Resistance to chemotherapeutic drugs overcome by c-Myc inhibition in a Lewis lung carcinoma murine model

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Chemotherapy resistance is a significant obstacle in lung cancer therapy, and has been found to frequently correlate with amplification and overexpression of the c-*myc* oncogene. Earlier studies have shown that c-Myc inhibition alone is not always effective in cancer models. The purpose of this study was to test different dosing regimen, which included commonly used chemotherapeutic drugs in combination with c-Myc inhibition in a Lewis lung syngeneic drug-resistant murine tumor model. Inhibition of c-*myc* was specifically achieved by using phosphorodiamidate Morpholino oligomer (PMOs), a novel, non-toxic antisense DNA chemistry for inhibition of gene expression by an RNase H-independent mechanism. When administration of cisplatin overlapped with c-*myc* PMO (AVI-4126) treatment there was no additional effect on tumor growth inhibition compared to cisplatin alone. In contrast, using a dosing regimen in which cisplatin or taxol treatment preceded AVI-4126, a dramatic decrease in tumor growth rate was observed with tumor areas less then 0.5 cm^2 in 60% of the animals at the end of the study. This effect was specific to c-Myc inhibition as other antisense PMOs against p21 or Rad51 showed no such effect in combination with chemotherapy. Immunoblot and HPLC-based analysis of tumor lysates at the end of the study confirmed c-Myc inhibition and detection of intact AVI-4126, respectively. In conclusion, AVI-4126 potentiates the efficacy of chemotherapeutic drugs in a manner that is schedule dependent. *Anti-Cancer Drugs* 14:39–47 © 2003 Lippincott Williams & Wilkins.

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Introduction

Lung cancer is the second most frequent cancer in men and women with an estimated 157 400 deaths in North America in 2001. Multiple drug resistance is a significant problem in lung cancer, particularly small cell lung carcinoma (SCLC), characterized by rapid development of resistance to cytotoxic agents, such as cisplatin and anthracyclines [1–3]. Amplification and overexpression of the c-*myc* oncogene has been consistently found to correlate with progression and chemotherapy resistance in lung cancer cells in culture, in the majority of SCLC and in a subset of non-small cell lung cancer (NSCLC) patients [4–6]. The role of c-*myc* in modulating the efficacy of cytotoxic drugs is controversial, since in some tumors, overexpression of c-Myc activates the apoptotic machinery [7–10] and in others, c-Myc overexpression causes chemoresistance [11–13].

In the present study we evaluated c-Myc expression and sensitivity of Lewis lung carcinoma, a syngeneic tumor model, to various cytotoxic agents. The Lewis lung tumors in C57BL/6 mice were treated with a combination of cytotoxic agents and c-*myc* phosphorodiamidate Morpholino-based antisense agent. Phosphorodiamidate Morpholino oligomers (PMOs) are novel third-generation antisense molecules, wherein the deoxyribose moiety of DNA is replaced with a six-membered morpholine ring and the charged phosphodiester internucleoside linkage is replaced with phosphorodiamidate linkages, thus rendering a novel non-ionic chemical structure [14,15]. Unlike the first (phosphodiester)- and second (phophorothioate)-generation oligonucleotides, the mechanism of action of PMO involves both steric blockade of ribosomal assembly (preventing translation) and the interference with intron–exon splicing of pre-mRNA (preventing appropriate translation of selected mRNA) [16,17]. 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 [18].

Our results demonstrate that the Lewis lung tumors are resistant to cisplatin, taxol, etoposide and 5-fluorouracil (5-FU) treatments. Inhibition of c-*myc* alone using the specific antisense PMO or co-administration of c-*myc* antisense PMO along with chemotherapy caused no decrease in tumor progression. In contrast, a significant decrease in tumor growth rate was observed in the Lewis

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lung tumor model with a dosing regimen in which the chemotherapy preceded treatment with the c-*myc* antisense agent.

Materials and methods

Oligomers

PMOs were synthesized and purified by AVI BioPharma (Corvallis, OR). Purity was greater than 90% as determined by reverse-phase HPLC and MALDI TOF mass spectrometry. Lyophilized PMOs were dissolved in sterile saline for injection. The base composition of the oligomers are shown in Table 1.

Cell culture

LLC1, murine Lewis lung carcinoma cells, were obtained from ATCC (Rockville, MD) and maintained in DMEF-12 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin (0.25 μg/ml) at 37°C in a 5% CO_2/95% air humidified incubator.

Animals

C57BL/6 mice (Simonsen, Gilroy, CA) weighing 22–24 g were housed in sterile plastic cages at the Laboratory Animal Resources Facility at Oregon State University (OSU; Corvallis, OR). Mice were given access to rodent chow (Harlan Teklad, Madison, WI) and tap water *ad libitum*, and exposed to 12-h light/dark cycles. 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 OSU.

