Efficacy of Antisense Morpholino Oligomer Targeted to c-*myc* in Prostate Cancer Xenograft Murine Model and a Phase I Safety Study in Humans

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ABSTRACT

Purpose: **The overexpression of c-*myc* associated with uncontrolled cell proliferation is a frequent genetic event in androgen-refractory prostatic neoplasia. The purpose of this study was to evaluate the bioavailability and efficacy of a novel antisense phosphorodiamidate morpholino oligomer directed against c-*myc*, AVI-4126, in PC-3 androgen-independent human prostate cancer xenograft murine model and its safety in a Phase I human clinical study.**

Experimental Design: **AVI-4126 administration in athymic mice bearing s.c. PC-3 xenografts was carried out to determine the bioavailability, tolerance, antitumor activity, and histological changes induced by targeted inhibition of c-Myc expression using a specific morpholine antisense oligomer. The Phase I safety study involved a single center, open label, dose-escalating design in healthy volunteers after i.v. administration of AVI-4126.**

Results: **The data reveal that AVI-4126 targets and inhibits c-*myc* translation in a sequence-specific manner and causes significant growth inhibition and apoptosis in prostate cancer cells and in s.c. tumor xenografts. A 75–80% reduction in tumor burden was observed in AVI-4126-treated animals compared with the scrambled oligomer and saline control groups. Histologically, tumors grown in the athymic mice treated with AVI-4126 were less cellular and vascular than those in control mice and showed an increased level of cellular degeneration, cytoplasmic vacuoles, and hyperchromatic nuclei. Phase I safety trials in humans via i.v. route of administration showed no toxicity or serious adverse events.**

Conclusions: **The present study demonstrates that inhibition of c-Myc expression by antisense phosphorodiamidate morpholino oligomer is a promising new and safe therapeutic strategy for prostate cancer.**

INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy other than superficial skin cancer and the second most common cause of mortality among men in the Western world (1). Progression to androgen independence remains one of the primary obstacles to improving quality of life and survival for patients with advanced prostate cancer because the androgen-independent cells are unresponsive to androgen deprivation therapy. It is clear that inappropriate gene expression is basic to the pathophysiology of cancer, including prostate cancer (2), which emphasizes the need for novel therapeutic strategies targeting these key molecular changes. The proto-oncogene c-*myc*, a key regulator of cell proliferation and differentiation, encodes a ubiquitously expressed nuclear phosphoprotein of 439 amino acids (c-Myc) found mainly in heterodimeric complexes with the related protein Max. The c-Myc/Max complexes bind to DNA in a sequence-specific manner and activate transcription and thereby induce cell transformation and cell cycle progression (3). Several studies have identified the significant role of c-*myc* in prostate carcinogenesis because high levels of c-*myc* overexpression have been reported in prostate cancer cells and in experimental animal models, as well as in human prostate adenocarcinomas (4–7). Fluorescence *in situ* hybridization analysis has identified c-*myc* oncogene amplification and chromosomal anomalies in metastatic prostatic carcinoma (8). Modulation of the expression levels of c-Myc protein could therefore represent a novel therapeutic approach for prostate cancer.

Antisense technology constitutes development of sequence-specific DNA or RNA strands that can inhibit gene expression by blocking DNA uncoiling, transcription, export of RNA, splicing, RNA stability, or translation (9). Antisense oligonucleotides, the most commonly used antisense approach, are unmodified or chemically modified single-stranded RNA or DNA molecules specifically designed to hybridize to corresponding RNA by Watson-Crick binding. PMOs[2] are one of the third-generation antisense molecules, wherein the deoxyribose moiety of DNA is replaced with a 6-membered morpholine ring, and the charged phosphodiester internucleoside linkage is replaced with phosphorodiamidate linkages, thus rendering a novel nonionic chemical structure (10, 11). Unlike the first (phosphodiester)- and second (phosphorothioate)-generation oligodeoxynucleotides, the mechanism of action of PMO involves both steric blockade of ribosomal assembly, thus pre-

[2] The abbreviations used are: PMO, phosphorodiamidate morpholino oligomer; HPLC, high-performance liquid chromatography; FBS, fetal bovine serum; PI, propidium iodide; i.t., intratumoral; CBC, complete blood count; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCI, National Cancer Institute; CTC, Common Toxicity Criteria.

Table 1 Various PMO sequences tested in the prostate cancer cells

c-*myc* mRNA target position is based on GenBank accession no. Y00396. The bold sequences represent the mismatch in the scrambled oligomers. AVI-4126 antisense sequence was chosen because it displayed most favorable solubility, efficacy, and potency in comparison with over 100 different antisense c-*myc* PMOs targeted to various sites along the c-myc 5′-untranslated region, splice acceptor of the first intron, and around the translational initiator AUG (12, 30).

Target	PMO	Sequence
c-*myc* AUG start site	AVI-4126 antisense	5′-ACGTTGAGGGGCATCGTCGC-3′
Mismatched control	AVI-144 scrambled	5′-AC**TG**TGAGGG**CG**ATCG**CT**GC-3′

venting translation, and interference with intron-exon splicing of pre-mRNA, thus preventing appropriate translation of selected mRNA (12, 13). The PMOs bind more strongly to complementary RNA than to congenic phosphodiester DNA and show excellent resistance to the action of purified nucleases and proteases found in serum and plasma (14).

The aims of the present study were 2-fold: (*a*) to investigate the efficacy of a PMO antisense directed against the c-*myc* gene, AVI-4126, for gene-specific therapy for prostate cancer; and (*b*) to evaluate the safety of AVI-4126 in a Phase I clinical study after i.v. route of administration. The data reveal that treatment of PC-3 androgen-independent cells with AVI-4126 specifically down-regulates the expression of c-Myc protein and causes significant growth inhibition and cell death. Safety analyses in human volunteers revealed no serious adverse effects.

MATERIALS AND METHODS
Oligomers

Morpholino oligomers were synthesized at AVI Bio-Pharma Inc. (Corvallis, OR) as described previously (10). Purity was >95% as determined by reverse-phase HPLC and matrix-assisted laser desorption ionization time-of-flight mass spectroscopy. The base composition of the oligomers is shown in Table 1.

