Novel Quinazoline-Based Compounds Impair Prostate Tumorigenesis by Targeting Tumor Vascularity

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Abstract

Previous evidence showed the ability of the quinazoline-based α1-adrenoreceptor antagonist doxazosin to suppress prostate tumor growth via apoptosis. In this study, we carried out structural optimization of the chemical nucleus of doxazosin and a subsequent structure-function analysis toward the development of a novel class of apoptosis-inducing and angiogenesis-targeting agents. Our lead compound, DZ-50, was effective at reducing endothelial cell viability via a nonapoptotic mechanism. Treatment with DZ-50 effectively prevented in vitro tube formation and in vivo chorioallantoic membrane vessel development. Confocal microscopy revealed a significantly reduced ability of tumor cells to attach to extracellular matrix and migrate through endothelial cells in the presence of DZ-50. In vivo tumorigenicity studies using two androgen-independent human prostate cancer xenografts, PC-3 and DU-145, showed that DZ-50 treatment leads to significant suppression of tumorigenic growth. Exposure to the drug at the time of tumor cell inoculation led to prevention of prostate cancer initiation. Furthermore, DZ-50 resulted in a reduced formation of prostate-tumor derived metastatic lesions to the lungs in an in vivo spontaneous metastasis assay. Thus, our drug discovery approach led to the development of a class of lead (quinazoline-based) compounds with higher potency than doxazosin in suppressing prostate growth by targeting tissue vascularity. This new class of quinazoline-based compounds provides considerable promise as antitumor drugs for the treatment of advanced prostate cancer. [Cancer Res 2007;67(23):11344–52]

Introduction

Prostate cancer is a major contributor to cancer mortality in American males, causing the death of ~30,000 men in 2006 (1). Therapeutic modalities such as radical prostatectomy and radiotherapy are considered curative for localized disease, yet no treatments for metastatic prostate cancer are available that significantly increases patient survival (2). Clinical and experimental evidence implicates two components as contributors toward the emergence of the androgen-independent phenotype: activation of survival (apoptosis suppression) pathways and increased tumor neovascularization (3, 4). Consequently, targeting of apoptotic players is of vital therapeutic significance because resistance to apoptosis is not only critical in conferring therapeutic failure to standard treatment strategies but anoikis [cell death on detachment from extracellular matrix (ECM)] also plays an important role in angiogenesis and metastasis of malignant cells (5, 6).

Angiogenesis is critical in tumor progression and metastasis because a functional vascular supply is required for the continued growth of solid tumors and the spread of cancer cells (7). Small nongrowing tumors may remain dormant for years and the angiogenic switch to aggressive metastatic phenotype involves a change in the local equilibrium between factors inducing blood vessel formation and those inhibiting the process (8, 9). During angiogenesis, cells are in a dynamic state, lacking firm attachment to the ECM, and exceedingly vulnerable to anoikis. Consequently, targeting tumor endothelial cell survival by triggering anoikis may provide a molecular basis for novel therapeutic strategies for metastatic prostate cancer. Two classes of angiogenesis-targeting agents consequently emerge: those preventing the development of neovascularature of tumors (via inducing apoptosis and/or inhibiting cell proliferation and migration) and those that directly target the existing tumor vasculature (via anoikis of tumor endothelial and epithelial cells; refs. 10, 11).

The quinazoline-based compounds doxazosin and terazosin are known α1-adrenoreceptor antagonists, which are clinically effective for the relief of benign prostate hyperplasia symptoms via their ability to selectively antagonize the α1A-adrenoreceptors, which are distributed in the bladder neck and prostate gland (12). Recent experimental and clinical evidence, however, documented additional antiproliferative effects of the quinazoline-based adrenoreceptor antagonists, with induction of prostate epithelial and smooth muscle cell apoptosis as one of the molecular mechanisms contributing to their overall long-term clinical efficacy in benign prostate hyperplasia patients (13, 14). Suppression of prostate tumor growth by these drugs proceeds via an α1-adrenoreceptor–independent mechanism, mediated by transforming growth factor-β1 (TGF-β1) apoptotic signaling (15, 16), receptor-mediated apoptosis involving death-inducing signaling complex formation and caspase-8 activity (17), and inhibition of Akt activation (17, 18).