Treatment protocols

After an acclimatization period of 5 days, mice were anesthetized with isoflurane, shaved and injected s.c. with approximately 2×10^5 LLC1 cells into the right rear flank. Injection sites were monitored daily to ensure that solid, homogeneous tumor growth was consistently obtained 4 days after LLC1 cell injections. Chemotherapy injections were prepared fresh daily before i.p. injection (see Table 1). PMO and cisplatin (Sigma, St Louis, MO) were dissolved in sterile, apyrogenic saline (Sigma) adjusted to an injection volume of 0.1 ml. Taxol stock solution (6 mg/ml in Cremophor EL and ethanol; Bristol Myers Squibb, Syracuse, NY) was diluted to 3 mg/ml in $1 \times$ PBS before injection. Etoposide (Sigma) stock solutions were prepared by dissolving in 70% ethanol at 11 mg/ml and then diluted with saline to a final concentration of 5 mg/ml. 5-FU (Calbiochem, San Diego, CA) was dissolved in saline at a concentration of 12.5 mg/ml. The experiment was started when the tumors were still in the latent period of growth and the mice were randomly assigned to different groups ($n = 6$–8).

- *Treatment with single agents.* Groups of mice received i.p. injections of saline, AVI-4126 (300 µg/day) or scrambled PMO (300 µg/day) on days 6–12 and 18–24. In case of the chemotherapy-treated groups, mice were administered cisplatin (83 µg/day), taxol (125 µg/day), etoposide (375 µg/day) or 5-FU (1250 µg/day) as single agents i.p. on days 2–4 and 14–16.
- *Co-administration.* One group of mice received two rounds of treatment in which cisplatin (days 2–4 and 14–16) treatment overlapped with AVI-4126 administration (days 2–8 and 14–20).
- *Cyclical administration.* The effect of cyclical administration of AVI-4126 (days 6–12 and 18–24) with the above-mentioned dosage of the chemotherapeutic agents on days 2–4 and 14–16 was studied in the LLC1 tumors. To test whether the effect was due to c-Myc inhibition or a general effect due to the PMO backbone, two other antisense sequences, p21 antisense PMO and RAD51 antisense PMO, were also tested utilizing a similar cyclical regimen in combination with cisplatin.

Therapeutic efficacy in each case was evaluated based on daily measurement of tumor area (length $\times$ width, cm^2) with digital calipers. 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 CBC counts, body weight and metabolic profile. A complete autopsy of the mice was done to rule out macroscopic side effects.

Protein expression

The tumors from the animals were lysed immediately after animal euthanization as described earlier [19]. Protein estimation was carried out using the Bradford protein assay kit (Bio-Rad, Hercules, CA). LLC1 protein

Table 1 **Various PMO sequences tested in the Lewis lung cancer cells**

Target	PMO	Sequence
c-*myc* AUG start site	AVI-4126 antisense	5′ ACG TTG AGG GGC ATC GTC GC 3′
Mismatched control	AVI-144 scrambled	5′ AC**T** **G**TG AGG G**CG** ATC G**CT** GC 3′
p21 AUG start site	PMO-27–30 antisense	5′ CAT CAC CAG GAT TGG ACA TGG 3′
Rad51 AUG start site	PMO-27–31 antisense	5′ CAA GCT GCA TTT GCA TAG CCA T 3′

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 to 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]. The p21 and Rad51 mRNA target positions are based on the accession no. U09507 and NM011234 mouse sequence, respectively.

lysate (300 μg) was analyzed on a 12% v/v sodium dodecylsulfate (SDS)–acrylamide separating gel. Gels were blotted, probed and visualized according to standard Western blotting protocols. Membranes were probed with rabbit anti-mouse c-Myc polyclonal antibody N-262 (sc-764) diluted 1:2000 in blocking buffer (Geno Technology, St Louis, MO) followed by goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (sc-2301). The relative amount of c-Myc protein was visualized by ECL Plus (Amersham, Piscataway, NJ) and analyzed on a Kodak 440 Image Station using 1D Image analysis software (Kodak, Rochester, NY). Membranes were then soaked in stripping buffer (Geno Technology) for 20 min at 25°C and reprobed with a 1:2000 dilution of goat anti-mouse β-actin polyclonal antibody (sc-1616), which served as a protein loading control, followed by donkey anti-goat HRP-conjugated antibody (sc-2304).

HPLC detection of PMO in tumor tissue

Tumor tissues from saline- and AVI-4126-treated animals were analyzed for the presence of PMO by HPLC analysis as described previously [20,21]. Briefly, a 10-μl aliquot (500 ng) of the internal standard PMO (15mer whose sequence was derived from a 5′ truncation of AVI-4126) was added to all 250-μl aliquots of tumor lysate (0.2 gm/ml). Then, 300 μl methanol was added to each sample and the tubes were vortexed. The tubes were centrifuged for 10 min using a high-speed centrifuge and supernatants transferred to new eppendorf tubes. The pellet was then washed with 100 μl 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 re-centrifuged 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 deionized water to each vial. The lyophilized samples were reconstituted using 100 μl aliquots of 5′-fluoresceinated DNA (1.0 OD U/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 of plasma) along with the internal standard. The standards were similarly extracted.