Cell Culture

The PC-3 human androgen-independent prostate carcinoma cell line was obtained from American Type Culture Collection (Manassas, VA). RPMI 1640 was purchased from Hyclone Laboratories (Logan, UT). FBS and the antibiotics were purchased from Life Technologies, Inc. (Gaithersburg, MD). Cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, and 75 units/ml streptomycin at 37°C under 5% CO_2.

Assisted PMO Delivery in Cells

The oligomers were delivered into PC-3 cells by a special DNA delivery protocol with slight modifications (15). Briefly, the PMOs were delivered into cells at a concentration of 1.4 μM according to manufacturer's protocol (Gene Tools, Corvallis, OR). Partially complementary DNA molecules with a 10-base adenine 5′ overhang were synthesized to serve as carriers for each PMO sequence. The partially complementary DNA used for delivery of antisense PMO AVI-4126 was 5′-ACACACA-CACGCGACGATGCCCCTCA-3′ and that used for delivery of the scrambled oligomer, AVI-144, was 5′-ACACACACACG-CAGCGATCGCCCTCA-3′. The PMO:DNA duplex was

formed by incubating 1 mM stocks of the PMO and partially complementary DNA in a 1.4:1 ratio for 10 min at room temperature; 16.8 μl of the duplex stock were diluted to 566.4 μl volume in deionized water. A total of 16.8 μl of the weakly basic delivery reagent, ethoxylated polyethylenimine, was added. The tube was vortexed and incubated for 20 min at room temperature. Serum-free media (5.4 ml) were added to bring the final volume to 6 ml. Subconfluent cultured cells were exposed to the above mixture for 3 h at 37°C (1.5 ml/well for 6-well plate and 150 μl/well for 96-well plate). This mixture was then aspirated and replaced with normal serum-containing media.

Annexin V Staining

PC-3 cells (1×10^5) were seeded in 4-well chamber slides, and the treatments were carried out when the cells were at 60% confluence. AVI-4126, the scrambled control oligomer (AVI-144), or vehicle alone was delivered by gene delivery technique. 6-Hydroxyurea (50 mM) was used as the positive control. The cells were stained with Annexin V-FITC (1:100) and 1:10 of 50 μg/ml PI 24 h after treatment using the Annexin V apoptosis detection kit (Santa Cruz Biotechnology, Santa Cruz, CA) protocol as suggested by the manufacturers. The cells were incubated with the stain for 15 min at room temperature in the dark, washed with $1 \times$ PBS, and visualized immediately under a fluorescence microscope. The photomicrographs were taken with a Nikon Diaphot 300 microscope connected to an Olympus (Melville, NY) Magnafire SP-brand digital camera.

Animals

Male athymic (Ncr nu/nu) nude mice, approximately 4 weeks old on arrival, were obtained from Simonson Laboratories. The animals were housed in laminar airflow cabinets in microisolator cages under pathogen-free conditions with a 12-h light/12-h dark schedule and fed autoclaved standard chow and water *ad libitum*. PC-3 cells (2×10^6) were suspended in 150 μl of RPMI 640 plus 10% FBS and 150 μl of Matrigel (Becton Dickinson, Palo Alto, CA) and injected via a 25-gauge needle into the s.c. space of the flank region of 5–6-week-old athymic male mice. All animals were anesthetized with isofluorane before injection of cells.

All animal protocols conformed to the ethical guidelines of the 1975 Declaration of Helsinki and were approved by the Institutional Animal Care and Use Committee of Oregon State University.
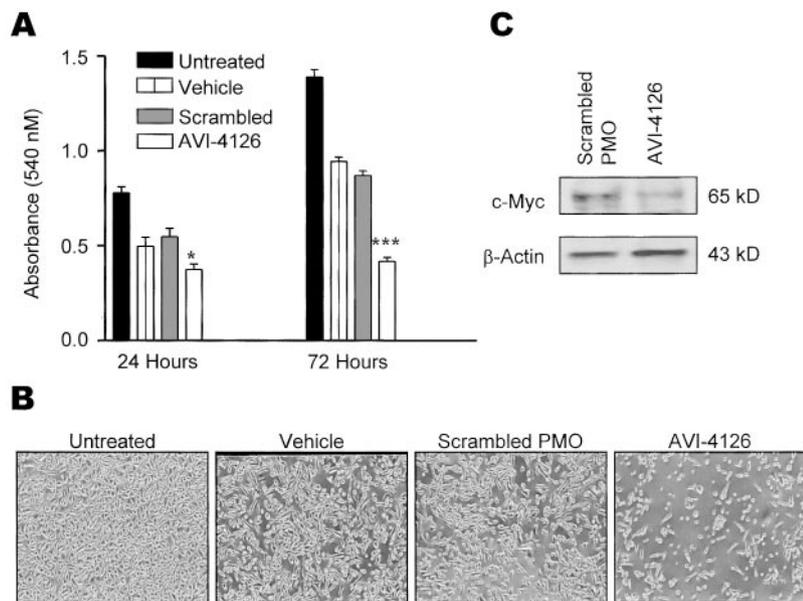
A

B

C

Fig. 1 Effect of AVI-4126 on c-Myc protein expression and cell proliferation in PC-3 androgen-independent human prostate cells. *A,* cell viability of untreated PC-3 cells and PC-3 cells treated with vehicle, 1.4 µM scrambled AVI-144 PMO, or AVI-4126 antisense PMO was analyzed 24 and 72 h after treatment by MTT assay. The data in the graph are expressed as mean ± SE ($n = 4$). ∗, $P < 0.05$; ∗∗∗, $P < 0.001$ AVI-4126 *versus* scrambled oligomer treatment. *B,* representative ×100 PC-3 cell photomicrographs 72 h after transfection. *C,* immunoblot analysis of the lysates from scrambled (AVI-144 PMO)- and antisense (AVI-4126 PMO)-treated PC-3 cells with anti-c-Myc polyclonal antibody, and the same immunoblot was stripped and reprobed to determine β-actin levels.