The separation of the effect of doxazosin on cancer cell apoptosis from its original pharmacologic activity in vascular cells provides an intriguing molecular basis to develop a novel class of apoptosis-inducing agents through lead optimization. Our recent pharmacologic exploitation of the quinazoline nucleus of doxazosin led to the development of novel compounds with and without the characteristic "classic" apoptotic activity but exhibiting potent antivascular activity (18). In this study, we report the targeting by the new lead quinazoline-based compounds of prostate tumor epithelial and endothelial cell survival, migration, neovascularization, and angiogenesis in vitro and in vivo.
Materials and Methods

Cell Lines and Reagents

The androgen-independent human prostate cancer PC-3 and DU-145 cell lines were obtained from the American Type Culture Collection and cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen) and antibiotics. Human umbilical cord endothelial cell line BPH-1 (a gift from Dr. Simon W. Hayward, Department of Urological Surgery, Vanderbilt University Medical Center, Nashville, TN) was cultured in RPMI 1640 (Invitrogen) containing 10% FBS and antibiotics. Human vascular endothelial cells (HUVEC) and human lung microvascular endothelial cells-lung (HMVEC-L) were cultured in endothelial medium (EGM-2; Cambrex) supplemented with EGM-2 and EGM-2MV (Cambrex). Recombinant human vascular endothelial growth factor (VEGF) was purchased from Lending Biotech. Doxazosin derivatives (DZ-1-23, DZ-38, DZ-40, DZ-42, and DZ-50) were synthesized as described previously (18).

Apoptosis and Cell Viability Evaluation

Hoechst staining. Cells were plated in six-well culture dishes at 5 × 10^5 per well and, at subconfluence, were treated with increasing concentrations of DZ-1-23, DZ-38, DZ-40, DZ-42, and DZ-50 (0–25 μmol/L). After 24 and 48 h of treatment, cells were fixed with 4% (vol/vol) paraformaldehyde (Sigma) and stained with 10 μg/mL Hoechst 33342 (B2661; Sigma) in the presence of 0.1% Triton X-100 (Sigma) as previously described. Cells were visualized under a Zeiss Axiowert S100 fluorescent microscope with a UV filter (365 nm), and cells with condensed chromatin were designated apoptotic (>100 magnification). The apoptotic index was determined by counting three random fields in duplicate wells per experiment. Each experiment was done twice.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Subconfluent cultures of cells were exposed to increasing concentrations of DZ-1-23, DZ-38, DZ-40, DZ-42, and DZ-50 (0–25 μmol/L). After treatment, the medium was removed and replaced with 250 μl of 0.4%(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma; 1 mg/mL) and incubated at 37°C to form blue crystals. After 2 h, the MTT was removed and replaced with DMSO (250 μL) and incubated overnight at 37°C. The DMSO-crystal solution absorbance was read at 540 nm in a microplate reader (Bio-Tek Instruments). Numerical data represent the average of three independent experiments done in triplicate.

Cell migration assay (wounding assay). Confluent monolayers of PC-3, DU-145, HUVEC, or HMVEC-L cells were wounded with a toothpick. After wounding, medium was changed to DZ-3 or DZ-50 (5 μmol/L). After incubation for 12 or 24 h, wounding areas were examined by light microscopy (Axiowert 10, Zeiss). Cells that had migrated to the wounded areas were counted under a microscope for quantification of cell migration. Migration was calculated as the average number of cells observed in five random high-power (400 ×) wounded fields per well in duplicate wells.

Tube formation assay: in vitro angiogenesis evaluation. In vitro formation of tubular structures was studied on ECM with an angiogenesis kit as described by the manufacturer (Chemicon International, Inc.). HUVEC or HMVEC-L (10 × 10^5 per well) in 96-well plates were seeded onto ECM-coated wells in the presence or absence of DZ-3 or DZ-50 and VEGF. Cells were treated with cytokines as single agents or in combination (e.g., DZ-50 and VEGF). Twenty-four hours after treatment, angiogenesis was assessed on the basis of formation of capillary-like structures of HUVEC according to the manufacturer's protocol. The capillary-like tubes were counted (Nikon Eclipse, TE2000-U) in each well.

