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Simultaneous Targeting of VEGF Message and VEGF Receptor Signaling as a Therapeutic Anticancer Approach

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Abstract. *Background: Vascular endothelial growth factor (VEGF) is one of the most important factors involved in tumor angiogenesis. Materials and Methods: Antisense phosphorothiolate oligodeoxynucleotides (PS-ODNs) were used to reduce VEGF production while the small molecule PD0203359-0002 (PD203359) was used to inhibit VEGF/bFGF receptor tyrosine kinase activity. Results: PD203359 exposure was found to profoundly impair the growth of human endothelial cells (HMVEC-L) at doses 20-fold less than those affecting human renal cell carcinoma (Caki-1) cell growth. In vivo, treatment with PD203359 inhibited tumor cell-induced angiogenesis and resulted in a significant tumor growth delay. Treatment with VEGF antisense PS-ODNs also significantly increased the time for tumors to grow to five times the starting size. Most importantly, when the PD203359 and VEGF antisense treatments were combined, a greater antitumor response than could be achieved with either therapy alone was observed. Conclusion: Simultaneously targeting VEGF production and VEGF receptor signaling enhances the anticancer efficacy of either therapy alone.*

It is widely accepted that tumor growth beyond a few cubic millimeters cannot occur without the induction of a new vascular supply (1). Consequently, inhibiting tumor vessel growth through the application of anti-angiogenic therapy has become a major focus of cancer research. Tumor angiogenesis is a complicated process that involves multiple, sequential and interdependent steps that include endothelial cell activation, proliferation and migration, basement membrane degradation, tube formation and maturation (2).

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Key regulatory growth factors involved in this process provide potential targets for angio-suppressive strategies. The predominant factor in the orchestration of blood vessel formation in a variety of human cancers is the cytokine vascular endothelial growth factor (VEGF) also known as vascular permeability factor (3). Its stimulatory effect on endothelial cell proliferation and migration is mediated through its interaction with a family of VEGF receptors and their associated high-affinity receptor tyrosine kinases, VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR) (4). The expression of these receptors is largely restricted to the vascular endothelium. VEGF-R2 is thought to be the dominant signal transduction pathway regulating angiogenesis (5). Effective blockade of the VEGF pathway has been demonstrated with multiple agents: neutralising antibody, receptor tyrosine kinase inhibitors, and ribozyme or antisense molecules targeting expression (6-10).

Despite positive tumor responses in preclinical investigations (11,12) and the promise of clinical utility (13-15), the complexity of tumor-induced angiogenesis poses potential serious limitations to the effectiveness of single agent anti-angiogenic therapy. In addition, the very nature of the angiogenic process suggests that, in order to achieve long lasting antitumor effects, anti-angiogenic agents will need to be administrated in a chronic fashion. However, the toxicity associated with such long-term exposures to therapeutic anti-angiogenic agents may be significant and become dose-limiting. One possible approach to overcoming such difficulties may be the use of combination anti-angiogenic agent therapy. Combining agents with different mechanisms of action has the potential not only lead to enhanced antitumor efficacy, but also to achieve such a result without an increase in normal tissue side-effects. Consequently, the evaluation of treatments combining different anti-angiogenic agents appears to be warranted.

In the present study, we evaluated the antitumor efficacy of simultaneously targeting two aspects of the VEGF signaling pathway; the tumor cell production of VEGF and the endothelial cell VEGF receptor-associated tyrosine kinase signaling.

Materials and Methods

Cell culture. The clear cell renal cell carcinoma cell line Caki-1 was a gift from Dr. Susan Knox (Stanford University, USA). Caki-1 cells were grown in Dulbecco's modified minimum essential medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% penicillin-streptomycin (Invitrogen) and 1% 200 mmol/L L-glutamine (Invitrogen). Human lung microvascular endothelial cells (HMVEC-L) were obtained from Cambrex (San Diego, CA, USA). HMVEC-L cells were grown in EBM-2-MV (Cambrex) supplemented with 5% FBS.