Treatment Protocols

The experiment was started when the tumors were well established, and the tumor volumes were between 125 and 175 mm³. Three groups of six mice each received 4 cycles (daily i.t. injections for 5 days and 2 days off) of vehicle (saline control) or 300 µg of scrambled oligomer (AVI-144) or AVI-4126. Tumors were measured using vernier calipers, and the tumor volume was calculated by using the formula $L \times W^2/2$, where L (length) $> W$ (width). At the end of the experiment, the animals were euthanized, tumors were excised and weighed, and tumor burden was calculated. In these experiments, the toxicity was evaluated on the basis of mortality rate, changes in CBCs, body weight, and metabolic profile. A complete autopsy of the mice was done to rule out macroscopic side effects. The tumor tissues were fixed in 10% formalin and embedded in paraffin. Six-µm-thick sections of paraffin-embedded tumor specimens were stained with H&E.

Protein Expression

PC-3 cell lysates were prepared by lysing the cells in solubilization buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 1% sodium deoxycholic acid, 1% Triton X-100, and 0.2% SDS]. The tissue samples from tumors, liver, and kidney were lysed immediately after animal euthanization as described previously (16). Protein estimation was carried out by Bradford Protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein in cell or tissue lysates were subjected to 12% SDS-PAGE under reducing conditions. Note that c-Myc was detectable only in fresh lysates that were never freeze-thawed. The protein was then transferred from the gel to methanol-soaked Immobilon-P transfer membranes (Millipore, Bedford, MA). The membranes were allowed to dry, resoaked in methanol, and then incubated with blocking buffer (20 mM Tris base, 150 mM NaCl, 3% nonfat milk, and 0.3% Tween 20) for 1 h at room temperature. The membranes were incubated for 2 h with primary antibodies

for c-Myc (clone N-262; Santa Cruz Biotechnology) at 1:1000 dilution in blocking buffer. The β-actin (clone AC-40) was obtained from Sigma Chemical Co. The membranes were washed three times with wash buffer (1× PBS and 0.3% Tween 20) and then incubated for 30 min with appropriate secondary antibody (1:5000) conjugated with horseradish peroxidase. The membranes were washed, and the immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). β-Actin immunodetection was performed to confirm that all lanes were loaded with similar amounts of protein by stripping the same blot in 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) for 1 h at 50°C, followed by the washing and blocking steps as described.

Cell Viability Assay

At different time intervals after treatment of cells with PMO or appropriate vehicle, 200 µl of 5 mg/ml MTT (Sigma Chemical Co.) were added to each well in a 6-well plate at 37°C until blue coloration started to appear in the cells. The medium was then aspirated and replaced with DMSO. The absorbance was read at 540 nm in a Molecular Devices plate reader and analyzed by SOFTmax Program.

HPLC Detection of PMO in Tumor and Organ Tissue

Tumor tissue, liver, and kidney lysates from AVI-4126-treated animals were prepared as described above and analyzed for presence of PMO by HPLC analysis as described previously (16, 17). Briefly, a 10-µl aliquot (500 ng) of the internal standard PMO (15-mer whose sequence was derived from a 5′ truncation of AVI-4126) was added to all 250-µl aliquots of tumor, kidney, and liver lysate (0.2 g/ml) samples. Methanol (300 µl) was added to each sample, and the tubes were vortexed. The tubes were centrifuged for 10 min using a high-speed centrifuge, and supernatants were transferred to new Eppendorf
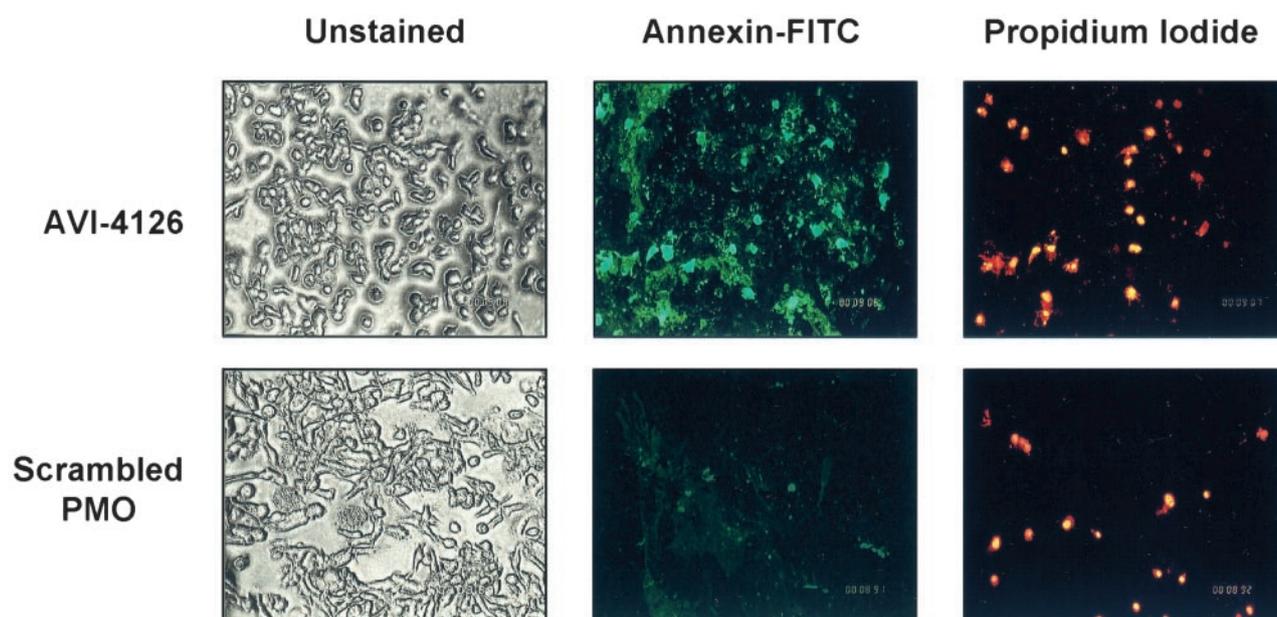
Unstained Annexin-FITC Propidium Iodide

Fig. 2 Induction of apoptosis and necrosis in AVI-4126-treated PC-3 cells. The cells were treated with AVI-4126 or scrambled PMO for 24 h and then stained with Annexin V-FITC and PI and analyzed by light and fluorescence microscopy. Photomicrographs of identical fields (×200) for each treatment are viewed as phase contrast with Chroma FITC filter and Chroma rhodamine filters.

tubes. The pellet was then washed with 100 μl of Tris, and the wash buffer was added to the supernatant. The supernatants were heated in a water bath at 70°C for 10 min. The samples were recentrifuged for 10 min, and the supernatants were transferred to new Eppendorf tubes. Methanol was removed using a speed vac, and the samples were finally transferred to clear shell vials and lyophilized after the addition of 100 μl of deionized water to each vial. The lyophilized samples were reconstituted using 100-μl aliquots of 5′-fluoresceinated DNA (1.0 absorbance units/ml) whose sequence was complimentary to that of AVI-4126 PMO. A set of AVI-4126 standards was prepared by spiking the PMO into 250-μl aliquots of blank rat plasma (10, 25, 50, 100, 250, 500, and 1000 ng/250 μl plasma) along with the internal standard. The standards were similarly extracted.