Chicken Chorioallantoic Membrane Assay

Fertilized chicken eggs were incubated at 37°C. At E8, a window was created to allow visualization of the egg shell membrane. Blank paper discs (6 mm; Becton Dickinson) were placed on the egg shell membrane along with VEGF (100 ng) or basic fibroblast growth factor (bFGF; 100 ng) and DZ-50. The windows were sealed with porous absorbent and allowed to incubate for 48 h. At E10, the adhesive was removed along with the egg shell membrane to expose the chorioallantoic membrane and 4% paraformaldehyde was added. Following excision, the number of vessels per chorioallantoic membrane was quantified by counting under a dissecting microscope.

Cell Attachment Assay

Prostate cancer PC-3 and DU-145 cells were treated for 3, 6, 9, 12, or 15 h with DZ-50 (5 μmol/L) and harvested. Cells (5 × 10^3) were added to each well of a six-well culture dish coated with either collagen or fibronectin and incubated for 30 min at 37°C. Following incubation, cells were fixed and the number of cells per well was recorded. Numerical data represent the average of three independent experiments in triplicate.

Transendothelial Migration Assay

Sterile (12-mm diameter) glass coverslips were coated with Matrigel (Becton Dickinson) at a dilution of 1:8 and air-dried at room temperature (1 h). Coverslips received 100 μm/L HMVEC-L to form a complete monolayer. The cells were allowed to spread on the Matrigel for 24 h before the experiment. PC-3 cells were resuspended in EGM-2MV (Cambrex) and added to the HMVEC-L monolayer at a concentration of 8 × 10^5 per coverslip. Cocultures were incubated at 37°C at 5% CO2 for 3, 6, 9, 12, and 24 h. Before the addition of prostate epithelial cells, cells were incubated with the lipophilic tracer 1,1'-dioctadeyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen) at 10 μg/mL for 20 min to stain cell membranes. To label F-actin, PC-3 cells, or cocultures of PC-3 cells, HMVEC-L were fixed for 10 min at room temperature in 2% paraformaldehyde in PBS and were permeabilized for 5 min with a buffer containing 15 μmol/L Tris, 120 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, and 0.5% Triton X-100 (pH 7.4). Cells were incubated for 1 h at room temperature with Alexa 488-conjugated phallolidin at a dilution of 1:50 in blocking solution, followed by 5 min of incubation with 10 mmol/L Hoescht 33342 (Sigma) in PBS. Coverslips were mounted with Vectashield (Vector Laboratories) on glass slides and analyzed by confocal microscopy.

Western Blot Analysis

Cultures of PC-3, DU-145, BPH-1, HUVEC, and HMVEC-L cells were treated with DZ-50 (10 μmol/L) for various time periods and cell lysates were subsequently generated in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 8.0), 0.5% deoxycholic acid, 1% NP40 with 1 mmol/L phenyl methyl-sulfonyl fluoride]. The total protein concentration in the lysates was quantified with BCA Protein Assay Kit (Pierce) and protein samples (30 μg) were subjected to SDS-PAGE and transferred onto Hybond-C membranes (Amersham Pharmacia Biotech.). After blocking with 5% dry milk in TBS-0.05% Tween 20 for 1 h at room temperature, membranes were incubated overnight at 4°C with antibodies against caspase-8, Akt, or phosphorylated Akt (Cell Signaling Technology). Following incubation with the respective primary antibody, membranes were exposed to species-specific horseradish peroxidase–labeled secondary antibodies. Signal detection was achieved with SuperSignal West Dura Extended Duration Substrate (Pierce) and visualized with a UV Bioimaging System (Upland). All bands were normalized to α-actin expression (Oncogene Research Products).

Fluorescence-activated cell sorting—(FACS) analysis. PC-3 cells were treated with DZ-50 (10 μmol/L) and harvested with 0.5 mmol/L EDTA solution. Prostate cancer epithelial cells were then incubated with HBSS supplemented with 2% bovine serum albumin and 0.01% sodium azide for 30 min at 4°C. Cells were subsequently fixed in 4% (wt/vol) formaldehyde, washed, and incubated with the designated integrin antibody followed by FITC-conjugated goat anti-mouse secondary. Flow-cytometry analysis was done on a Partec FlowMax (Partec).

