0026-895X/95/040738-09$3.00/0

MOLECULAR PHARMACOLOGY, 48:738–746 (1995).

Contribution of Sequence and Phosphorothioate Content to Inhibition of Cell Growth and Adhesion Caused by c-*myc* Antisense Oligomers

CHRISTINE CHAVANY, YVETTE CONNELL, and LEN NECKERS

Clinical Pharmacology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Received March 28, 1995; Accepted May 26, 1995

SUMMARY

c-*myc* is overexpressed in glioblastoma multiforme, the most common form of brain tumor. To find a suitable target for *in vivo* antisense therapy of gliomas, we investigated the biological effects on the human glioma cell line, U87MG, of antisense oligonucleotides targeted against the translation start site of c-*myc* mRNA. Parameters examined included c-*myc* protein level, cell proliferation, and cell adhesion to substratum. Oligonucleotides were administered by electroporation as capped phosphorothioates. Antisense oligomers caused a reduction in c-*myc* protein expression, loss of cell adhesion to plastic, and complete growth inhibition. Various control sequences, including sense, scrambled, and three-base mismatched oligomers, were also tested. Some of the controls retained a dG quartet found in the antisense sequence. Reduction in c-*myc* protein and cell growth and loss of cell adhesion were specific to the antisense sequence. Surprisingly, fully thioated antisense and scrambled sequences, either containing or lacking a dG quartet, were equally inhibitory to both cell growth and adhesion. Loss of cell adhesion was observed with only phosphorothioate-containing oligomers, not with either their phosphodiester or nuclease-resistant PA congeners, and was completely reversed when cells were plated onto fibronectin. These results demonstrate that a commonly used c-*myc* antisense oligomer also displays dramatic, sequence- but not antisense-specific effects on cell proliferation and cellular adhesion, depending on the backbone.

Synthetic ODNs have been used successfully to inhibit numerous cell functions. Recent studies suggest that ODNs may also be used as inhibitors of gene expression in animals (1–3). In general, because of their nuclease sensitivity, PO ODNs have been replaced by their PS counterparts. The *in vivo* distribution and stability of these congeners have also been studied (4).

Despite their utility, PS ODNs have been shown to exibit nonantisense-mediated effects. Binding of PS ODNs to proteins such as CD4, gp120, or HIV-1 reverse transcriptase and DNA polymerase has been described (5–8). Bergan *et al.* also reported the inhibition of $p210^{bcr\text{-}abl}$ and PDGFR tyrosine kinase activity with certain PS sequences containing closely spaced GGC repeats (9). Recently, Perez *et al.* described the sequence-independent induction of a nuclear SP1-like transcription factor activity by both PS and PO ODNs (10). Interaction of PS ODNs with certain drugs, interfering with their tumoricidal activity, has also been reported. Thus, binding of PS ODNs to adriamycin, daunomycin, quinacrine, and actinomycin D was able to protect cells from the cytotoxic effects of these drugs (11).

Certain thioated sequences targeting the retinoblastoma gene product have been reported to stimulate keratinocyte growth (12). Also, nonantisense-mediated antiproliferative effects of PS ODNs containing a dG quartet have been recently demonstrated by Yaswen *et al.* (13). Identical sequences with a mixed PO and PS backbone or an all-PO backbone had little or no effect on cell growth. Moreover, an antisense PS ODN targeted to the translation start site of c-*myc* mRNA (and also containing a dG quartet) was found to inhibit adhesion onto plastic of the human breast cancer line MCF-7 (14). Loss of cell adhesion was related to neither cell growth nor c-*myc* protein inhibition.

In searching for a suitable target for *in vivo* antisense therapy of gliomas, we also investigated the biological effects of a commonly used c-*myc* antisense ODN on the human glioma cell line U87MG. c-*myc* has been found to be overexpressed in many tumors, including glioblastoma (15–18). To avoid both nonspecific effects and nuclease digestion, this ODN was synthesized with a PO backbone containing four PS linkages on both 3′ and 5′ end (POPS). POPS oligomers

Y.C. is supported by Lynx Therapeutics, Inc., Hayward, CA. This study was partially supported by a cooperative research and development agreementwith Lynx Therapeutics.

ABBREVIATIONS: ODN, oligonucleotide; PO, phosphodiester; PS, phosphorothioate; PDGFR, platelet-derived growth factor receptor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PA, phosphoroamidate; TTBS, Tris-buffered saline/Tween 20.

are in general more stable to both exonucleases and endonucleases, are able to inhibit *in vitro* translation in wheat germ extracts, and exhibit a decreased binding capacity to human serum albumin (19).

We observed that electroporation of U87MG cells with POPS c-*myc* antisense ODNs caused a reduction in c-*myc* protein expression, loss of cell adhesion, and complete growth inhibition. Control POPS sequences containing the dG quartet (as found in the antisense sequence) had little or no effect on cell adhesion, c-*myc* protein expression, or cell growth. Loss of cell adhesion onto plastic and growth inhibition were dependent on the DNA backbone because with a fully thioated (PS) backbone, these phenomena were observed with both antisense and scrambled sequences, containing or lacking the dG quartet, whereas with the POPS (53% PS) backbone, these phenomena were observed only with the antisense sequence. Loss of cell adhesion, but not growth inhibition, induced by c-*myc* antisense ODNs was reversed by exogenous fibronectin, indicating that these events were independent of each other. These results demonstrate that a commonly used c-*myc* antisense ODN also displays dramatic, nonantisense effects on cell proliferation and cellular adhesion, which can be both sequence and backbone dependent.

Materials and Methods

Cell lines and culture conditions. The human glioma cell line U87MG and murine monoclonal c-*myc* 1–9E10.2 antibody-producing hybridoma cells were purchased from the American Type Culture Collection (Rockville, MD). U87MG cells were maintained in DMEM containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), 1 mM glutamine (Biofluids, MD), and 10 mM HEPES (Biofluids, MD) and were maintained at 37° in an atmosphere of 6% CO_2.

ODN synthesis. ODNs were synthesized by cyanoethyl phosphoramidite chemistry on an Applied Biosystems Model 380B DNA synthesizer. Sulfurizing reagent (Glen Research, Sterling, VA) was used according to the manufacturer's instructions. ODNs were ethanol precipitated, washed with 70% ethanol, and resuspended in sterile water. Aliquots were resolved on a denaturing polyacrylamide gel to check for homogeneity. Phosphoramidate ODNs were supplied by Lynx Therapeutics (Hayward, CA).