Caki-1 xenografts. Female nude mice (NCR, nu/nu), age 6 - 8 weeks were obtained from the National Cancer Institute (Frederick, MD, USA) and maintained under specific pathogen-free conditions (University of Florida Health Science Center, USA) with food and water supplied *ad libitum*. Animals were inoculated subcutaneously in a single flank with 5 x 10^6 tumor cells. When the tumors reached a size ~200 mm^3, the animals were randomly assigned to different treatment groups or kept as untreated controls.

Phosphorothioate oligodeoxynucleotides (PS-ODNs). Antisense and control PS-ODNs (20-mers) were custom synthesized by Geno Mechanix (Alachua, FL, USA). PS-ODNs V515 was complementary to 5' UTR just up-stream of the translation start site (AUG codon) of VEGF mRNA: 5'-CTC ACC CGT CCA TGA GCC CG-3'. A scramble sequence: 5'-CAC CCT GCT CAC CGC ATG GC-3'; sense sequence: 5'-CGG GCT CAT GGA CGG GTG AG-3' and an inverted sequence: 5'-GCC CGA GTA CCT GCC CAC TC-3', were used as PS-ODNs controls. All PS-ODNs were suspended in sterile and endotoxin-free water at a concentration of 1 mM, aliquoted and stored at -20˚C (8).

DOTAP:DOPE liposome preparation. Cationic liposomes were prepared using the method described by Tang (16). Briefly, cationic lipid 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) was dissolved in chloroform and mixed with a helper lipid 1,2-dioleoyl-3-sn-phosphatidylethanolamine (DOPE) (Avanti Polar-Lipids, Alabaster, Al, USA) at a molar ratio of 1:1. The mixture was evaporated to dryness in a round-bottomed flask using a rotary evaporator at room temperature. The resulting lipid film was dried by nitrogen for an additional 10 min to evaporate any residual chloroform. The lipid film was re-suspended in sterile water to a final concentration of 1 mg/ml based on the weight of cationic lipid. The resultant mixtures were shaken in a water bath at 35˚C for 30 min. The suspensions then were sonicated using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, USA) for 1 min at room temperature to form homogenized liposomes. The particle-size distribution of liposomes was measured using a NICOMP 380 ZLS instrument (Santa Barbara, CA, USA). The average particle diameter was 144.0±77.0 nm. Liposomes were stored at 4˚C and used within 3 months.

PD0203359-0002 (PD203359) drug preparation. PD203359 was received as a gift from Pfizer (New York, NY, USA). For *in vitro* experiments, stock solutions (10 mM) were made in 100% DMSO and diluted with culture media before use. Culture media containing an equivalent DMSO concentration served as vehicle controls. For *in vivo* experiments, PD203359 was prepared in 0.05 M sodium lactate buffer and administered by gavage.

Cell growth curve. Caki-1 or HMVEC-L cells were plated in 60-mm dishes at 5 x 10^4 or 2 x 10^4, respectively, and allowed to attach overnight. Different doses of PD203359 were then added to each well. At various times thereafter the cells were trypsinized and counted using a hemocytometer.

Cell cycle assay. HMVEC-L and Caki-1 cells were plated in 60-mm dishes at 1 x 10^5 cells per dish and allowed to attach overnight. The cells then were treated with different doses of PD203359. Seventy-two h later, the cells were trypsinized, counted and fixed in 50% ethanol overnight. The cells then were treated with 1 mg/ml RNase in PBS for 30 min, washed twice in PBS and resuspended in 25 mg/ml propidium iodine in PBS at a density of 1 x 10^6 cells/ml. The cells were stained in the dark for a period of 15 min prior to FACS analysis for cell cycle distribution on a Beckman Dickson flow cytometer (University of Florida Flow Cytometry Core Facility).