Tumorigenicity studies. Human prostate cells (PC-3 and DU-145) suspended in PBS were inoculated s.c. (2.5 × 10^3 per site) in the flank of male nude mice 4 to 6 weeks of age. Tumors were measured every 48 h with a digital caliper, and tumor volumes were calculated using the following formula:

\[ V = \frac{1}{2} \times \text{width} \times \text{length} \times \text{height} \]

When tumors reached ≥50 mm³, mice were stratified into treatment groups of six mice per treatment. DZ-50 was administered at doses of 50, 100, and 200 mg/kg in 0.5% methylcellulose (wt/vol) + 0.1% Tween-80 (vol/vol) in water by oral gavage using a 22-gauge, 1.5-in. gavage needle. Animals were sacrificed after 2 weeks of treatment unless otherwise indicated. In a separate experiment, human prostate cells

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(PC-3) were inoculated as described above and dosing began (200 mg/kg) concurrently for 2 weeks. On termination of the experiment, tumors were surgically excised and tissue specimens were fixed in a 10% (vol/vol) formalin solution (Sigma) and subsequently embedded in Paraplast X-tra paraffin (VWR). Blocks were sectioned (6 μm) on a Finesse Microtome (ThermoShandon).

Spontaneous metastasis assay. Human prostate cells (PC-3) were injected (2 × 10⁶/80 μL of PBS) in the tail vein of male nude mice 4 to 6 weeks of age; mice were maintained in a pathogen-free environment. At 10 days postinoculation, 200 mg/kg DZ-50 was given daily (via oral gavage as described above). After 2 weeks of treatment, DZ-50–treated and vehicle control mice were sacrificed and lungs, spleen, kidneys, and prostate organs were excised and subjected to examination for metastatic tumor lesions.

Apoptosis evaluation. Apoptotic cells were detected with the ApopTag Peroxidase In Situ Apoptosis Kit (Chemicon). Briefly, paraffin-embedded sections were treated with Protease K (DAKO) and were subsequently incubated with terminal deoxynucleotidyl transferase enzyme. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive cells were counted in five different fields (×400) and the apoptotic index was determined based on the number of apoptotic cells over the total number of cells.

Vascularity evaluation. CD31 staining was done for endothelial cells by enzymatic digestion with Protease K (DAKO). The primary antibody used was the mouse anti-human CD31 specific for endothelial cells from DAKO (overnight incubation at 4°C). CD31–positive endothelial cells were counted in five different fields (×400).

Statistical Analysis
One-way ANOVA was done using the StatView statistical program to determine the statistical significance between values. P < 0.05 was considered statistically significant.

Results
DZ-50 is effective at inducing cell death via a nonapoptotic mechanism. Pharmacologic exploitation of the quinazoline nucleus of doxazosin led to the development of several novel agents with varying effects on apoptosis (Fig. 1A). Functional characterization of these compounds revealed two classes of agents: those that are not effective at inducing apoptosis but elicit their effects by an alternative cell-death mechanism (DZ-50) and those that trigger

![Figure 1](https://cancerres.aacrjournals.org/issue/Cancer%20Research/76/11/12196/12196_F1.jpg)

Figure 1. Effect of novel lead quinazoline-derived compound DZ-50 on human prostate cancer cells. A, chemical structure of DZ-50: the 2,3-dihydrobenzo[1,4]dioxane-carbonyl moiety of doxazosin was replaced with the biphenyl aryl sulfonyl substituent, whereas the methoxy side chains were replaced with isopropyl propoxy functions. B, apoptosis induction by novel quinazoline compounds. PC-3 cells were treated (10 μM/L) for 24 h and apoptosis was measured by Hoechst staining. C, apoptosis induction by DZ-3. Fluorescence-activated cell sorting analysis of propidium iodide and bromodeoxyuridine staining was done on PC-3 cells treated with DZ-3 (10 μM/L) and a negative control, DZ-50 (10 μM/L). D, cell death following DZ-50 treatment. Cell death was evaluated in endothelial and epithelial cell lines following 24 and 48 h (inset) of treatment with DZ-50 (5, 10, 15, 20, and 25 μM/L) as described in Materials and Methods.
The most intriguing compound from the first category was DZ-50, which reduced cell viability in a number of endothelial and epithelial cell lines at both 24 and 48 h without induction of classic apoptosis (Fig. 1D).