Antisense, scrambled, sense, and mismatch ODNs targeting c-*myc* mRNA were synthesized either as complete POs, complete PSs, or PS capped (POPS), as indicated. Three antisense congeners (AS1, AS2, and AS3) were synthesized. Their sequences are complementary to different regions of c-*myc* mRNA containing the AUG start codon of translation: AS1, 5′-AACGTTGAGGGGCAT-3′ (+1 to +15); AS2, 5′-GGCATCGTCGCGGGA-3′ (−10 to +5); and AS3, 5′-TGAGGGGCATCGTCG-3′ (−5 to +10), respectively. Control sequences to AS1 (three-base mismatched, scrambled, sense, or inverse sequences containing [ASM2, SCR2] or lacking [ASM1, SCR1, SE1, INV1] a dG quartet as in AS1) were also tested (Fig. 1). POPS ODNs contain four interbase PS linkages at each end.

Electroporation of cells with ODNs. Electroporation was performed with a GIBCO-BRL Cell-Porator Electroporation System 1. U87MG cells were electroporated in DMEM containing 10% serum at a capacitance of 1180 μF and 350 V. Cell concentration during electroporation was 1–4 × 10^6 cells/ml. After electroporation, cells were plated in 100- × 20-mm dishes with 6 ml of medium (Western blot experiments) or diluted 1:12 in medium and plated in 24-well plates (cell adhesion and growth experiments) precoated or not precoated with 1 μg/cm^2 fibronectin (Sigma Chemical Co.), as indicated in the text. At the appropriate time, the medium containing nonadherent cells was removed. Adherent cells were washed once with PBS and detached by trypsin treatment. At various times after electroporation, viable adherent and nonadherent cells were determined by the Trypan blue exclusion method. In Fig. 2, cell growth is expressed as a ratio between final and initial cell number (a ratio of 1 is equal to complete cell growth inhibition). In the other experiments described, the following formula was used to express cell growth in percent of control: $[(n - \text{No. treated})/(n - \text{No. untreated})] \times 100$, where n is the final cell number, and No. is the initial cell number.

c-*myc* and PDGFα receptor protein analysis. After electroporation, adherent and nonadherent cells were pooled and centrifuged (4500 rpm). The cellular pellet was washed once with PBS and then lysed at 4° in TNESV buffer (50 mM Tris, pH 7.5, 1% Nonidet P40, 2 mM EDTA, 0.1% SDS, 100 mM NaCl, and 1 mM vanadate) supplemented before use with protease inhibitors: 20 μg/ml aprotinin and leupeptin and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was then centrifuged at 14,000 rpm in the cold (4°) for 15 min. The supernatant was removed, and protein content was determined with a bicinchroninic acid-based protein assay (BCA, Pierce). Total cell proteins were separated on 8% sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membranes (0.45 μm; Schleicher and Schuell) for immunoblot analysis. c-*myc* and PDGFRα proteins were detected with a chemiluminescence-based Western blotting kit (DuPont) according to the supplier's instructions. Briefly, the nitrocellulose membrane was incubated for 1 hr with 5% nonfat dry milk in TTBS (20 mM Tris·HCl, pH 7.6, 0.05% Tween 20, and 0.9% sodium chloride). After 25-min washes in TTBS, the membrane was incubated with a 1:1000 dilution of human-specific c-*myc* mouse monoclonal antibody for 1 hr, washed again for 25 min, incubated with a 1:1000 dilution of sheep anti-mouse horseradish peroxidase-linked whole antibody (Amersham) for 1 hr, and washed for 1 hr in TTBS buffer. For human PDGFRα detection, a dilution of 1:3000 of rabbit anti-human PDGFRα (UBI) and a 1:1000 dilution of donkey anti-rabbit horseradish peroxidase-linked whole antibody (Amersham) were used. Last, membranes were incubated for 1 min with a luminol-based detection solution. Membranes were then exposed for various times to Kodak X-Omat AR film. Densitometric analysis of films was performed to quantify p65 c-*myc* or p170 PDGFRα expression.

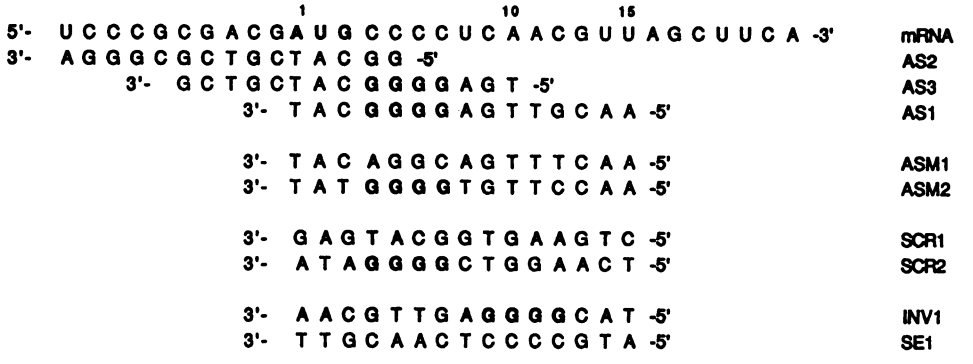


Fig. 1. Oligodeoxynucleotide sequences.