The samples were analyzed by injection onto a Dionex DNA PacPA-100 column (4 × 250-mm column; Dionex Corp., Sunnyvale, CA) using a Varian autosampler (AI-200) connected to a Varian HPLC pump (model 9010 inert) equipped with a Varian fluorescence detector (model 9075). The mobile phases [A, 0.025 M Tris (pH 8); B, 0.025 M Tris (pH 8)/1.0 M NaCl] were prepared using HPLC-grade water and reagents and filtered through a 0.2 μm filter before use. The gradient program used was (90–10%B) at 0 min and (55–45%B) at 20 min, whereas the pump was held at a flow rate of 1.5 ml/min. The runs were monitored at excitation and emission wavelengths of 494 nm and 518 nm, respectively.

Photomicrography

Tumor slices were placed in plastic molds filled with embedding medium for frozen tissue processing (Sakura Finetek, Torrance, CA) as described previously, with slight modifications (17). The molds were wrapped in tin foil to protect from light and frozen in a −80°C freezer. Cryostat sections (5 μm) were cut at Oregon State University Veterinary Diagnostic Laboratory (Corvallis, OR). Slides were air dried in the dark for 30 min and coverslip-mounted using Fluoromount-G mounting medium (Southern Biotechnology Associates, Birmingham, AL). The photomicrographs were taken with a Nikon Diaphot 300 microscope connected to an Olympus (Melville, NY) Magnafire SP-brand digital camera. The exposure times were kept constant for all fluorescent pictures at 25 s.

Phase I Safety Trials

Subjects and Sampling Schedule for i.v. Administration. This study was a single-center, open label study using a single-dose-escalating design (FDA IND-59,255). The study was conducted at MDS Harris (Phoenix, AZ). The study was conducted in compliance with the applicable Code of Federal Regulations, Good Clinical Practices, including guidelines set forth by the International Conference on Harmonization. Protocol specifications and a detailed time and events schedule were prepared by MDS Harris with local ethics committee (institutional review board) approval. AVI-4126 was supplied by AVI BioPharma, Inc. as single-dose vials containing 10 mg/ml in sterile PBS solution. The objectives of this study were to evaluate the safety of AVI-4126 administered i.v. in normal healthy male and female subjects. Informed consent was obtained from each enrolled volunteer. Five dose levels were evaluated in a single-dose escalation fashion in five cohorts. Six normal healthy subjects were enrolled in each cohort after a screening of medical history, examination, and laboratory tests had shown no clinically significant abnormalities. The weight of the subjects ranged between 52 and 88 kg, with the mean weight of 70
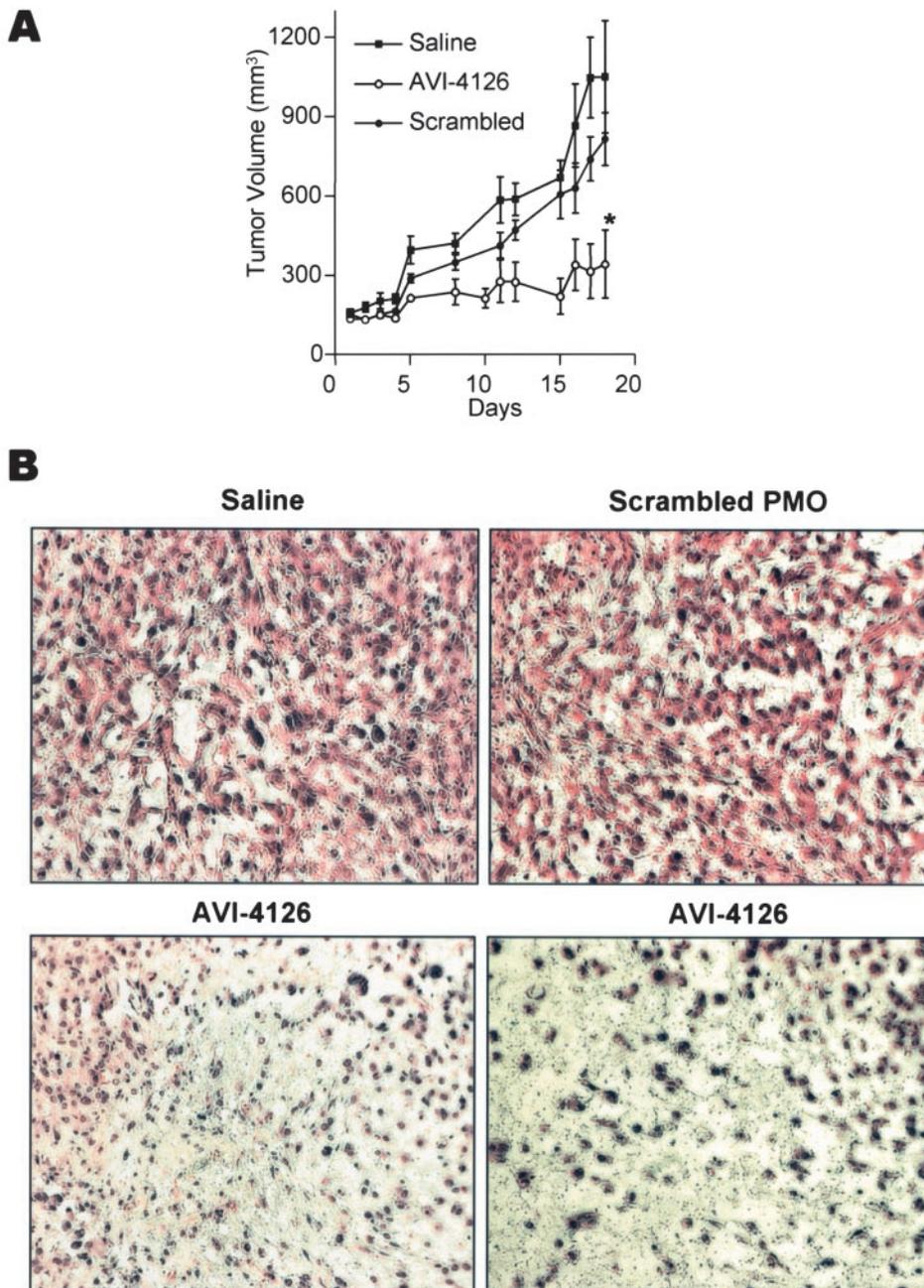
Fig. 3 A, effect of antisense PMO AVI-4126 on the growth of s.c. xenografts of PC-3 human androgen-independent prostate cancer in athymic mice. The i.t. injections of the indicated agents were given for 4 cycles (daily i.t. injections for 5 days and 2 days off) when the tumors were about 150 mm³. No deaths occurred during this period. Tumors were measured using calipers, and the tumor volume was calculated by using the formula $L \times W^2/2$, where L (length) $> W$ (width). The values indicated are the mean $\pm$ SE. *Asterisk* indicates significant difference ($P < 0.05$) between scrambled oligomer or saline *versus* AVI-4126. *B,* histological sections of s.c. xenografts of PC-3 human androgen-independent prostate cancer treated with saline, scrambled PMO, or AVI-4126 at the end of the above-mentioned tumor study. The representative photomicrographs are at 200×2.65 intermediate magnifier lens in a Nikon Diaphot 300 inverted microscope.