Anoikis effect of DZ-50: inhibition of cell migration and cell adhesion. The ability of DZ-50 to potentially trigger anoikis of tumor epithelial and endothelial cells was subsequently investigated. Treatment with DZ-50 at (noncytotoxic doses 5 and 10 \( \mu \)mol/L) led to a significant inhibition of migration of endothelial cells and prostate cancer epithelial cells (PC-3 and DU-145; Fig. 2A). Inhibition of prostate cancer cell migration was not due to drug-induced loss of cell viability (Fig. 2B).

Figure 2. DZ-50 prevents cell migration and adhesion to ECM of human prostate tumor epithelial cells and vascular endothelial cells. A, wounding assays were done on endothelial and epithelial cells and the number of migratory cells was quantified as described in Materials and Methods. There was a significant reduction in the migratory capacity detected in the vascular endothelial and tumor epithelial cells analyzed (\( *, P < 0.0001; **, P < 0.001; ***, P = 0.004 \)). B and C, DZ-50 partially inhibits prostate tumor epithelial cell attachment to ECM components. The ability of PC-3 prostate cancer cells to adhere to ECM protein components was evaluated after exposure to DZ-50 for 6, 9, and 12 h at concentrations of 5 and 10 \( \mu \)mol/L. Attached prostate cancer cells were counted on fibronectin- or collagen-coated culture dishes (columns, mean; bars, SD). DZ-50 reduced the ability of PC-3 cells to attach to either fibronectin or collagen, but this effect was not statistically significant. D-I and II, DZ-50 prevents prostate cancer epithelial cell attachment to endothelial cells. Transendothelial migration assays were done to assess the ability of PC-3 prostate cancer cells to attach and migrate through a confluent monolayer of HMVEC-L following exposure to DZ-50. D-I, PC-3 cells were stained with the lipophilic tracer Dil (red) and were subsequently added to a confluent monolayer of HMVEC-L. Epithelial cell adhesion to the endothelial cell monolayer was prevented following 9 h of exposure to the drug (10 \( \mu \)mol/L). Cell viability assays were done on PC-3 and HMVEC-L cells treated with DZ-50 (10 \( \mu \)mol/L). No death was detected within the first 24 h of treatment, indicating that blocking of transendothelial tumor migration was not due to drug-induced loss of cell viability (D-II).
monolayer of HMVEC-L following exposure to DZ-50. PC-3 cells were stained with the lipophilic tracer DiI (red) and subsequently added to a confluent monolayer of HMVEC-L and exposed to DZ-50 for 3 and 9 h (Fig. 2D). 4',6-Diamidino-2-phenylindole (DAPI) staining identified the nuclei (blue). As shown in Fig. 2D, tumor epithelial cell adhesion to the endothelial cell monolayer was prevented following 9 h of exposure to the drug (10 μmol/L). There was no effect on cell viability or cell death in either cell population (PC-3 or HMVEC-L cells) in response to the drug DZ-50 (10 μmol/L) within the first 24 h of treatment (Fig. 2D), indicating that the effect on transendothelial tumor cell migration was not due to drug-induced cell death.

We subsequently investigated the direct effect of our lead drug DZ-50 on angiogenesis in vitro by the tube formation assay. As shown in Fig. 3A and B, after treatment with DZ-50, vascular endothelial cell tube formation was significantly inhibited. Furthermore, exposure to DZ-50 led to a significant suppression of angiogenesis or vascularity in the in vivo chorioallantoic membrane blood vessel development assay (Fig. 3C and D). Simultaneous presence of a potent angiogenic factor, VEGF and/or bFGF (data not shown), was not able to rescue the cells from the antiangiogenic effect of DZ-50.

**Reduction of integrin β1 surface expression by DZ-50.** To explore the potential mechanism underlying that action of DZ-50 against prostate tumor epithelial cells, analysis of the integrin expression profile was done. PC-3 untreated control cells were found to express integrin subunits α2, α3, αV, h1, and h3 (Supplementary Fig. S2). Exposure to DZ-50 did not affect the surface expression of integrins α2, α3, αV, and h3 (data not shown). As shown in Fig. 4A, integrin β1 subunit was undetectable in cells treated with DZ-50 for 12 to 24 h, compared with vehicle control (Fig. 4A). DU-145 prostate cells exposed to DZ-50 exhibited a significantly smaller shift in integrin β1 expression intensity (Fig. 4B).