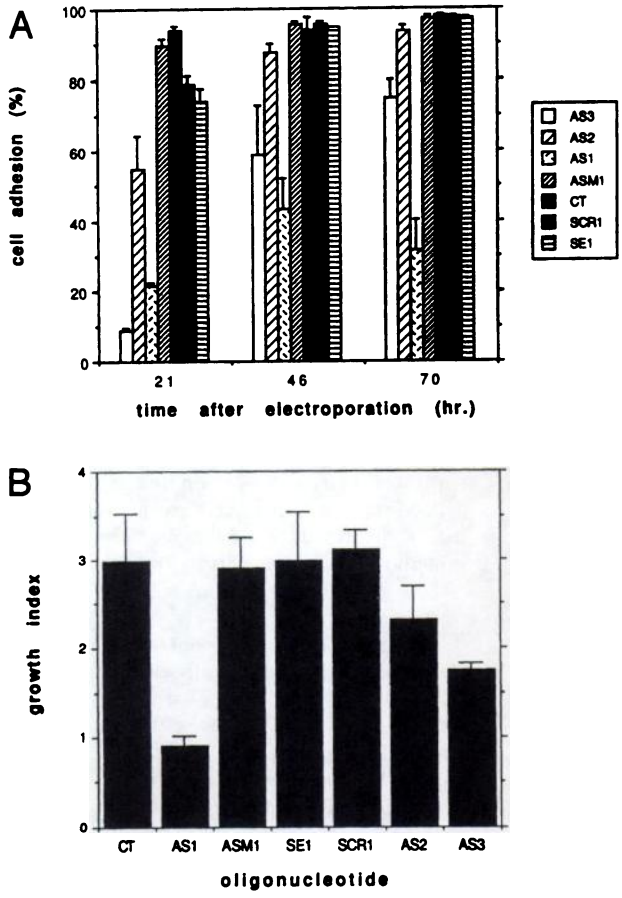


Fig. 2. Inhibition of cell adhesion and growth after electroporation with POPS antisense c-*myc* ODNs. U87MG cells were electroporated in the absence (*CT*) or presence of 20 μM antisense ODNs (*AS1*, *AS2*, *AS3*) or control sequences (*SCR1*, *MIS1*, *SE1*) and plated onto plastic dishes for different periods of time as indicated. A, Relative adhesion was determined after counting viable adherent and nonadherent cells; B, cell growth was determined 48 hr after treatment as described in Materials and methods; C, cell morphology was observed 24 hr after treatment. Data in A and B represent the mean ± standard deviation of one representative experiment performed in triplicate.

Results

Inhibition of cell adhesion and growth after electroporation with POPS antisense c-*myc* ODNs. U87MG cells were electroporated with various POPS antisense ODNs (AS1, AS2, AS3) targeted against c-*myc* mRNA (Fig. 1). In addition, a scramble ODN (SCR1), a three-base mismatched antisense (ASM1), and a sense sequence (SE1) were tested. Both cell adhesion as a function of time and cell growth were monitored. As seen in Fig. 2A, we observed that U87MG cells electroporated with 20 μM c-*myc* antisense ODNs (AS1, AS3) lost their ability to adhere onto plastic, as monitored 21 hr after treatment. The effects of AS1 on adhesion could be observed as early as 3 hr after electroporation (data not shown). The total number of viable cells (adherent plus nonadherent) was not altered by antisense treatment compared with control (data not shown). AS2 was a less-efficient inhibitor of adhesion, with 55% of cells adherent after 21 hr. At 70 hr after electroporation, only 30% of viable cells treated with AS1 were adherent, whereas at the same time period, 70% and 90% of cells treated with AS2 and AS3, respectively, were adherent (Fig. 2A).

We also monitored the effect of these different ODNs on the growth of U87MG cells (Fig. 2B). As seen with respect to cell adhesion, the three antisense ODNs could be ranked in terms of growth inhibition as AS1 > AS3 > AS2. At 70 hr after electroporation, cell growth was still completely inhibited by AS1. No effect on U87MG growth was observed with any of the three control ODNs tested (ASM1, SCR1, SE1).

In addition to inhibition of cell growth and adhesion, a marked change in morphology could be observed in AS1- and AS3-treated cells (Fig. 2C). Antisense-treated cells were more spherical and formed clumps. This change in cell morphology was not observed with any of the control ODNs or with AS2.

To determine whether the effects seen on cell adhesion and growth were related to the presence of a dG quartet in the active sequences (AS1, AS3), we tested control sequences (ASM2, SCR2) retaining the same dG quartet. As seen in Fig. 3A, both ASM2 and SCR2 were only moderate inhibitors of cell proliferation (30%) and adhesion (20–40%), and they had no effect on cell morphology (Fig. 3B).

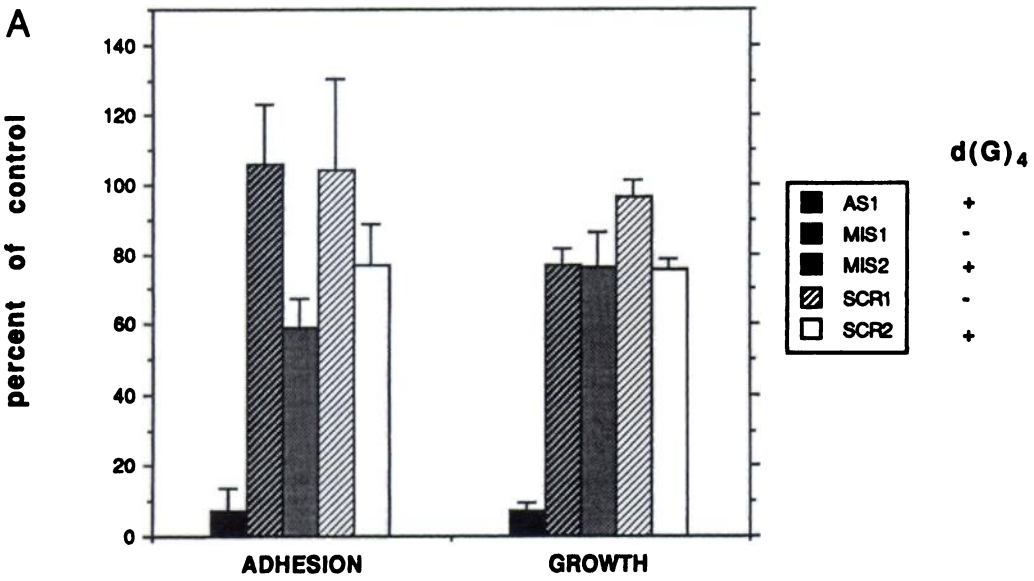
The effect on cell adhesion and growth induced by AS1 was dose dependent (Fig. 4), with an EC_{50} of 5 μM. Neither the inverse of AS1 (INV1) nor a 21-mer containing the motif GAGGGG as in AS1 (GA[G_4]) showed any effects on cell adhesion or growth when tested at 20 μM (Fig. 4).