kg. Cohorts 1 (three males and three females), 2 (two males and four females), 3 (three males and three females), 4 (two males and four females), and 5 (four males and two females) received 1, 3, 10, 30, and 90 mg AVI-4126, respectively, administered as a slow injection i.v. bolus/push. The subjects in all five dose levels were in the fed state. They were confined to the clinic through the 72-h postdose events and returned for the week 1 and week 2 safety events.

Safety Analyses. Laboratory safety screens, CBC with differential and platelet count, blood biochemistry, coagulation profile, and urinalysis were performed on day 1 (predose); 24, 48, and 72 h after dose; and on follow-up week 1 and 2 visits. Complement (C3a) was measured at 24, 48, and 72 h after dose. Serum pregnancy tests were performed in the female subjects during screening, day 1 predose, and at 2-week follow-up.

Statistical Analysis

The data are expressed as the mean $\pm$ SE. The statistical analyses were performed using GraphPad InStat Student's two-tailed *t* test and ANOVA (Tukey-Kramer multiple comparison test). Differences were considered significant at $P < 0.05$.

Table 2 Effect of saline, scrambled PMO, and AVI-4126 on the growth of PC-3 human androgen-independent prostate cancer s.c. xenografts in athymic mice

Treatment[a]	Tumor volume (mm³)		Tumor doubling (days)	Tumor weight (mg)	Tumor burden (% inhibition) (mg/g)
	Initial (day 1)	Final (day 18)			
Saline	157 ± 16	1050 ± 212.5	4.7 ± .2	560 ± 98	18.4 ± 3.31
Scrambled	150 ± 11	815 ± 98.2	4.8 ± .2	380 ± 25	12.6 ± 0.82
AVI-4126	135 ± 9.1	342 ± 129.2[b]	9.25 ± 1.2[b]	140 ± 68[b]	4.58 ± 2.22[b] (75% *vs.* saline)

[a] i.t. injections of 300 µg/mouse (5 days on and 2 days off cycle) of each compound for a period of 18 days. Values are mean ± SE ($n = 6$).
[b] Statistical analysis: two-tailed *t* test ($P < 0.05$ *versus* saline or scrambled).

RESULTS

AVI-4126 Treatment Inhibits PC-3 Cell Proliferation and Viability. The antisense c-*myc* oligomer (AVI-4126) and the corresponding scrambled oligomer, AVI-144, paired to partially complementary single-stranded DNA oligonucleotides were transfected using ethoxylated polyethylenimine into PC-3 cells using special DNA delivery protocol (see "Materials and Methods"). Cell viability was evaluated at 24 and 72 h after transfection in the absence or presence of oligomers by MTT cell viability assay (Fig. 1*A*), trypan blue cell count (data not shown), light microscopy (Fig. 1*B*), and labeling with annexin V and PI (Fig. 2). The antiproliferative effects of AVI-4126 antisense PMO were evident within 24 h, with about 30% growth inhibition compared with the vehicle and scrambled PMO. After 72 h, there was complete growth arrest in the AVI-4126-treated cells with about 40% survival. The light microscopy pictures in Fig. 1*B* also show evidence of cell death and altered morphology in AVI-4126-treated cells. Loss of membrane asymmetry, an early apoptotic marker, was detected by staining with annexin V in the AVI-4126-treated cells (Fig. 2) compared with scrambled oligomer (AVI-144)-treated cells (Fig. 2).

Immunoblot analyses were performed to determine c-Myc protein levels at 24 h in the untreated, vehicle-treated, scrambled oligomer-treated, or AVI-4126 oligomer-treated PC-3 cell lysates. A specific inhibition of Myc protein levels was seen in the AVI-4126-treated PC-3 cell lysates (Fig. 1*C*). β-Actin immunodetection on the same blots was used as loading control.

AVI-4126 Inhibits *in Vivo* Tumor Growth and Alters Tumor Histology. The antitumor activity of the c-*myc* antisense PMO (AVI-4126) was evaluated in PC-3 s.c. xenografts in athymic mice. The oligomers or saline were administered i.t. (18–20) consisting of 3 cycles of 5 day injections at 300 µg/mouse dose in well-established tumors (average initial tumor volume, 150 mm³). Changes in PC-3 tumor growth in saline, scrambled control, and AVI-4126 groups are compared in Fig. 3*A* and Table 2. At the end of the treatment cycles, tumor volumes of the animals injected with AVI-4126 were significantly smaller (341 ± 129.2) than those in saline (1049.8 ± 212) or scrambled (815 ± 98.2) control groups, corresponding to a 60–70% inhibition ($P < 0.05$). In the AVI-4126-treated group, tumors completely regressed in two animals, whereas in the others, the tumor doubling time was longer (9.25 ± 1.2 days) in comparison with the control groups (average, 4.5 days). The AVI-4126-treated tumor weights were also significantly lower as compared with those of the controls (Table 2).