Intradermal angiogenesis assay. Caki-1 cells (5 x 10^4) were inoculated intradermally at a volume of 10 ml at 4 sites on the ventral surface of nude mice. One drop of 0.4% trypan blue was added to the cell suspension, making it lightly colored and thus simplifying subsequent location of the sites of injection. Three days later the mice were killed, the skin carefully separated from the underlying muscle and the number of vessels counted using a dissecting microscope. Scoring of all of the reaction areas was carried out at the same magnification (x5) and only vessels readily detected at this magnification were counted (17). The sites of injection, recognized by local swelling and blue staining, were exposed by carefully removing fat or other tissue covering the area. All vessels that touched the edge of the tumor inoculates were counted. All the animals in the experiments were pre-coded and vessel counts in each animal were scored twice. The data from each treatment group were pooled for statistical analysis (Wilcoxon rank sum test).

Tumor growth delay assay. Once the Caki-1 xenografts reached a size of ~200 mm^3, animals were assigned randomly to various treatment groups. V515 or control PS-ODNs were administered *via* the tail vein with DOTAP:DOPE liposomes at a dose of 5 mg/kg or 10 mg/kg on day 1 and day 4. PD203359 or sodium lactate buffer was administered daily by gavage for 14 days. Tumors were measured using calipers and volumes were approximated by the formula, volume=$^1/_6$ (πab^2), with a and b representing two perpendicular tumor diameters. The times for the tumors in the various treatment groups to grow from 200 mm^3 to 1000 mm^3 were recorded and compared (Wilcoxon rank sum test).

Results

PD203359 is a small molecule VEGF/bFGF receptor tyrosine kinase inhibitor. The direct effect of PD203359 on cell growth was examined *in vitro* in both tumor (Caki-1) and endothelial (HMVEC-L) cells. Exponential phase HMVEC-L cells were found to be very sensitive to treatment with this agent; inhibition of cell growth was achieved at a dose of about 0.5 nM (Figure 1). In contrast, significant inhibition of Caki-1 cell proliferation could be achieved only at much higher doses (>250 nM) (Figure 1). The marked sensitivity of endothelial cells appeared, at least in part, to be due to a block in the cell cycle. This is

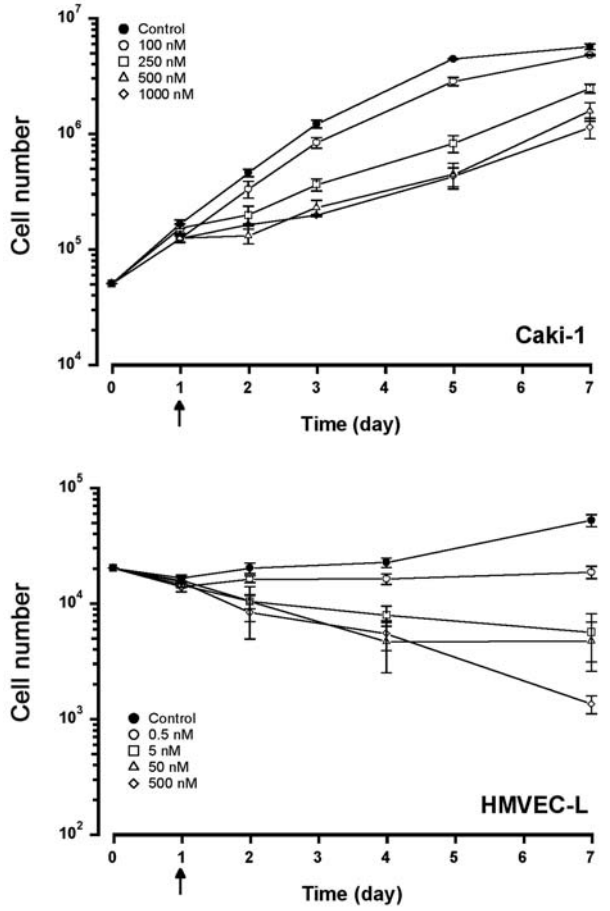

Figure 1. *Effect of PD203359 treatment on Caki-1 (upper panel) and HMVEC-L (lower panel) cell proliferation.*