Effect of c-*myc* antisense on c-*myc* protein expression. Expression of c-*myc* protein and a control protein, PDGFRα, after electroporation with ODNs was monitored. AS1 (20 μM) significantly reduced c-*myc* protein when measured 24 hr after treatment (50% of control, Table 1, treatment A). Similar to their effects on growth and adhesion, the three antisense ODNs could be ranked in terms of potency of c-*myc* inhibition as AS1 > AS3 > AS2. Inhibition of c-*myc* expression by AS1 was dose dependent, with an EC_{50} of 10 μM (Table 1, treatment C). At this concentration, no inhibition of PDGFRα protein was observed. Nevertheless, after treatment with 30 μM AS1, a 32% reduction in PDGFRα was observed, indicating the possibility of nonspecific effects of AS1 at higher concentrations. Neither c-*myc* nor PDGFRα protein was significantly inhibited by any of the control sequences (Table 1, treatments A and B).

Involvement of extracellular matrix proteins in c-*myc* antisense inhibition of cell adhesion but not in inhibition of cell growth. We wanted to determine whether AS1-induced loss of cell adhesion and growth inhibition were two independent phenomena. Because adherent cells produce and secrete their own extracellular matrix components, we hypothesized that treatment with AS1 might be interfering, either specifically or nonspecifically, with this process. To test this hypothesis, we precoated culture dishes with fibronectin (1 $\mu g/cm^2$) and tested the effects of AS1 after the placement of electroporated cells in these dishes. When cells electroporated with AS1 (20 μM) were plated onto fibronectin-coated dishes, the effects of AS1 on cell adhesion were totally abolished (data not shown), although cell growth was still completely inhibited (Fig. 5A). These data demonstrate that AS1 effects on adhesion and cell growth are clearly separable. Although exogenous fibronectin could overcome the adhesion defect caused by AS1, synthesis and secretion of fibronectin by AS1-treated U87MG cells, as determined by fibronectin immunoprecipitation after a 2-hr pulse with [^{35}S]methionine, was normal (data not shown). These data suggest that the effects seen with AS1 on cell adhesion might be associated with perturbation of fibronectin interaction with its cellular receptor, plastic substratum, or both.

Involvement of extracellular ODN in inhibition of cell adhesion but not growth inhibition. When AS1-electroporated cells were centrifuged and resuspended in medium before plating to remove noninternalized ODNs, effects on adhesion but not on growth inhibition were reversed (Fig. 5B). Moreover, the simple addition of ODNs to cells before plating was able to produce a similar loss in cell adhesion without affecting cell growth (result not shown). These results, combined with those discussed, show that the reduction caused by AS1 is mediated by extracellular ODNs, whereas growth inhibition is mediated by intracellular ODNs.

Effect of backbone and PS content on loss of cell adhesion and growth inhibition caused by c-*myc* antisense ODNs. To know whether the ODN backbone contributes to the effects observed on cell adhesion and growth, we compared the effects of AS1 and SCR2 sequences synthesized as either complete POs, complete PSs, POPS, or as complete PAs. As seen in Fig. 6, both PS and POPS AS1 had a dramatic effect on cell adhesion, whereas PA and PO AS1 were ineffective. The control sequence containing a dG quartet (SCR2) inhibited cell adhesion and growth as well as AS1 in its PS but not in its POPS form. The effect on cell adhesion and growth induced by fully thioated ODNs was also dose dependent (Fig. 7). EC_{50} values for AS1 and SCR2 were 5 and 15 μM for growth and 2 and 6 μM for adhesion, respectively. These results show that the SCR2 sequence, in its fully PS version, is only one third less inhibitory than the AS1 sequence. Moreover, a third sequence, which did not contain a dG quartet (SCR1), was almost as inhibitory to growth and adhesion as SCR2 and AS1. These results indicate that ODN effects on cell adhesion and growth are complex but are strongly dependent on PS content.