No mortality or significant change in body weight was observed in any of the groups during the course of the experiment. CBC analysis and metabolic profile at the end of the experiment showed no significant differences in the different treatment groups, except there was an increase in eosinophil counts in the AVI-4126-treated group (292 ± 34.5) compared with the scrambled oligomer group (72 ± 33). No mortality was associated with any treatment groups, and no macroscopic damage was evident in the area surrounding the s.c. tumor, liver, lungs, heart, and kidneys of the animals in this experiment.

Representative H&E-stained histological sections from the center of the AVI-4126-, saline-, and scrambled oligomer-treated tumors are shown in Fig. 3*B*. The AVI-4126-treated tumor sections have fewer and smaller cells and more hyperchromatic nuclei, and a major part of the section has degenerate cells with large cytoplasmic vacuoles. Very few mitotic figures were identified compared with the scrambled oligomer- and saline-treated tumor sections under similar power field (data not shown). A uniform staining pattern with no substantial changes in morphological indices was observed in tumors treated with saline or scrambled oligomer (Fig. 3*B*).

***In Vivo* Bioavailability of AVI-4126.** Quantitation of AVI-4126 PMO levels in the tumor tissue was carried out in the tumor lysates by HPLC analysis. The elution order for each chromatogram is AVI-4126, the internal standard and the excess fluoresceinated DNA probe are the last to elute (Fig. 4, *A–D*). Representative chromatograms showing separation of peaks at different time points are presented in Fig. 4*A*. The peak corresponding to full-length (20-mer) AVI-4126 was readily observed up to 24 h after administration of a single dose of 300 µg of AVI-4126 in tumor samples from the s.c. PC-3 tumors. Quantitation of the PMO concentration/g tumor tissue revealed the presence of 44 µg/g at 4 h, 31 µg/g at 8 h, and 9 µg/g at 24 h. The liver and kidney were predominantly the other organs that showed PMO bioavailability. This analytical technique is capable of resolving peaks resulting from (N-1)-mers and truncated versions of AVI-4126, but neither was detected in the tumor or organ lysates.

The tumor tissue lysates were also run on an immunoblot and probed with anti-c-Myc antibody (Fig. 4*E*). The data show specific reduction of c-Myc levels in the AVI-4126-treated PC-3 xenograft tumor lysates.

Cryostat sections (5 µm) of PC-3 tumors (200 mm³) treated i.t. with a single dose of 100 µg of fluorescein-labeled AVI-4126 were examined by phase-contrast (Fig. 5*A*) and fluorescence microscopy (Fig. 5*B*). The micrographs revealed widely dispersed intracellular fluorescence that was absent in
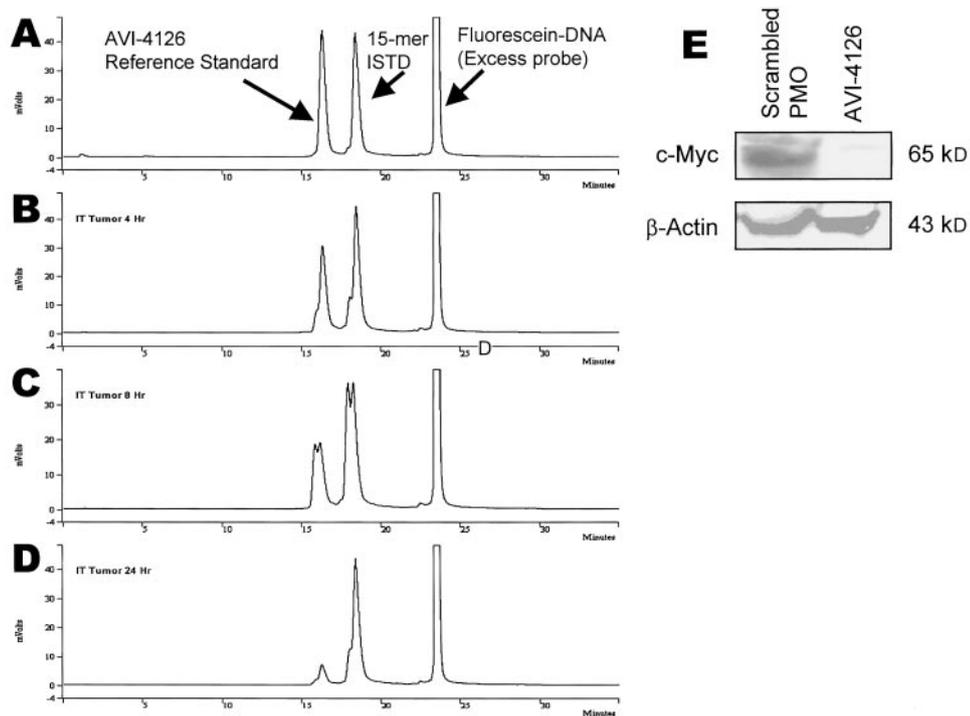
Fig. 4 Representative anion-exchange HPLC chromatograms showing detection of the oligomer AVI-4126 in (*A*) control plasma of untreated rat spiked with AVI-4126 stock solution and (*B–D*) tumor lysates of athymic PC-3 xenograft mice 4, 8, and 24 h, respectively, after i.t. administration of 300 µg of AVI-4126. *E,* effect of AVI-4126 on c-Myc protein expression in PC-3 s.c. xenograft tumor in athymic mice. A representative immunoblot showing probing of the lysates from scrambled oligomer (AVI-144 PMO)-treated and antisense (AVI-4126 PMO)-treated PC-3 tumor lysates with anti-c-Myc polyclonal antibody, and the same blot was stripped and reprobed with β-actin.
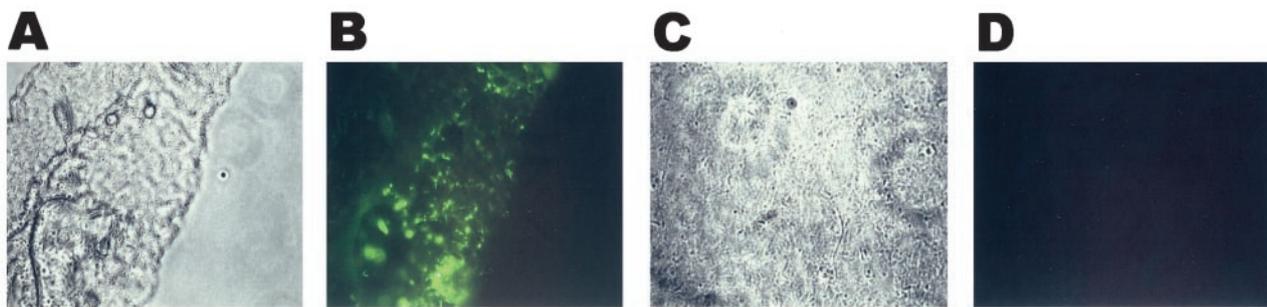