The samples were analyzed by injecting on to a Dionex DNA Pac PA-100 column (4 × 250 mm column; Dionex, Sunnyvale, CA) using a Varian autosampler (AI-200) connected to a Varian HPLC pump (model 9010 inert) equipped with a Varian fluorescent 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 prior to use. The gradient program employed was [90–10% B] at 0 min and [55–45% B] at 20 min while the pump was held at a flow rate of 1.5 ml/min. The runs were monitored at excitation and emission wavelengths of 494 and 518 nm, respectively.

Inductively coupled plasma-mass spectrometry (ICP-MS) detection and quantitation of platinum/cisplatin

A 200 μl aliquot from cisplatin- and cisplatin + AVI-4126-treated tumor lysates (0.2 g/ml) were dissolved in 1.33 ml of aqua regia followed by a 10-fold dilution. The samples were then analyzed by the ICP-MS technique for the presence of platinum according to the method of Long *et al.* [22] (Anatek, Moscow, ID).

Statistical analysis

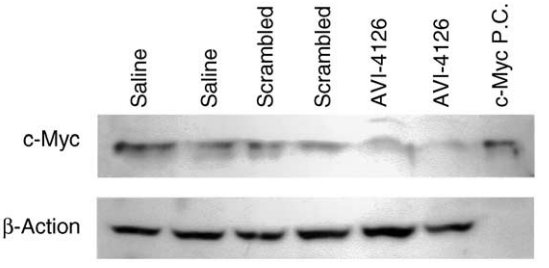
The data are expressed as the mean ± SE. The statistical analyses were performed using GraphPad InStat Student's two-tailed *t*-test and ANOVA (Tukey–Kramer multiple comparison test). Differences were considered significant at $p < 0.05$.

Results

Antisense-mediated inhibition of c-Myc expression in LLC1 tumors

In order to determine the effect of c-*myc* antisense PMO (AVI-4126) on c-Myc expression in LLC1 tumors, mice with well-established tumors (2–2.5 cm^2) were treated i.p. with a single injection of saline, scrambled control PMO (AVI-144) or antisense c-*myc* oligomer (AVI-4126) at 100 μg dose. Tumor lysates were prepared 24 h post-treatment and immunoblot analyses revealed that a specific inhibition of c-Myc protein levels was seen in the AVI-4126-treated LLC1 tumor lysates compared to the saline or scrambled PMO-treated groups (Fig. 1). β-Actin immunodetection on the same blot resulted in similar band intensities in all lanes, confirming identical loading of total protein in all lanes.

Fig. 1

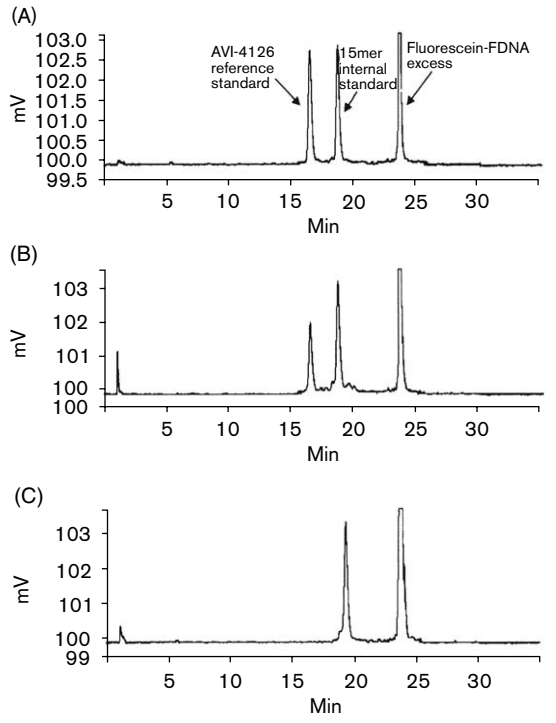


Immunoblot analysis of c-Myc and β-actin protein in LLC1 tumor lysates prepared 24 h post i.p. administration of saline or 100 μg of scrambled control (AVI-144) or c-*myc* antisense PMO (AVI-4126). The 65-kDa c-Myc recombinant protein produced in *Escherichia coli* (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control to ascertain molecular weight and antibody specificity. The immunoblots were probed with c-Myc polyclonal antibody (P.C.), and the same immunoblot was stripped and reprobed to determine β-actin levels.