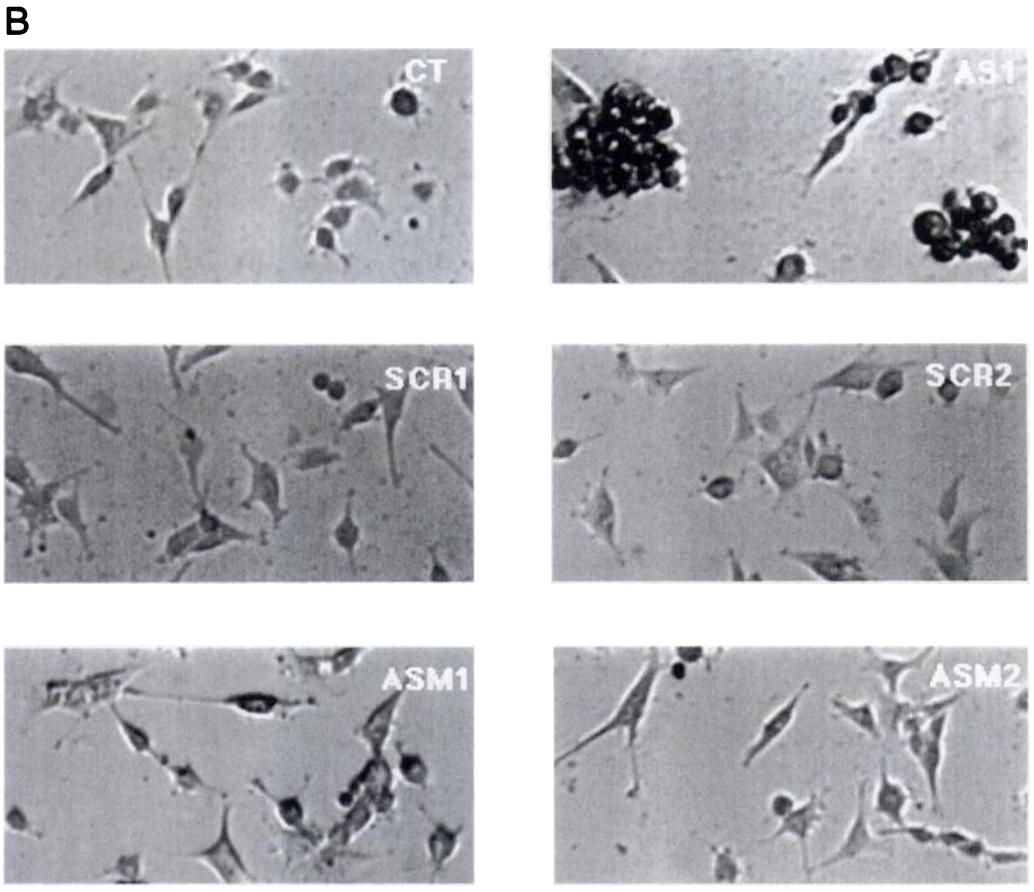


Fig. 3. Relative cell adhesion and growth after electroporation with antisense c-*myc* ODNs or control sequences containing a dG quartet. U87MG cells were electroporated in absence or presence of 20 μM POPS ODNs containing (*AS1, MIS2, SCR2*) or not containing (*MIS1, SCR1*) a dG quartet. A, Relative adhesion and cell growth were determined as in Fig. 2, 4 and 72 hr, respectively, after electroporation. B, Cell morphology was observed 24 hr after electroporation. Data in A represent the mean ± standard deviation of results pooled from two independent experiments, each done in triplicate.

Discussion

The c-*myc* gene, identified as the cellular homologue of the v-*myc* oncogene found in several transforming retroviruses, is expressed at elevated levels in a variety of tumors, indicating that its deregulation may be an important component of carcinogenesis (20). Moreover, c-*myc* appears to be an essential gene involved in the regulation of proliferation, mitogenesis, differentiation, and programmed cell death (21, 22). Inhibition of c-*myc* expression by a pentadecamer antisense PO oligomer targeted against the translation start site of human c-*myc* mRNA was found to inhibit mitogen-stimulated human peripheral blood lymphocytes from entering S phase (23). The same oligomer was also able to inhibit the growth of the human promyelocytic leukemia cell line HL-60 while inducing granulocytic differentiation of these cells (24–26). Using chemically modified versions of this oligomer (PS or PA), others have shown specific inhibition of vascular smooth muscle cell proliferation and c-*myc* protein expression both *in vitro* and *in vivo* (3, 27, 28). In our laboratory, we

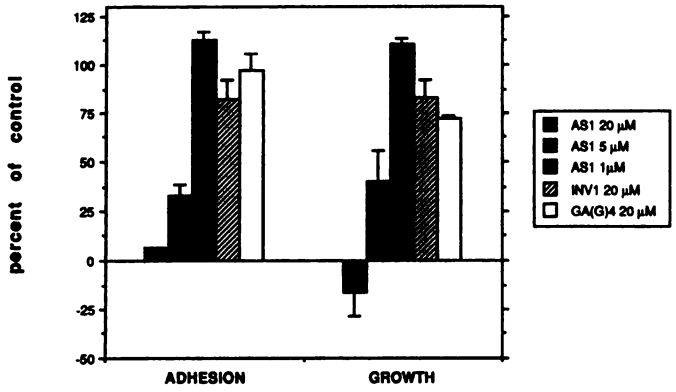


Fig. 4. Dose-dependent inhibition of cell adhesion and growth after electroporation with POPS AS1. U87MG cells were electroporated with 1, 5, or 20 μM POPS AS1 or with 20 μM of either the inverse sequence (*INV 1*) or a 21-mer containing the motif GAGGGG (*GA[G]4*). Relative cell adhesion and growth were determined 4 or 72 hr, respectively, after electroporation. Data represent the mean ± standard deviation of results pooled from two independent experiments, each done in triplicate.

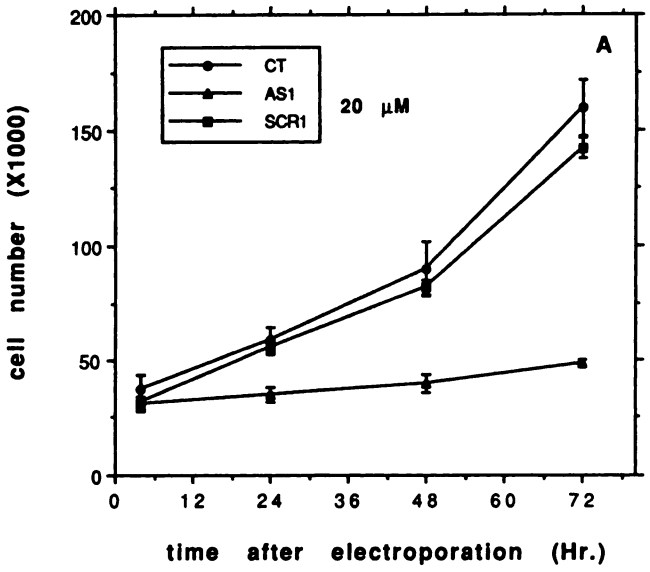
TABLE 1

Dose-dependent inhibition of c-*myc* protein synthesis after electroporation with capped c-*myc* antisense ODNs

U87MG cells were electroporated with 20 μM capped antisense c-*myc* ODNs (AS1, AS2, and AS3) or control sequences containing (SCR2, ASM2) or not containing (SCR1, ASM1) a dG quartet (A and B). Dose-dependent inhibition of c-*myc* protein with AS1 (C) 24 hr after electroporation cells were lysed in TNESV and identical amounts of protein were loaded on a sodium dodecyl sulfate-polyacrylamide gel. The immunodetection of c-*myc* or PDGFRα proteins was performed as described in Materials and Methods.

Treatment	$d(G)_4$	PDGFRα	C-*myc*
		Proteinsynthesis(%control)	
A			
AS1	+	80	49.8
AS2	−	95	86.8
AS3	+	112	60.4
SCR1	−	94	86.8
ASM1	−	92	95
B			
AS1	+	ND	50
ASM2	+	ND	115
SCR2	+	ND	103
C			
AS1 = 5 μM	+	112	67.6
AS1 = 15 μM	+	82	34.2
AS1 = 30 μM	+	68	35

ND = not done.