**DZ-50 treatment suppresses prostate tumor growth in vivo.** To assess the ability of DZ-50 to suppress prostate cancer growth, we subsequently investigated the in vivo antitumor efficacy in human prostate cancer xenografts growing in nude mice. Our

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**Figure 3.** DZ-50 prevents angiogenesis in vitro and in vivo. A and B, in vitro angiogenesis is blocked following exposure to DZ-50. Endothelial cells were seeded in Matrigel in the presence or absence of either DZ-50 or doxazosin at 10 μmol/L concentration and tube formation was visualized and quantified in the presence or absence of VEGF, as described in Materials and Methods. Control (top) shows HUVEC tube formation with decisive branch points whereas DZ-50 shows severely abrogated branch point formation. B, quantitative analysis of the data; a significant reduction in tube formation is detected in the presence of DZ-50 compared with controls, whereas the novel quinazoline DZ-10 (no effect on cell viability-negative control) does not change the ability of HUVEC cells to form multibranched tubular networks. VEGF cannot reverse the antiangiogenic effect of DZ-50. C and D, in vivo angiogenesis is blocked by DZ-50. Chorioallantoic membrane assays were done in the presence or absence of DZ-50, as described in Materials and Methods, and the number of blood vessels was counted.
initial toxicity studies revealed no change in the animal's behavioral pattern and weight (data not shown). Both gross and histologic examination of lung, liver, spleen, and prostate showed no apparent changes compared with control animals (data not shown). The tumorigenicity studies showed a significant reduction in tumor volume in both androgen-independent human prostate cancer PC-3 and DU-145 tumor xenografts following treatment with DZ-50 (200 mg/kg; Fig. 5A and B). The efficacy of DZ-50 to hinder the growth initiation of prostate tumors was examined by inoculation of nude mice with PC-3 prostate cancer cells with simultaneous treatment with DZ-50 (200 mg/kg). As shown in Fig. 5C, prostate tumor development was dramatically suppressed with drug exposure (2 weeks).

*In situ* detection of apoptosis in prostate tumors revealed no significant change in the apoptotic index of DZ-50 of prostate cancer xenografts from treated tumor-bearing mice compared with control (Table 1; Supplementary Table S3), further verifying that this compound does not induce apoptosis. Also shown in Table 1 is that there are no significant changes in the proliferative index of human prostate tumor xenografts from PC-3 and DU-145 cells derived from untreated and DZ-50–treated tumor-bearing hosts. In contrast, treatment with DZ-50 led to a significant suppression of vascularity and angiogenesis, as detected by the reduced CD31 immunoreactivity in both PC-3– and DU-145–derived prostate tumor xenografts compared with the untreated prostate tissue (control mice; Table 1; Supplementary Table S2). The results from the immunohistochemical analysis of prostate tumor apoptosis, vascularity, and cell proliferation indicate that the DZ-50–mediated reduction in prostate tumor growth is, at least in part, consequential to targeting and reduction of angiogenesis.

The ability of DZ-50 to directly affect tumor cell metastasis was evaluated with the *in vivo* spontaneous metastasis assay. Following 21 days of DZ-50 treatment, there was a significant reduction in the number of metastatic foci to the lungs compared with the untreated control mice (Fig. 6). These results indicate the ability of DZ-50 to prevent and reduce prostate tumor growth, as well as inhibit invasion and metastatic potential *in vivo*.
Discussion

This study shows that our lead drug, DZ-50, a novel quinazoline-based compound, effectively targets human prostate tumor epithelial cells as well as vascular endothelial cells without inducing classic apoptosis. This unique feature of the antitumor action of the new drug, inducing a pattern of cell death that is independent of caspase-activation characteristic of apoptotic signaling, is mechanistically intriguing. The invasion process requires a range of cell-to-cell interactions, primarily through the association of adhesion complexes between tumor cells and the adjacent endothelial cells. The present findings indicate that DZ-50 triggers the anoikis phenomenon, as it interferes with prostate tumor cell migration and attachment to ECM components fibronectin and type I collagen (most abundant protein in bone). Examination of the ability of tumor cells to extravasate by an in vitro model of transendothelial migration revealed that prostate tumor cells, on treatment with DZ-50, lost their ability to attach to the monolayer of endothelial cells; our results indicate that attachment of tumor epithelial cells to an endothelial monolayer was significantly inhibited after 6 h of exposure to DZ-50 and was completely abrogated after 9 h of treatment at nontoxic doses. These in vitro data indicate that the lead compound can effectively minimize the possibility of transendothelial invasion and metastatic behavior of prostate cancer cells.

