptosis in prostate cancer cells (20). Whereas these data provide an intriguing mechanistic insight, the precise cellular pathway that signals this effect remains to be elucidated. One could speculate that the apoptotic activity of the quinazoline-derived α1-adrenoceptor antagonists could reflect either a direct effect on cellular dynamics or could arise secondarily to an action on cellular apoptotic factors, such as transforming growth factor-β.

The present findings suggest that the cellular sensitivity to quinazoline-induced apoptosis in the androgen-sensitive prostate cancer cells, LNCaP, was not modified by androgens (at physiological levels). Interestingly enough, both doxazosin and terazosin treatment reduced PSA expression and secretion. This is consistent with recent clinical studies from our center that documented the ability of terazosin to reduce tissue PSA expression in patients with prostate cancer (27). Because androgens had no effect on the apoptotic response of LNCaP cells, the data imply that doxazosin-mediated reduction in PSA expression represents a result of the cell killing, rather than being a molecular target for the quinazoline-apoptotic action at the transcriptional level. The effect of quinazoline on VEGF expression is supported by our earlier observations that terazosin inhibits prostate tissue vascularity and VEGF protein levels in clinical specimens of prostate cancer (27).
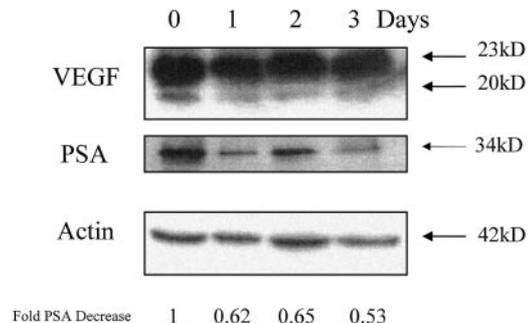

Fig. 6. Effect of treatment with quinazoline-derived α1-antagonists on VEGF and PSA protein expression in prostate cancer cells. LNCaP cells were treated with doxazosin for 1, 2, and 3 days, and cell lysates were subjected to Western blot analysis as described in "Materials and Methods." Aliquots of 60 μg of protein were subjected to electrophoretic analysis through 12% (w/v) SDS-PAGE gels, transferred to nitrocellulose, and probed with specific antibodies. Changes in protein expression were determined by semiquantitation using the Scion Image program and normalized to actin expression. The sizes/molecular weights of the respective proteins (VEGF, PSA, and α-actin) are shown on the *right*. Data are representative of three independent experiments.

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The *in vivo* antitumor efficacy of the α1-adrenoceptor antagonists, against prostate cancer growth via apoptosis induction (20), resembles the *in vivo* effect in the model of intimal hyperplasia (30). Considering this evidence, it is tempting to speculate on a potentially clinically relevant concept that there may be substantive analogies between the effects of α1-adrenoceptor antagonists on cell growth in the cardio-vascular system and in the prostate gland. Attractive as this argument might be, one should also consider the differential effects of doxazosin (apoptotic *versus* antiproliferative) demonstrated in the two cell types (present findings and Refs. 28 and 29, respectively).

In conclusion, the present study indicates that the quinazoline-derived α1-antagonists, doxazosin and terazosin, but not the sulfonamide-based tamsulosin, activate apoptosis in prostate cancer cells via a mechanism independent of androgens and without interfering with cell cycle progression. More significantly, this potent apoptotic activity of doxazosin and terazosin occurs through a novel pathway unrelated to their ability to block the α1-adrenoceptor. Current studies are exploring the apoptotic signaling triggered by the quanizoline-based α1-antagonists, doxazosin and terazosin, within the context of anoikis regulation (31). Preliminary evidence suggests that the quinazolines cause severe perturbation in cell attachment to the extracellular matrix leading to induction of anoikis via direct activation of the transforming growth factor-β signaling pathway.[4]

These findings highlight the emerging therapeutic significance of the use of the class of quinazoline-derived α1-antagonists, not only for the management of BPH but also as potential antitumor agents for the treatment of androgen-dependent and androgen-independent prostate cancer. Because this apoptotic effect is independent of an action on the α1-adrenoceptor, assuming that the molecular mechanism can be unraveled (32), this could represent an exciting new starting point for quinazoline-based drug design for targeting prostate cancer cell apoptosis.

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Quinazoline-derived α1-Adrenoceptor Antagonists Induce Prostate Cancer Cell Apoptosis Via an α 1-Adrenoceptor-independent Action

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