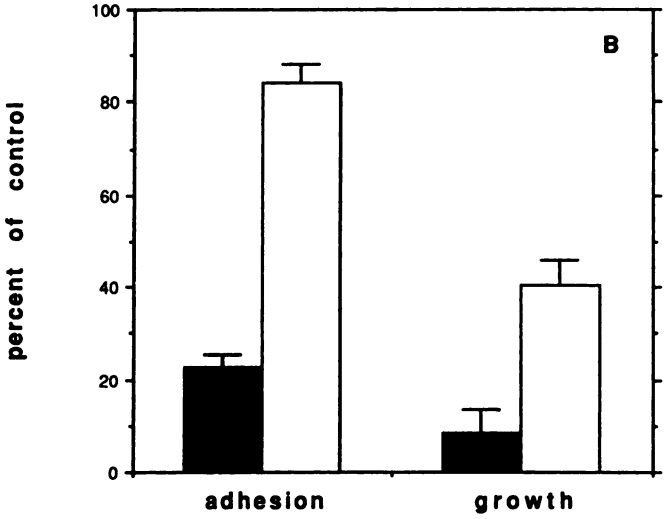


Fig. 5. Addition of exogenous fibronectin or removal of extracellular ODN after electroporation reverses inhibition of cell adhesion but does not reverse the cell growth inhibition obtained with POPS AS1. A, U87MG cells were electroporated in the absence (*CT*) or presence of 20 μM AS1 or SCR1 and plated into 24-well plates previously coated with fibronectin (1 μg/ml). At various times after electroporation, viable adherent and nonadherent cells were counted as described in Fig. 2. The loss of cell adhesion induced by AS1 was completely reversed by treating culture dishes with fibronectin. B, After electroporation with 20 μM AS1, extracellular ODN was removed (□) or not removed (■) by centrifugation and resuspension of cells in ODN-free medium before plating. Relative cell adhesion and growth were determined 4 or 72 hr, respectively, after electroporation. Data represent mean ± standard deviation of one representative experiment done in triplicate.

have shown c-*myc* antisense ODN, delivered to the human monocytic leukemia cell line U937 by electroporation, to specifically inhibit c-*myc* protein expression and *in vivo* tumorigenesis (29).[1]

The same antisense oligomer was also able to inhibit c-*myc* expression and proliferation of human breast cancer lines MCF-7 and MDA-MB-231 (30). Surprisingly, when these authors delivered the ODN to MCF-7 by electroporation, they observed no effect on cell growth and c-*myc* protein expression but rather found that cell adhesion was dramatically reduced (14). PS ODNs have been reported to cause a number of nonspecific effects, depending on their ability to bind to both proteins (5–9, 31) and drugs (11). Others have attempted to avoid this problem by the use of alternative chemical modifications (32) or by reducing the phosphorothioate content of ODNs (i.e., POPS copolymers) (19).

In the present study, we used a POPS c-*myc* oligomer containing 53% PS linkages distributed on both 5′ and 3′ ends and used electroporation as method of cellular delivery. Among the three POPS antisense oligomers tested, AS1, which has been used before by others (23–26), was the most effective. A dose-dependent inhibition of cell growth and c-*myc* protein expression was obtained with an EC_{50} value of

[1] R. Bergan, Kyle, E., Schwartz, G. N., Hakim, F., Fowler, D., Cepada, R., Szabo, J. M., Gress, R., and Neckers, L. Electroporation of synthetic oligodeoxynucleotides: a novel technique for *ex vivo* bone marrow purging, submitted for publication.

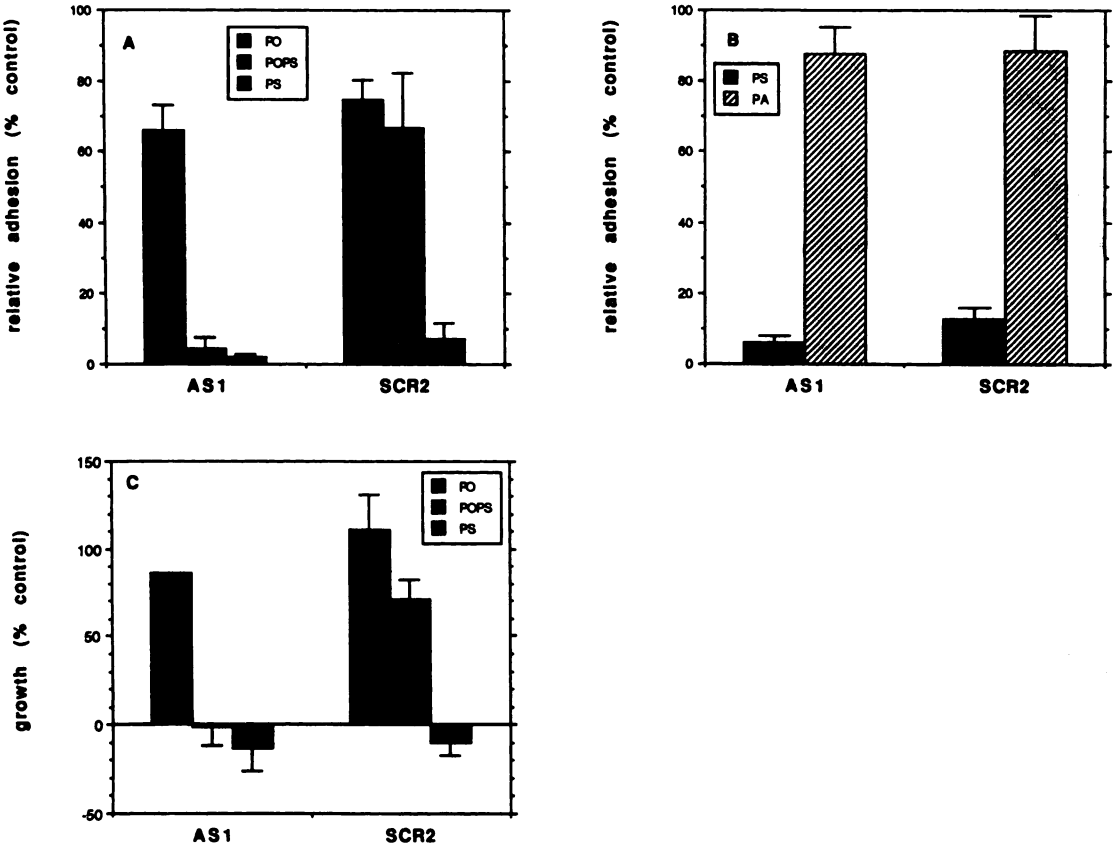


Fig. 6. Effect of ODN backbone and PS content on cell adhesion and growth. U87MG cells were electroporated in the absence or presence of 20 μM AS1 or a control sequence (*SCR2*) containing a PO, POPS, PS, or PA backbone. A and B, Relative cell adhesion onto plastic determined 4 hr after treatment; C, cell growth determined 72 hr after treatment. Data represent mean ± standard deviation of results pooled from two independent experiments, each done in triplicate.