Collagen I binds the integrin pairs α1β1, α2β1, and α3β1 (19), and although we were unable to detect α1 expression in PC-3 and DU-145 prostate cells, there was strong expression of integrins α2β1 and α3β1. Following exposure to DZ-50, the PC-3 prostate cancer cells (originally isolated from a prostate tumor bone metastasis) exhibited complete loss of integrin β1 surface expression, whereas DU-145 prostate cancer cells had a minimal loss. Interestingly, human prostate cancer cells, characterized by a specific ability for bone metastasis, migrate toward collagen type I in an α2β1-dependent manner, leading to increased in vivo growth within the bone (20). Thus, one could argue that down-regulation of integrin β1 could provide the molecular basis for the response of prostate cancer cells to DZ-50. The regulation of β1 integrin expression has been shown to be altered by TGF-β1 signaling (21).

Figure 5. Suppression of primary tumor growth in human prostate cancer xenograft model by DZ-50. A and B, tumor volume of prostate xenografts is reduced following DZ-50 treatment. Following s.c. inoculation of nude mice (n = 6 per group) with either PC-3 (A) or DU-145 (B) human prostate cancer cells, DZ-50 (100 and 200 mg/kg) was administered p.o. (via oral gavage) to tumor-bearing hosts for 14 d (subsequent to palpable tumor formation). Tumor volume was measured daily as described in Materials and Methods; DZ-50 treatment significantly suppressed prostate tumor volume compared with the vehicle control (P < 0.001). C, primary inhibition of androgen-independent human prostate tumor growth by DZ-50. To determine the ability of the new lead drug to interfere with prostate cancer development, nude mice were s.c. inoculated (n = 6 per group) with PC-3 cells with concurrent exposure (p.o.) to DZ-50 (200 mg/kg) for 2 wk. D, prostate cancer xenografts were excised from DZ-50–treated and vehicle control tumor-bearing mice, paraffin embedded, and then tissue sections (6 μmol/L) were subjected to immunohistochemical analysis for apoptosis, cell proliferation, and tumor vascularity (A and B). The three images represent TUNEL staining for apoptosis, CD31 immunoreactivity for vascularity, and Ki67 expression for cell proliferation (magnification, ×400).
transcriptional level by its attachment to the ECM, at posttranscriptional or translational level (22, 23), and during differentiation (24) and cancer progression (25). Moreover, integrin α2β1 mediates PC-3 cell adhesion to collagen and fibronectin, both major components of bone microenvironment (19), with some therapeutic promise. Thus, ionizing radiation leads to a significant reduction in β1 integrin levels and a decrease in cell adhesion to fibronectin (26).

The present findings indicate that in vivo administration of the novel lead drug DZ-50 (at well-tolerated doses) not only significantly inhibits the growth of established human xenograft prostate tumors but also prevents the initiation of prostate cancer development in this model. Moreover, exposure to DZ-50 resulted in a considerable suppression of the metastatic capacity of human prostate cancer cells, potentially by targeting their invasion and migration potential. Initial mechanistic dissection pointed to integrins as primary candidates of drug targeting. Integrin β1–knockout mice fail to develop a vasculature (27); thus, a direct functional link between reduced tumor growth and a lack of integrin β1 is an attractive possibility. Furthermore, VEGF directly activates integrins α5β1 and α2β1, which are both implicated in angiogenesis (28). One could easily argue that loss of integrin β1 expression by DZ-50 (as detected in the present study) could interfere with VEGF signaling and thus lead to reduced tumor vascularity without affecting tumor cell death. VEGF has been specifically targeted by strategies such as

### Table 1. Effect of DZ-50 treatment on apoptosis, cell proliferation, and vascularity of human prostate cancer xenografts