In vivo AVI-4126 bioavailability in LLC1 tumors

The saline- and AVI-4126-treated tumor lysates from the above-mentioned experiment were also processed and run on HPLC as described in Materials and methods. Representative chromatograms showing separation of peaks are presented in Figure 2. The elution order for each chromatogram is c-*myc* antisense PMO, AVI-4126, the internal standard and the excess fluoresceinated DNA probe is last to elute (Fig. 2A). The peak corresponding to full-length (20mer) AVI-4126 was readily observed following administration with a single dose of 100 μg AVI-4126 in tumor samples from the s.c. LLC1 tumors (Fig. 2B) and this specific peak was not observed in the saline-treated tumor lysates (Fig. 2C). Quantitation of the PMO concentration per gram tumor tissue from various experiments revealed the presence of about 0.15–0.4 μg PMO/g tissue at 24 h. 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 lysates.

Fig. 2



Representative anion-exchange HPLC chromatograms showing detection of the c-*myc* antisense oligomer AVI-4126 in tumor tissue from LLC1 tumor-bearing mice treated with a single i.p. injection of saline or (100 μg) AVI-4126. (A) Control: untreated rat plasma spiked with AVI-4126 stock solution. (B) LLC1 tumor lysates at 24 h following administration with a single i.p. dose of 100 μg AVI-4126. (C) LLC1 tumor lysates at 24 h following i.p. administration with saline.

Antitumor efficacy of cisplatin alone or in combination with c-*myc* antisense PMO

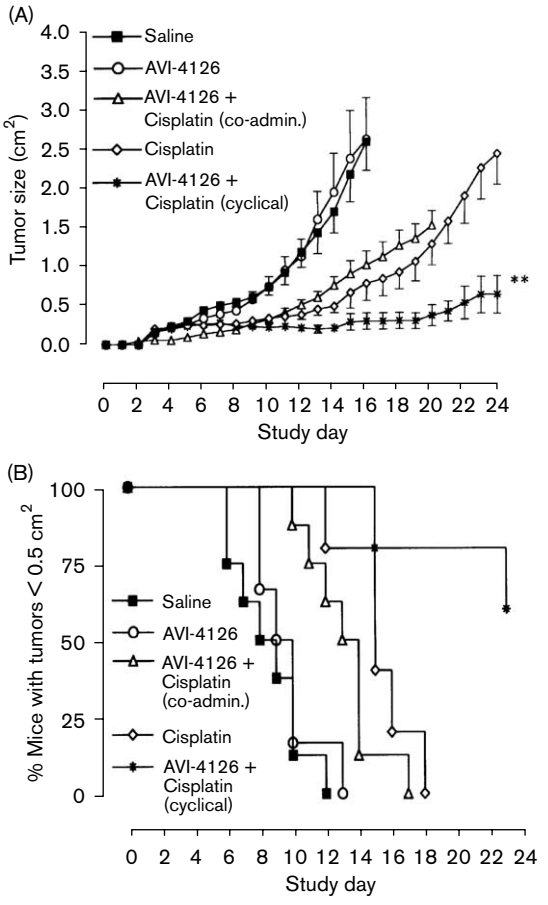
The antitumor effect of cisplatin was evaluated in C57BL/6 mice bearing the LLC1 s.c. tumors. Cisplatin was administered alone (3.3 mg/kg i.p) according to two repeated cycles, on days 2–4 and 14–16 as described by earlier studies, which showed that repeated administrations were more effective than a single bolus [23,24]. The interval of 10 days gave sufficient time for the animals to recover from the chemotherapy-related lethargy and weight loss.

It is evident in Figure 3(A) that there was a significant delay in tumor growth after the first cycle of cisplatin administration (0.4 ± 0.05) compared to saline-treated animals (1.2 ± 0.2). However, the tumors became more aggressive after the second treatment cycle with cisplatin and achieved a similar tumor size as the saline-treated animals by day 24, suggesting chemoresistance (Fig. 3A and B). Treatment with 300 μg/day i.p. of the c-*myc* antisense PMO, AVI-4126, alone was ineffective in reducing the rate of tumor growth. The tumor area at the end of the study in the AVI-4126-treated group (2.6 ± 0.5) was similar to the saline-treated group (2.7 ± 0.4). These animals had to be euthanized by day 17 of the study since the tumors were quite large and morbid.

In a combination dosage schedule in which cisplatin and AVI-4126 treatments overlapped, no significant change in tumor area (1.6 ± 0.3) was observed at the end of the study compared to the cisplatin-only-treated animals (1.5 ± 0.2). In contrast, in the group of tumor-bearing mice in which cisplatin was administered prior to AVI-4126 according to two repeated cycles such that the animals received cisplatin (3.3 mg/kg i.p.) on days 2–4 and 14–16 and AVI-4126 (300 μg/day i.p.) on days 6–12 and 18–24, a dramatic decrease in tumor area (0.6 ± 0.2) and mass (0.1 ± 0.05) was observed ($p < 0.01$). In addition, at the end of the treatment regimen, 60% of the animals that received the cyclical combination of cisplatin and AVI-4126 had a tumor size below 0.5 cm^2 compared to none in the other treatment groups (Fig. 3B). No mortality or significant change in body weight was observed in the saline- or AVI-4126-treated groups. There was one death in the cisplatin- and cisplatin + AVI-4126-treated groups between days 4 and 7 before administration of AVI-4126 attributable to cisplatin toxicity.