Fig. 5 Representative ×200 tumor photomicrographs of athymic PC-3 s.c. xenograft mice 6 h after i.t. administration of 100 µg/mouse AVI-4126. *A* and *B,* phase-contrast and fluorescent images, respectively, of an identical field from fluorescein-labeled AVI-4126-treated mice. *C* and *D,* phase-contrast and fluorescent images, respectively, of an identical field from unlabeled AVI-4126-treated mice.

similar sections from mice treated with unlabeled AVI-4126 (Fig. 5, *C* and *D*).

Phase I Safety Study. Thirty subjects (16 females and 14 males) of Caucasian, Hispanic, African-American, or American-Indian origin entered the trial involving single i.v. administration of AVI-4126. The subjects ranged in age from 22–63 years (mean age, 42 years). All of the subjects completed the trial procedures. No serious adverse events (graded according to the NCI CTC) were observed during the treatment or at the follow-up periods in any subjects (Table 3). Few cases of headaches (five cases), backaches (six cases), or leg cramps (eight cases) were reported, which were graded as mild according to NCI CTC and regarded as either unrelated or possibly related to AVI-4126 treatment. Neck pain (one subject), acid indigestion (one subject), nausea (one subject), herpetic lesion

in mouth (one subject), fever blister in lip (one subject), or chest wall pain (one subject), each of mild intensity, were reported and considered to be unrelated to the AVI-4126 administration. One subject had mildly elevated complement C3a levels. None of these effects were dose related. The problems were resolved without any therapy, except in the case of the neck pain and one case of headache, where a moist heat pack or ice bag was applied, respectively, to the area. Uninalysis, serum chemistry, and complete hematology screens revealed no clinically significant deviations from the normal range in the cohorts (data not shown). Clotting screen and serum complement split products (C3a) revealed no significant changes due to AVI-4126 administration compared with predose screening. Representative graphs of activated partial thromboplastin time and C3a levels are shown in Fig. 6 to highlight this feature.

Table 3 Adverse events during AVI-4126 administration

The adverse events were graded according to the NCI (CTC). SEV (severity): 1, mild; 2, moderate; 3, severe. SER (serious): 1, not serious; 2, medical event; 3, congenital anomaly; 4, disability; 5, hospitalization; 6, life-threatening; 7, death. OUT (outcome): 1, resolved; 2, improved; 3, unchanged; 4, worse; 5, fatal; 6, unknown. ACT (action): 1, none; 2, drug discontinued due to adverse events; 3, drug dosage adjusted; 4, drug stopped-restarted; 5, therapy; 6, hospitalization. FRE (frequency): 1, single episode; 2, intermittent; 3, continuous. REL (relationship): 1, not related; 2, possibly related; 3, probably related; 4, definitely related.

Adverse events	Dose (no. of cases)	SEV	SER	OUT	ACT	FRE	REL
Headache	1 mg (3)	1	1	1	1	3	2
	10 mg (1)	1	1	1	1	3	2
	30 mg (1)	1	1	1	1	3	1
Back ache	1 mg (1)	1	1	1	1	1	1
	10 mg (5)	1	1	1	1	2	2
Leg cramps	3 mg (1)	1	1	1	1	3	1
	10 mg (7)	1	1	1	1	2,3	2
Elevated C3a	3 mg (1)	1	1	1	1	3	2
Acid indigestion	90 mg (1)	1	1	1	1	3	1
Chest wall pain	90 mg (1)	1	1	1	1	2	1
Neck ache	1 mg (1)	1	1	1	5	3	1

DISCUSSION

At the time of clinical diagnosis, most prostate cancers present themselves as a heterogeneous population of androgen-dependent and androgen-independent cells. Androgen ablation, the common treatment strategy, causes rapid apoptosis of the population of androgen-dependent cells. However, androgen deprivation rarely cures patients because there is frequent recurrence due to takeover of the tumor mass by androgen-independent tumor cells, and apoptosis-resistant clones emerge as a result of various genetic alterations (21). Systemic cytotoxic chemotherapy has limited antitumor activity in hormone-refractory prostate cancer (22). Other treatment options such as external beam and radioisotope radiotherapy offer only symptom palliation (23). Clearly, the development of novel therapies that interfere with the expression levels of essential growth-regulatory molecules is a high priority for the development of more efficacious treatment modalities.