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<th></th>
<th>PC-3</th>
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<th>DU-145</th>
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<td></td>
<td>Control</td>
<td>100 mg/kg</td>
<td>200 mg/kg</td>
<td>Control</td>
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<tr>
<td>TUNEL (apoptotic index)</td>
<td>1.4 ± 0.3</td>
<td>2.0 ± 0.8</td>
<td>1.4 ± 0.4</td>
<td>3.2 ± 0.8</td>
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<td>CD31+ cells (vasularity)</td>
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<td>13.5 ± 1.9</td>
<td>6.5 ± 0.6*</td>
<td>18.5 ± 0.9</td>
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<tr>
<td>Ki67 (proliferation index, %)</td>
<td>43.7</td>
<td>42.6</td>
<td>45.0</td>
<td>51.2</td>
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NOTE: Quantitative analysis of immunostaining of tumor sections (Fig. 5) revealed significant decrease in the number of CD31-positive cells was detected in both PC-3–, and DU-145– derived prostate tumors following treatment with DZ-50. Neither the apoptotic nor the proliferative index of the prostate tumor cell populations were affected by DZ-50 treatment.

*P < 0.01.

Figure 6. Inhibition of metastasis of human prostate cancer cells by DZ-50. In the experimental metastasis assay, nude mice (n = 7 per group) were injected with prostate cancer cells PC-3 (2 × 10⁶) through the tail vein. DZ-50 treatment (200 mg/kg) was initiated at 10 d postinoculation for 21 d. Evaluation of the lungs (under dissecting microscope) revealed a significant reduction in the number of metastatic lesions to the lungs in the DZ-50–treated group compared with vehicle control mice; P < 0.05. Arrows, metastatic foci on the lungs.
monoclonal antibodies (bevacizumb) and inhibitors of endothelial cell receptor–associated tyrosine kinase activity (9). Other approaches including targeting basement membrane degradation, endothelial cell migration, and endothelial cell proliferation have also been clinically evaluated, but success has been variable (29, 30).

In increases patient survival in response to any antiangiogenic therapy have yet to be reported and current antiangiogenic therapy has been clinically ineffective. Phase III clinical trial data are lacking for any novel antiangiogenic compound, thus the immediate need for new targeted therapies for metastatic prostate cancer. Ongoing studies focus on dissecting the ability of the lead DZ compounds to target the interactions between integrin β1 and its intracellular signaling partners. Decreased surface expression of integrin β1 might result from down-regulation at the transcriptional or translational level. Alternatively, integrin β1 deregulation in response to DZ-50 might be an indirect effect from alterations in the focal adhesion complex (talin, focal adhesion kinase) and other key components of the actin microfilaments that determine cell motility and migration. From a therapeutic standpoint, either mechanism could prove beneficial because by reducing the migratory capacity of tumor epithelial cells and/or inducing anoikis of endothelial cells, we could effectively prevent their ability to metastasize.

Because analytic measurement of DZ-50 levels in plasma and/or tissues has yet to be developed, we cannot conclude that the drug concentrations shown to be effective in vitro (10 μmol/L) could be achieved in vivo. While recognizing this as a caveat that might affect the translational value of the present findings, one has to also consider the significant tumor growth suppression detected in the prostate tumor xenograft models and the associated intratumoral biomarker modulation consistent with the in vivo findings collectively suggesting that well-tolerated, effective concentrations of DZ-50 can be achieved in vivo after p.o. administration. Ongoing studies focus on the evaluation of the drug levels in biological samples as well as subsequent biodistribution and pharmacokinetic analyses.

The observed effect of DZ-50 in preventing prostate tumor development in the xenograft model implies a prophylactic value for these compounds. Indirect support for such a concept stems from the recent epidemiologic cohort study indicating that exposure to doxazosin significantly decreases the incidence of prostate cancer among men (31), thus suggesting a chemopreventive role for the quinazoline-based compounds.

Finally, a combination of DZ-50 (targeting vascularity) with an apoptosis-inducing regimen for the treatment of metastatic prostate cancer emerges as an attractive therapeutic possibility begging pursuit.

References

23. Meléady P, Clunes M. Bromodeoxyuridine induces in vivo expression at transcriptional (α2 subunit) and post-transcriptional (β1 subunit) levels, and alters the adhesive properties of two human lung tumour cell lines. Cell Commun Adh 2001;8:45–59.
30. Eskens FA. Angiogenesis inhibitors in clinical development—where are we now and where are we going? Br J Cancer 2004;89:1–7.
Novel Quinazoline-Based Compounds Impair Prostate Tumorigenesis by Targeting Tumor Vascularity

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