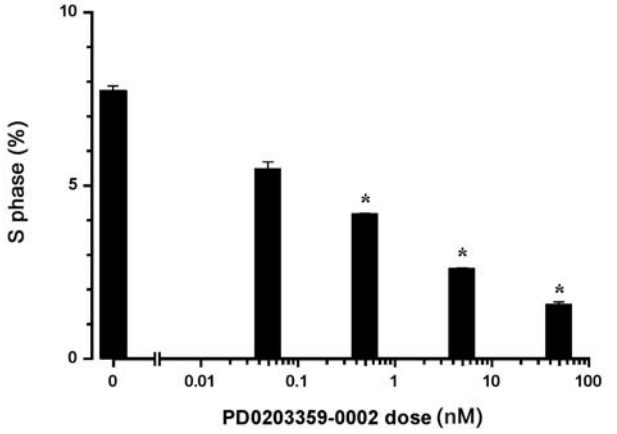

Figure 2. *Percentage of HMVEC-L cells in the S-phase of the cell cycle 3 days after treatment with various doses of PD203359 as determined by flow cytometric analysis. Stars indicated statistical significance compared to untreated cells ($p<0.05$, Student's t-test).*

illustrated in Figure 2, which shows that doses of 0.5 nM and higher resulted in significant and dose-dependent arrest of HMVEC-L cells in the S-phase of the cell cycle. In the tumor cells no effects on cell cycle progression were observed with doses as high as 100 nM (data not shown).

Although inhibition of endothelial cell proliferation by PD203359 *in vitro* suggested the potential of *in vivo* anti-angiogenic activity, its effect on tumor-induced angiogenesis was evaluated directly *in situ*. Caki-1 cells were injected intradermally and the animals were treated daily with either PD203359 or the sodium lactate buffer vehicle. The numbers of vessels induced were then counted. The results showed (Figure 3) that the angiogenic potential of Caki-1 cells growing in mice treated with PD203359 was significantly impaired (26 and 16 vessels at the doses of 20 mg/kg and 40 mg/kg) compared to that attained in mice treated with the sodium lactate buffer (34 vessels). This result was in concordance with our previous findings in mice treated with VEGF antisense (8). Taken together these data further confirm the important role of VEGF and its receptors in Caki-1 tumor-induced angiogenesis.

In the case of VEGF antisense such treatments lead to demonstrated antitumor efficacy in the Caki-1 renal cell carcinoma model (8). Enhanced tumor responses also were observed when mice bearing $\sim$200 mm^3 Caki-1 xenografts were treated daily with 20 or 40 mg/kg doses of PD203359 for a period of 2 weeks (Figure 4). The results showed that the median times for the tumors to grow to five times starting size were significantly prolonged in the drug treatment groups. Although the higher dose resulted in a greater growth delay, this dose also led to significant toxicity as indicated by animal body weight losses approaching 20%.

In order to evaluate the antitumor efficacy of simultaneously targeting the VEGF message and receptors, VEGF antisense PS-ODNs (V515) were administered together with PD203359. Based on previous efficacy studies (8), two 10 mg/kg doses of V515 were administered 1 and 4 days after the tumors reached a size of $\sim$200 mm^3. This treatment was combined with a 2-week treatment regimen consisting of daily 20 mg/kg doses of PD203359 initiated after the first dose of V515 had been given. The time for the tumors to grow from 200 mm^3 to 1000 mm^3 was then recorded and analyzed (Figure 5). The results showed that single agent treatment with V515 or PD203359 led to tumor growth delays of $\sim$7.5 or 3.5 days, respectively. However, when the two agents were combined, the resultant tumor response was significantly enhanced compared to that achieved in animals treated with either agent alone ($p<0.05$, Wilcoxon rank sum test).