In order to determine whether the ability of AVI-4126 to modulate LLC1 tumor cell sensitivity to cisplatin is specific to c-Myc inhibition and not due to the PMO backbone, two other antisense PMO agents targeted to p21 and Rad51 were synthesized (Tables 1 and 2). The p21 and Rad51 PMOs were tested in the LLC1 tumor

Fig. 3



Effects of c-*myc* antisense AVI-4126 in combination with cisplatin in syngeneic LLC1 tumors in C57BL/6 mice. (A) Intraperitoneal injections of saline, AVI-4126 alone (days 6–12 and 18–24), cisplatin alone (2–4 and 14–16), AVI-4126 + cisplatin co-administration (cisplatin, days 2–4 and 14–16 with AVI-4126 on days 2–8 and 14–20) or cyclical administration of cisplatin and AVI-4126 (cisplatin on days 2–4 and 14–16 followed by AVI-4126 on days 6–12 and 18–24) were given to tumor-bearing mice. Tumors were measured using calipers and the tumor area was calculated (length × width). The values indicated are means ± SE. Asterisk indicates significant difference between cisplatin alone versus cisplatin + AVI-4126 (cyclical treatment). (B) The percentage of animals in each treatment group from the above-mentioned experiment with tumor size below 0.5 cm^2 during the period of treatment.

model utilizing the same cyclical treatment regimen that was effective in the case of AVI-4126. The data in Table 3 reveals that the other antisense PMOs failed to enhance the sensitivity of the LLC1 tumors to cisplatin. The tumor growth and mass in animals treated with p21 and Rad51 antisense alone or in combination with cisplatin were similar to saline-treated and cisplatin-treated animals, respectively.

To ascertain that the c-*myc* antisense PMO effect was due to downregulation of the c-Myc protein, lysates from tumors excised a day after the end of the treatment were immunoblotted and probed with anti-c-Myc antibody. The data in Figure 4 reveals a specific inhibition of c-Myc levels in the AVI-4126-treated animals and in cisplatin + AVI-4126-treated animals. No statistically significant differences in the c-Myc levels in the saline compared to cisplatin-only-treated tumors were observed. Cisplatin levels in the cisplatin-treated versus cisplatin + AVI-4126-treated tumor lysates, as measured by ICP-MS, showed no significant differences (data not shown).

c-Myc inhibition sensitizes the LLC1 tumors to taxol, etoposide but not 5-FU

The sensitivity of LLC1 tumor cells to other commonly used cytotoxic agents was studied in the murine tumor model. The data in Figure 5(A) reveals that the LLC1 tumors were resistant to taxol, etoposide and 5-FU treatments; however, cyclical treatment of these agents with AVI-4126 increased the tumor cell sensitivity to taxol and etoposide (Fig. 5B). The efficacy of taxol (5 mg/kg) and etoposide (15 mg/kg) administered on days 2–4 and 14–16 was significantly enhanced by addition of AVI-4126 (300 μg) on days 6–12 and 18–24 in the cyclical treatment regimen compared to the single-agent treatment or saline. AVI-4126 inclusion sensitized the cells more effectively to cisplatin treatment followed by taxol and etoposide. 5-FU (50 mg/kg) was, however, ineffective as a single agent or in combination with AVI-4126 in the LLC1 tumors (Table 4).

Discussion

We report herein the potential of a novel c-*myc* antisense agent to enhance the potential of various chemotherapeutic agents in a widely used lung cancer syngeneic murine model. Our results show that the LLC1 tumors in the C57BL/6 mice are initially sensitive to cisplatin treatment but gradually gain resistance, and are overall insensitive to taxol, etoposide and 5-FU treatments. Cisplatin treatment followed by targeted inhibition of c-*myc* significantly decreased tumor growth rate and induced tumor regression. c-Myc inhibition also enhanced the sensitivity of these tumors to two other commonly used chemotherapeutic agents, taxol and etoposide, in a similar cyclical treatment regimen, albeit the etoposide combination was less remarkable compared to the cisplatin and taxol combination with c-Myc downregulation.