We report herein that an antisense PMO directed against c-*myc* (AVI-4126) causes specific decrease of c-Myc protein levels in PC-3 prostate cancer cells in culture and in xenografts. Amplification of the proto-oncogene c-*myc* has been identified as one of the most frequent genetic alterations in prostate cancer, thus making it an attractive therapeutic target. Studies have shown that one of the most common chromosomal alterations associated with prostate carcinogenesis is gains at chromosome 8q, where *myc* (8q24) is also located (6). Additionally, c-*myc* consensus sites have been identified in the androgen receptor, and these seem to be involved in androgen-mediated up-regulation of androgen receptor mRNA (24). Androgen deprivation therapy has been observed to cause c-*myc* and androgen receptor gene amplifications associated with increased cell proliferation (8). Distinct changes in gene copy number before and after androgen ablation suggest possible involvement of the c-*myc* gene in the escape from androgen control, making c-*myc* a rational target for treatment of this disease. Overexpression of c-*myc* in transgenic mice prostates led to development of ab-
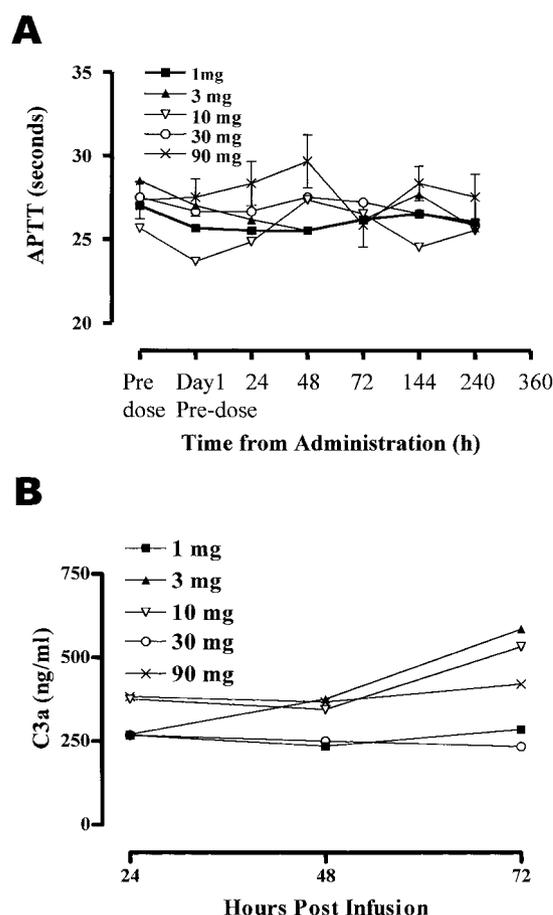
Fig. 6 Summary of APTT (*A*) and serum complement C3a (*B*) levels after i.v. administration of AVI-4126 in normal human volunteers in a Phase I dose-escalating clinical trial (normal range, 26–43 s for APTT and 100–400 ng/ml for C3a).

normalities similar to those seen in low-grade prostate intraepithelial neoplasia in humans (25). Down-regulation of c-*myc* has been found to cause a decrease in neuroendocrine differentiation in various cancer cell lines, particularly small cell lung carcinoma cells, neuroblastoma, and colon cancer cell lines (26, 27). A recent study identified that only a 50% reduction in c-Myc expression resulted in a >10-fold reduction in susceptibility to transformation by oncogenic Ras or Raf proteins (28).

Antiproliferative effect of a phosphorothioate antisense oligonucleotide against c-*myc* has been reported in prostate cancer cell lines (29). This study, however, was unable to identify whether the mode of cell death was due to decrease in c-Myc levels at the translational and/or transcriptional level or to a nonantisense aptameric effect. An earlier study using c-*myc* phosphorothioate antisense oligomer in smooth muscle cells revealed that the inhibition could be due to the presence of four contiguous guanosine residues in the oligonucleotide sequence (30).

PMOs represent a novel antisense oligomer chemistry in which the backbone is neutral at physiological pH and consists of a six-membered morpholine ring. The lack of internucleoside

charge allows the PMOs to avoid nonspecific effects such as G-quartet or interaction through the CpG motifs observed with the commonly used phosphorothioate oligonucleotides that bind to cellular and extracellular proteins (31, 32).

AVI-4126 sequence was chosen because it displayed the most favorable solubility, efficacy, and potency in comparison with over 100 different antisense c-*myc* PMOs targeted to various sites along the c-myc 5′-untranslated region, splice acceptor of the first intron, and around the translational initiator AUG (33). Another study has shown that in addition to steric blockage, a 28-mer c-Myc antisense PMO whose sequence overlaps with that of AVI-4126 causes mis-splicing of pre-mRNA in cancer cells (12). In a syngeneic Lewis lung carcinoma murine model, a combination regimen in which cisplatin was administered on days 2–4 and 13–15, followed by AVI-4126 treatment on days 6–12 and 17–23, inhibited tumor growth significantly more than cisplatin alone (34). This potentiation of antitumor activity of AVI-4126 was also observed in combination with β-human chorionic gonadotropin in DU145 prostate cancer cells (35). In addition, AVI-4126 has demonstrated *in vivo* efficacy in polycystic kidney disease (36), liver regeneration (16), and vascular restenosis after balloon injury associated with angioplasty (37) and is currently undergoing Phase Ib and Phase II clinical trials for polycystic kidney diseases and vascular restenosis, respectively.

In this study, we have shown three methods to qualitatively (fluorescence photomicrography and Western immunoblot) and quantitatively (HPLC analysis) demonstrate *in vivo* bioavailability of AVI-4126 in the tumor tissue. This will be of great value in future studies involving delivery and routes of administration in tumor models. The PMOs are stable in serum and plasma but are sensitive to degradation after prolonged exposure to low pH. Previous studies have shown no evidence of truncated versions of AVI-4126 in plasma or liver tissue of Sprague Dawley rats (16, 17).

The i.t. injection of the oligomer at a dose of 300 μg/mouse decreased tumor burden by about 75% compared with the control treatments. In addition, the PMO seem to be very safe for daily administration because treatment of the mice for 3 weeks with AVI-4126 did not cause any mortality, untoward changes in metabolic profile, or macroscopic damage in the area surrounding the s.c. tumor, liver, lungs, heart, and kidneys of the animals. Phase I safety trials in humans via i.v. route of administration showed no toxicity or serious adverse events. The commonly observed side effects such as rise in APTT, complement C3a, anemia, decrease in platelet count, and hypotension related to various phosphorothioate-based antisense agents (32, 38, 39) were not observed with the AVI-4126 PMO.

In summary, we have demonstrated potent growth-inhibitory effects of the c-*myc* antisense PMO AVI-4126 on *in vivo* prostate cancer cell growth. Because c-*myc* is differentially expressed in tumor cells compared with normal cells, c-*myc*-directed therapy is expected to show stringent tumor specificity. This novel treatment approach has potential practical clinical application in patients with advanced hormone-refractory prostate cancer who have failed androgen ablation (40–42). This compound needs to be examined in other tumor models, and delivery strategies/formulations are currently being worked out

to define the scope of AVI-4126-directed therapy for prostate cancer.

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