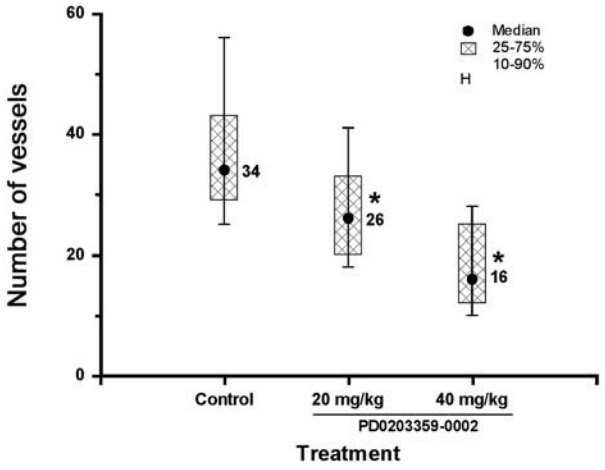

Figure 3. *Number of blood vessels induced 3 days after injecting 5 x 10^4 Caki-1 cells intradermally in nude mice. The animals were treated with 20 or 40 mg/kg PD203359 or sodium lactate buffer daily starting one day before Caki-1 cells injection. Caki-1 cells in PD203359-treated mice induced significantly fewer blood vessels than in control animals ($p<0.05$, Wilcoxon rank sum test).*

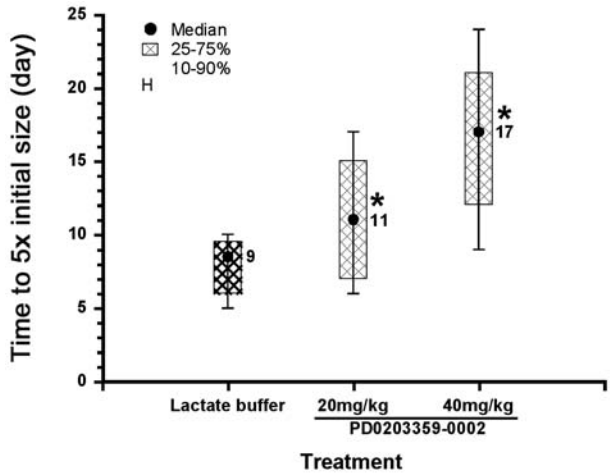

Figure 4. *Response of Caki-1 xenografts to daily treatments with 20 or 40 mg/kg doses of PD203359 administered for a period of 14 days. Each group contained 10 animals. Stars indicate statistical significance compared to vehicle (sodium lactate buffer)-treated control animals. ($p<0.05$, Wilcoxon rank sum test).*

Discussion

Since their introduction in the mid 1980s, anti-angiogenic therapies have offered the possibility of new cancer treatment strategies directed against both primary tumor growth and secondary metastatic spread (1). In adults, angiogenesis is typically relatively dormant (2). Consequently, when used to treat cancer, anti-angiogenic approaches would be predicted to have few side-effects, particularly when compared to conventional anticancer drug approaches (18). However, in general, the application of angio-suppressive agents is likely to require chronic administration of the agent in order to achieve optimal therapeutic effects. This raises the possibility that side-effects associated with such prolonged treatments may be significant. By combining anti-angiogenic agents with each other and/or other modalities used in the treatment of cancer, such potential limitations may not only be reduced but also lead to enhanced antitumor efficacy in the presence of diminished toxicity (19,20).

The complexity of regulatory factors and steps involved in tumor-induced angiogenesis make combining anti-angiogenic agents a logical treatment strategy. The VEGF signaling pathway represents a promising target given its important function in the development and maintenance of angiogenesis in many solid tumors (3,5). VEGF expression may be up-regulated in tumor cells as a consequence of both microenvironmental conditions, especially hypoxia, and genetic mutations (21). Increase of VEGF expression by tumor cells in turn leads to a concomitant increase in the expression of VEGF-R1 and VEGF-R2 on the tumor endothelium (21). Thus, the simultaneous inhibition of VEGF production and VEGF-R signal transduction should provide a more efficient block of this pro-angiogenic pathway than targeting each of the components individually.