Chemotherapy, particularly platinum-based drugs, is the first line of care for lung cancer patients. However, the majority of the patients inevitably develop resistance to chemotherapy with disease progression. One of the common genetic alterations observed in lung cancer which is correlated with poor prognosis, is amplification and overexpression of c-*myc* oncogene [1,25,26]. It is unclear as to the exact mechanism by which c-*myc* influences tumor progression, but the ability of this oncogene to effect cellular processes such as proliferation,

Table 2 Effect of AVI-4126 (c-*myc* antisense PMO), cisplatin alone or in combination on the growth of Lewis lung carcinoma s.c. tumors in C57BL/6 mice

Treatment[a]	Tumor area (cm^2)			Tumor weight (g)	Tumor growth rate (% of saline)
	Initial day	After first cycle	Final day		
Saline (days 6–12 and 18–24)	0.2±0.02	1.2±0.2	2.6±0.4	1.4±0.3	100
AVI-4126 (days 6–12 and 18–24)	0.3±0.02	1.1±0.23	2.7±0.5	1.6±0.6	103.9
Cisplatin (days 2–4 and 14–16)	0.2±0.01	0.4±0.05*	2.4±0.4**	0.9±0.3	60
Cisplatin + AVI-4126 (cyclical)	0.2±0.01	0.2±0.04	0.6±0.2***	0.1±0.05	18.6

[a]Groups of mice received i.p. injections of either saline, 300 μg/day of AVI-4126 or cisplatin (83 μg/day) as a single agent. The effect of cyclical administration of cisplatin (83 μg/day i.p) on days 2–4 and 14–16 and AVI-4126 (300 μg/mouse/day i.p. on days 6–12 and 18–24) was studied in the LLC1 tumors. Values are means±SEM ($n=5$–8). Tumor growth rate was calculated using linear regression to analyze the change in tumor area from day 14 to 24 when the change was linear. The data presented is percent of saline (slope±SD). Statistical analysis (two-tailed *t*-test):
*cisplatin versus saline or AVI-1426 (end of first cycle), $p<0.01$ or $p<0.05$, respectively;
**cisplatin versus saline or AVI-4126 (end of second cycle), $p<0.05$ (NS);
***cisplatin versus AVI-4126 + cisplatin (end of second cycle), $p<0.01$.

Table 3 Effect of cisplatin, p21 antisense PMO or Rad51 antisense PMO as single agents or in combination on the growth of Lewis lung carcinoma s.c. tumors in C57BL/6 mice

Treatment[a]	Tumor area (cm^2)		Tumor weight (g)	Tumor growth rate (% of saline)
	Day 13	Day 24		
Saline (days 6–12 and 18–24)	0.6±0.07	2.9±0.2	1.5±0.15	100
Cisplatin (days 6–12 and 18–24)	0.3±0.02	1.4±0.1	0.5±0.1	50
p21AS PMO (days 6–12 and 18–24)	0.4±0.1	2.4±0.4	1.3±0.4	90
Cisplatin + p21AS (cyclical)	0.4±0.06	1.6±0.3	0.6±0.2	51
Rad 51AS PMO (cyclical)	0.4±0.04	2.5±0.3	1.2±0.2	97
Cisplatin + Rad51AS (cyclical)	0.4±0.06	1.2±0.2	0.4±0.1	44

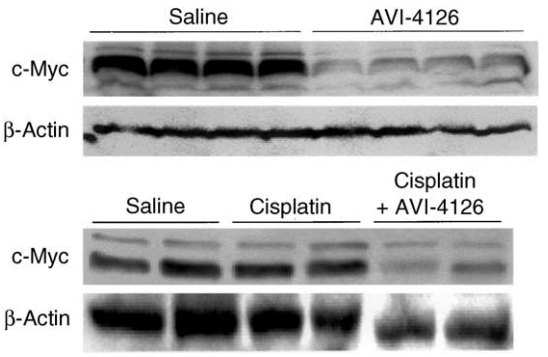
[a]Groups of mice received i.p. injections of saline, 300 μg/day of p21AS PMO, Rad51AS PMO or cisplatin (83 μg/day) as a single agent. The effect of cyclical administration of antisense PMO (300 μg/mouse/day i.p. on days 6–12 and 18–24) with cisplatin (83 μg/day i.p) on days 2–4 and 14–16 was also studied. Values are means±SEM ($n=6$–8). Tumor growth rate was calculated using linear regression to analyze the change in tumor area from day 14 to 24 when the change was linear. The data presented is percent of saline (slope±SD).

differentiation and apoptosis is well established [27]. *In vitro* resistance to cisplatin in various cancer cellular models has been shown to correlate with c-Myc expression [11,12,28] and prior chemotherapy has also been reported to induce c-*myc* amplification [29]. These studies suggest that c-Myc inhibition could have therapeutic potential for cancer [30,31]. Inhibition of c-*myc* using phosphorothioate oligonucleotides has been observed to increase the sensitivity of cisplatin-resistant melanoma xenografts [32].