5–10 μM (Fig. 4, Table 1). At the concentration of 15–20 μM necessary for maximal effect on c-*myc* level, the synthesis of a control protein, PDGFRα, was only slightly affected (Table 1). Moreover, none of the control sequences tested, either containing (SCR2, ASM2, INV1, GA(G)$_4$) or not containing (SCR1, SE1, ASM1) a dG quartet, altered either growth or c-*myc* protein expression. These results demonstrate the specificity of AS1 when used in its POPS form. Nevertheless, at the concentration of 30 μM, a 30% reduction of PDGFRα was observed, indicating the possibility of nonspecific effects of AS1 at higher concentration. Because the proto-oncogene c-*myc* is involved in regulation of gene expression at both a transcriptional and posttranscriptional level (33, 34), it is also possible that down-regulation of c-*myc* expression may affect synthesis of other proteins, such as PDGFRα.

In addition to inhibiting cell growth and c-*myc* protein expression, a dose-dependent effect on cell adhesion and cell morphology was also observed after administration of POPS antisense ODNs but not with any of the control POPS sequences tested. This effect could be duplicated when cells were mixed with the ODNs before plating (result not shown) and was reversed when treated cells were either plated onto plastic dishes coated with fibronectin or washed before plating to remove noninternalized ODNs (Fig. 5). Watson *et al.* (14) have reported similar findings using fibronectin to reverse loss of cell adhesion after electroporation. These results demonstrate, on the one hand, that loss of cell adhesion and growth inhibition are independent phenomena and, on the other hand, that the former is due to an extracellular ODN effect. Because fibronectin synthesis and secretion were not affected by AS1, our results suggest a direct interaction of POPS AS1 with membrane ECM receptors, such as β-integrin. Alternatively, Shi *et al.* (3) observed that AS1 caused a marked decrease in type I collagen synthesis in smooth muscle cells. The reduction of collagen synthesis by the c-*myc* antisense ODN was seen in density-arrested cells (confluent) and was therefore independent of any growth-inhibitory effects after antisense treatment. However, because the reduction of cell adhesion in our experiments appears to be an externally mediated event, it is unlikely to be related directly to c-*myc* down-regulation by antisense ODNs. Rather, the sequence-specific effects of AS1 on cell adhesion appear to represent another example of a direct sequence-dependent interaction of ODN with protein.

The inhibition of adhesion and growth by the ODNs used in the present study is particularly complex, appearing to be dependent not only on sequence but also on the amount of PS content. When synthesized with either a nuclease-sensitive PO or nuclease-resistant PA backbone, AS1 was markedly less able to affect adhesion (Fig. 6). Increasing the PS content of AS1 to 53% or more resulted in inhibition of adhesion; however, for POPS ODNs, the AS1 sequence was the only one able to affect adhesion and growth. When PS content is increased to 100%, scrambled sequences containing (SCR2) or not containing (SCR1) a dG quartet become equally inhib-

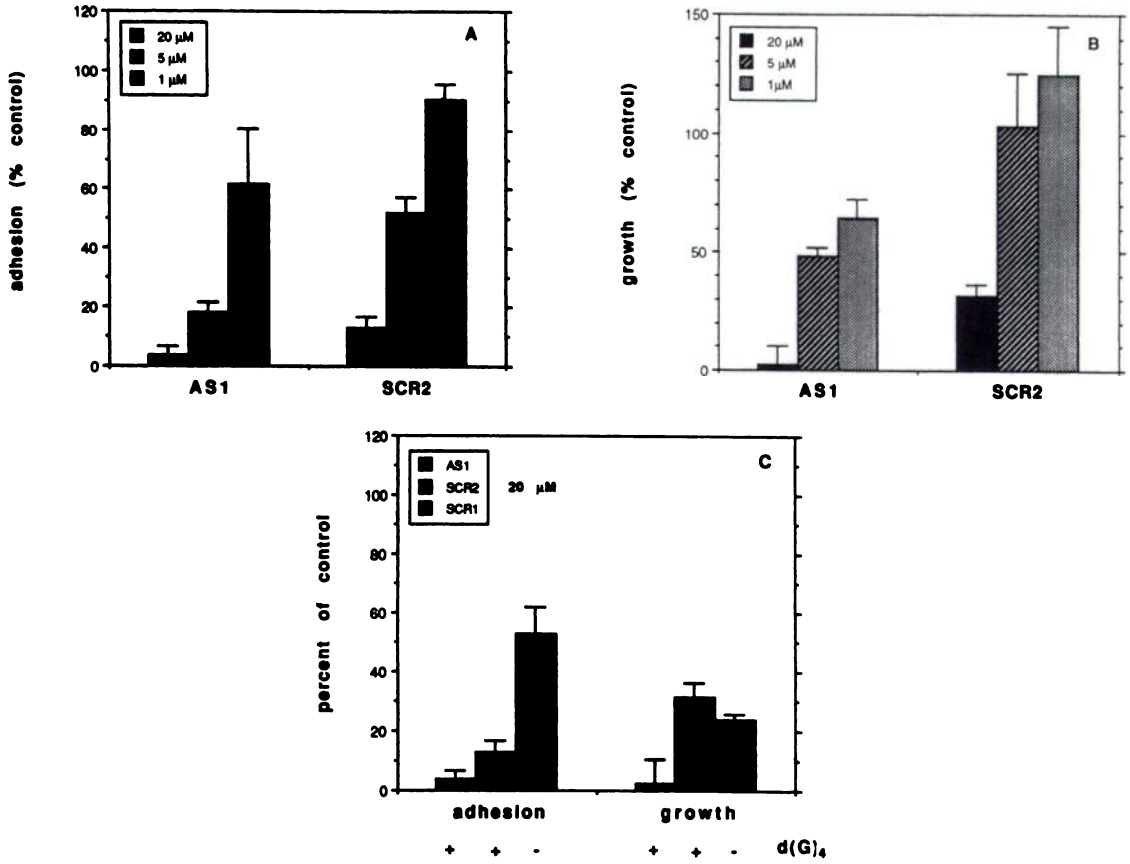


Fig. 7. Inhibition of cell growth and adhesion by PS c-*myc* ODN is dose dependent but not dG quartet dependent. U87MG cells were electroporated with 1, 5, or 20 μM PS AS1 or SCR2 (A and B) or with 20 μM PS ODNs containing (*AS1, SCR2*) or not containing (*SCR1*) a dG quartet (C). Relative cell adhesion and growth were determined 4 or 72 hr, respectively, after electroporation. Data represent the mean ± standard deviation of one representative experiment done in triplicate.

itory to cell growth and adhesion as AS1 (Figs. 6 and 7). Thus, at 100% PS content, sequence specificity is lost.