Antisense PS-ODNs technology provides an approach for inhibiting gene expression with target specificity as a particular advantage (22). VEGF antisense PS-ODNs V515 was developed in our laboratory and its efficacy has been tested *in vitro* and *in vivo* (8). Because of its demonstrated suppression of VEGF production in Caki-1 xenografts (8), V515 was used in the present study. The application of inhibitors of VEGF receptor-associated tyrosine kinase activity represents another approach to target the VEGF signaling cascade (5). Many small molecules with such functions have been developed and lead agents, including ZD6474 and SU5416 (9,10), are undergoing preclinical and clinical evaluation. PD203359 is a small molecule receptor tyrosine kinase inhibitor that, in addition to its anti-VEGF, can also inhibit FGFR signaling in cells. The FGF receptor can be activated by a number of structurally related ligands, like aFGF and bFGF (23). Of these, bFGF is believed to be the more important in regulating angiogenesis given its role as a potent pro-angiogenic growth factor (24) and the observation that high bFGF expression in tumor cells may correlate with poor prognosis in a variety of malignancies (25,26).

PD203359 showed strong proliferation inhibition in endothelial cells at very low doses (Figure 1). This result is not surprising given the selective expression of VEGF receptors in endothelial cells. Some growth inhibitory effects were also

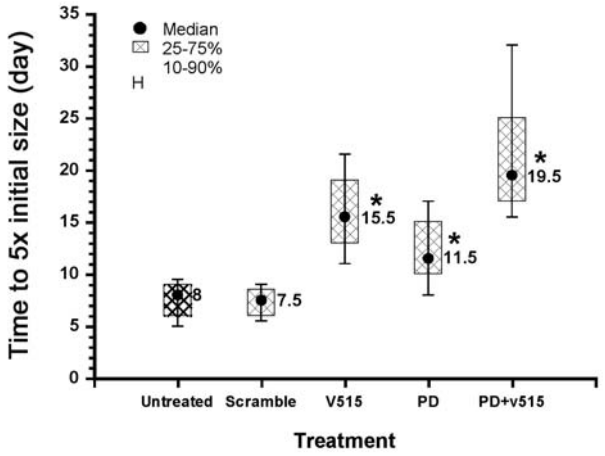

Figure 5. *The response of Caki-1 xenografts to treatment with VEGF antisense PS-ODNs and VEGF tryrosine kinase inhibitor administered either alone or in combination. V515 was given as 2 doses of 10 mg/kg administered on days 1 and 4. PD203359 (20 mg/kg) was given daily, starting on day 1 for a period of 14 days. Each group comprised 10 animals. Stars indicate statistical significance compared to untreated control ($p<0.05$, Wilcoxon rank sum test).*

observed in Caki-1 tumor cells but only at significantly higher doses (Figure 1). Since Caki-1 cells express FGFR1, 2 and 3 but not VEGF-R (27), this effect probably is mediated by the blocking of FGF-R tyrosine kinase activities. Most importantly, a highly selective growth inhibition was observed in endothelial cells treated with low doses of PD203359. This effect was at least in part due to a direct inhibition of the S-phase transition of endothelial cells (Figure 2).

When applied *in vivo*, PD203359 not only inhibited Caki-1 tumor cell-induced angiogenesis (Figure 3) but also resulted in xenograft growth inhibition (Figure 4). When VEGF antisense was administered in conjunction with PD203359, significantly enhanced anti-tumor effects were observed (Figure 5). It is important to note that, in the combination studies, a low non-toxic dose of PD203359 was used. These data demonstrate that simultaneously targeting the VEGF molecule and its receptor can lead to greater antitumor efficacy than either treatment alone without increasing treatment toxicities.

While Caki-1 is a VEGF-R-negative tumor model, other solid tumors, including Kaposi's sarcoma and certain types of breast, gastric and head and neck cancer, can be VEGF-R-positive (28-31). In such cases, VEGF antisense and VEGF/bFGF tyrosine kinase inhibitor therapies would have the potential to elicit not only an anti-angiogenic response but also a direct antitumor effect.

In summary, the simultaneous targeting of both VEGF production and VEGF receptor signal transduction by means of VEGF antisense and VEGF/bFGF receptor-associated tyrosine kinase inhibition provided more proficient antitumor activity than could be achieved with non-toxic doses of either therapy alone. These findings suggest that combining different anti-angiogenic agents or strategies may prove to be an effective and safe means to deliver long-term angio-suppressive therapy.

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