In the present study we used a PMO to inhibit c-Myc in LLC1 murine syngeneic tumors. We observed that following administration of two rounds of a combination regimen in which cisplatin and AVI-4126 treatments overlapped, no significant change in tumor growth rate was observed compared to mice administered two rounds of the cytotoxic agent or saline alone. In contrast, in a dosing regimen in which administration of the chemotherapeutic agents cisplatin, taxol or etoposide preceded AVI-4126, a dramatic decrease was observed in tumor growth rate, with tumor areas below 0.5 cm^2 in 60% of the cisplatin + AVI-4126-treated animals at the end of the study. This decrease in tumor area was associated with a specific decrease in c-Myc protein levels and intact AVI-4126 was detected in the tumor lysates by immunoblot and HPLC-based analysis, respectively. Necropsies of tumors revealed small, non-invasive tumor nodules in the cyclical combination regimen compared to highly invasive and vascular tumors in the other groups.

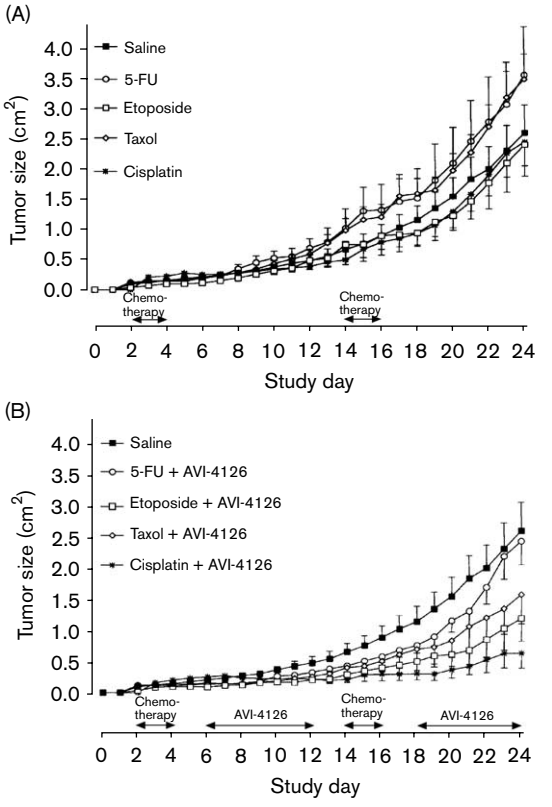
Treatment of the LLC1 tumors with the c-*myc* antisense agent, AVI-1426, alone caused a significant decrease in c-Myc levels; however, this decrease did not cause any change in the tumor growth rate compared to the saline-treated animals. This is not surprising due to the fact that cancer is a result of multiple genetic events and inhibition of a single gene product may not be sufficient to reverse a malignant phenotype. Results from various studies from our laboratory and others with antisense compounds suggest that the practical use of an agent that inhibits c-*myc* expression will be as part of a combination chemotherapeutic regimen [21,33,34]. Studies by D'Cruz *et al.* [34] utilizing a conditionally expressed c-*myc* gene in transgenic mice found that deregulation of c-*myc*

Fig. 4



Immunoblot analysis of representative tumor lysates from saline, AVI-4126, cisplatin and cisplatin + AVI-4126 combination groups. The tumor lysates from the experiment as described in Figure 3 were run on a 12% SDS–PAGE and subjected to Western transfer. The immunoblots were probed with a c-Myc polyclonal antibody and the same immunoblot was stripped and reprobed to determine β-actin levels.

Fig. 5



Effects of c-*myc* antisense AVI-4126 in combination with cytotoxic agents in syngeneic LLC1 tumors in C57BL/6 mice. (A) Effect of the chemotherapeutic agents as indicated on LLC1 tumor growth. Saline or various chemotherapeutic agents were administered i.p. on days 2–4 and 14–16. Tumors were measured using calipers and the tumor area was calculated (length × width). The values indicated are means ± SE. (B) Saline or the chemotherapeutic agents were administered to the tumor-bearing mice in combination with c-*myc* antisense PMO AVI-4126 in a cyclical manner (chemotherapy on days 2–4 and 14–16 staggered with AVI-4126 on days 6–12 and 18–24). Tumors were measured using calipers and the tumor area was calculated (length × width). The values indicated are mean ± SE.

expression results in the formation of invasive mammary adenocarcinomas. In this test system, tumors maintained by high c-*myc* expression regress when c-Myc levels are reduced except for a subset in which spontaneous mutations occurred in the K-*ras2* or N-*ras* genes. We recently reported that in prostate cancer cells and xenograft tumors, inhibition of c-Myc alone differentially regulated the proliferation and tumorigenicity of two cell lines based on the expression of the retinoblastoma (Rb) protein [21]. Treatment of the LNCaP prostate cells ($Rb^{+/+}$) with AVI-4126 caused decreased cell proliferation. In contrast, the DU145 prostate cancer xenograft tumors, which are $Rb^{-/-}$, were unresponsive to a decrease in c-Myc levels alone. However, tumor regression in the refractory DU145 cells was achieved by blocking both c-Myc and a growth factor β-hCG using a combination antisense strategy. Although synergy between oncogenic

Table 4 Effect of AVI-4126 (c-*myc* antisense PMO), chemotherapy alone or in combination on the growth of Lewis lung carcinoma s.c. tumors in C57/BL6 mice

Treatment[a]	Tumor area (cm^2)		Tumor weight (g)	Tumor growth rate (% of saline)
	Day 13	Day 24		
Saline ($n=20$)	0.5 ± 0.1	2.6 ± 0.2	1.5 ± 0.1	100
Cisplatin ($n=6$)	0.3 ± 0.09	1.2 ± 0.3	0.5 ± 0.1	49
Taxol ($n=6$)	0.8 ± 0.14	3.5 ± 0.4	1.8 ± 0.3	114.7
Taxol + AVI-4126 ($n=6$) (cyclical)	0.3 ± 0.1	1.6 ± 0.5	0.5 ± 0.3	51
Etoposide ($n=6$)	0.5 ± 0.1	2.6 ± 0.5	1.26 ± 0.4	75
Etoposide + AVI-4126 ($n=6$) (cyclical)	0.2 ± 0.06	1.2 ± 0.4	0.5 ± 0.2	37
5-FU ($n=6$)	0.8 ± 0.2	3.5 ± 0.8	2.7 ± 0.9	115
5-FU + AVI-4126 ($n=6$) (cyclical)	0.4 ± 0.04	2.4 ± 0.4	1.0 ± 0.2	88

[a]Groups of mice received cisplatin (83 μg/mouse/day i.p.), taxol (125 μg/mouse/day i.p.), etoposide (375 μg/mouse/day i.p.) or 5-FU (1250 μg/day/mouse i.p.) as single agents on days 2–4 and 14–16. The effect of cyclical administration of cytotoxic agents on days 2–4 and 14–16 and AVI-4126 (300 μg/mouse/day i.p. on days 6–12 and 18–24) was studied in the LLC1 tumors. Tumor growth rate was calculated using linear regression to analyze the change in tumor area from day 14 to 24 when the change was linear. The data presented is percent of saline (slope ± SD).

pathways has been demonstrated previously, these studies demonstrate that deregulation of c-*myc* expression selects for preferred secondary oncogenic pathways. In the present study, about 75% inhibition of c-Myc protein levels was achieved when treatment included AVI-4126; however, this may not be sufficient to inhibit tumor growth without the additional cellular damage at the appropriate time that a rigidly scheduled combination therapy provides. This is consistent with a model in which c-*myc* expression influences the transformation process, but is not the only factor involved in maintaining a transformed phenotype. Studies by Bazarov *et al.* [35] have demonstrated that a modest reduction in c-Myc levels can dramatically reduce the susceptibility to *Ras* and *Raf* transformation without causing significant cell cycle defects. Our results suggest that c-*myc* may also be important in maintaining the transformed phenotype since LLC1 tumors, which are inherently resistant to cisplatin, exhibited increased sensitivity to cisplatin in a treatment regimen that included AVI-4126 in a schedule-dependent manner. Tumors were significantly more sensitive to cisplatin and taxol treatment, and, to a lesser extent, etoposide when AVI-4126 treatment followed chemotherapy.

PMOs represent a novel antisense oligomer chemistry in which the backbone is neutral at physiological pH and consists of a six-membered morpholine ring. PMOs are resistant to a variety of nucleases and proteases, bind with higher affinity to RNA than congenic phosphodiester DNA, and act as steric inhibitors of translation inhibition. The lack of inter-nucleoside charge allows the PMOs to avoid non-specific effects like G-quartets or interaction through the CpG motifs observed with the commonly used phosphorothioate oligonucleotides that bind to cellular and extracellular proteins and cause serious side effects *in vivo* [36,37]. AVI-4126 sequence was chosen because it displayed most favorable solubility, efficacy and potency in comparison to 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 [38]. The rational for using c-*myc* antisense PMO to treat cancer is based on results that it inhibits c-*myc* translation in a sequence-specific manner by steric blockade and mis-splicing of pre-mRNA in cancer cells, and causes significant cell growth inhibition [16]. In earlier studies, AVI-4126 has demonstrated *in vivo* efficacy in polycystic kidney disease [39], liver regeneration [19] and vascular restenosis following balloon injury associated with angioplasty [40], and has successfully completed a phase I safety clinical trial in healthy human volunteers by the i.v. route of administration with no serious adverse events [41]. In the present study too, no mortality or significant change in body weight was observed in the AVI-4126-treated groups.

In summary, the c-*myc* antisense PMO potentiates the efficacy of chemotherapeutic drugs in a manner that is schedule dependent in a lung cancer murine model. These data suggest that treatment with AVI-4126 would provide enhanced antitumor activity from drugs such as cisplatin, which has a narrow therapeutic index and can cause fatal toxicities at higher doses [42]. The biochemical pathways that control proliferation and apoptosis are complex, and this new generation of antisense compounds not only provides a useful tool in understanding the pivotal role of c-*myc* in cancer, but also a corresponding non-toxic therapeutic entity.

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