The 15-mer c-*myc* antisense ODN has often been used in the past with success to inhibit c-*myc* expression without any apparent toxic effect. These studies used mainly nonadherent cell lines, such as human promyelocytic leukemia cells or human T lymphocytes (23, 24, 26), and unmodified (PO) ODNs (4–30 μM) added directly to cell suspensions. In our laboratory, the same c-*myc* antisense ODN was synthesized with a complete PS backbone to increase nuclease resistance. We had previously used such an ODN to inhibit c-*myc* expression in human histiocytic lymphoma cells, with electroporation used as a delivery system (29). Specific inhibition of c-*myc* protein synthesis and cell viability were observed with AS1 at concentrations at which a scrambled sequence lacking a dG quartet was not inhibitory. Because these cells grow in suspension, the possible loss of cell adhesion after electroporation was not investigated. Previously, Watson *et al.* (14) observed a dramatic reduction in MCF7 breast tumor cell line adhesion after electroporation but at a lower concentration of fully thioated c-*myc* antisense ODNs (2.5 μM). This was obtained without inhibition of cell growth or c-*myc* protein synthesis. In addition, contrary to our findings, sense or irrelevant sequences had no effect on these parameters. These results stress the variety of toxic effects that can be observed with the same thioated sequence depending on the cell line used. To further complicate matters, we saw no involvement of dG quartets in either growth inhibition or loss of adhesion. This is in contrast to results reported by Yaswen *et al.* (13). As these authors found, "the dG effect" on growth also appears to be cell type dependent, with normal mammary epithelial cells being more sensitive than immortalized or normal breast fibroblasts.

In conclusion, our findings demonstrate that a commonly used c-*myc* antisense ODN must be used with caution because, depending on its backbone composition, it can cause multiple effects in certain cells. These effects include those mediated by sequence-specific antisense inhibition, sequence-specific nonantisense (aptameric) inhibition, and nonsequence-specific, nonantisense inhibition. Furthermore, one should certainly be conservative in the interpretation of the biological effects of such antisense ODNs.

References

1. Higgins, K. A., Perez, J. R., Coleman, T. A., Dorshkind, K., McComas, W. A., Sarmiento, U. M., Rosen, C. A., and Narayanan, R. Antisense inhibition of the p65 subunit of NF-kB blocks tumorigenicity and causes tumor regression. *Proc. Natl. Acad. Sci. USA* **90**:9901–9905 (1993).
2. Bennett, M. R., Anglin, S., McEwan, J. R., Jagoe, R., Newby, A. C., and Evan, G. I. Inhibition of vascular smooth muscle cell proliferation *in vitro* and *in vivo* by c-*myc* antisense oligodeoxynucleotides. *J. Clin. Invest.* **93**:820–828 (1994).
3. Shi, Y., Fard, A., Galeo, A., Hutchinson, H. G., Vermani, P., Dodge, G. R., Hall, D. J., Shaheen, F., and Zalewski, A. Transcatheter delivery of c-*myc* antisense oligomers reduces neointimal formation in a porcine model of coronary artery balloon injury. *Circulation* **90**:944–951 (1994).
4. Agrawal, S., Temsamani, J., and Tang, J. Y. Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice. *Proc. Natl. Acad. Sci. USA* **88**:7595–7599 (1991).
5. Majundar, C., Stein, C. A., Cohen, J. S., Broder, S., and Wilson, S. H.

Stepwise mechanism of HIV reverse transcriptase: primer function of phosphorothioate oligodeoxynucleotide. *Biochemistry* **28**:1340–1346 (1989).
6. Stein, C. A., Neckers, L. M., Nair, B. C., Mumbauer, S., Hoke, G., and Pal, R. Phosphorothioate oligodeoxycytidine interferes with binding of HIV-1 gp120 to CD4. *J. Acquired Immune Defic. Syndr.* **4**:686–693 (1991).
7. Gao, W. Y., Han, F. S., Storm, C., Egan, W., and Cheng, Y. C. Phosphorothioate oligonucleotides are inhibitors of human DNA polymerases and RNase H: implications for antisense technology. *Mol. Pharmacol.* **41**:223–229 (1992).
8. Yakubov, L., Khaled, Z., Zhang, L., Truneh, A., Vlassov, V., and Stein, C. A. Oligodeoxynucleotides interact with recombinant CD4 at multiple sites. *J. Biol. Chem.* **268**:18818–18823 (1993).
9. Bergan, R., Connell, Y., Fahmy, B., Kyle, E., and Neckers, L. M. Aptameric inhibition of $p210^{bcr\text{-}abl}$ tyrosine kinase autophosphorylation by oligodeoxynucleotides of defined sequence and backbone structure. *Nucleic Acids Res.* **22**:2150–2154 (1994).
10. Perez, J. R., Li, Y., Stein, C. A., Majumder, S., van Oorschot, A., and Narayanan, R. Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides. *Proc. Natl. Acad. Sci. USA* **91**:5957–5961 (1994).
11. Blagosklonny, M. V., and Neckers, L. M. Oligonucleotides protect cells from the cytotoxicity of several anticancer chemotherapeutic drugs. *Anticancer Drugs* **5**:437–442 (1994).
12. Koike, M., Ishino, K., Ikuta, T., Huh, N., and Kuroki, T. Growth enhancement of normal keratinocytes by the antisense oligonucleotide of the retinoblastoma susceptibility gene. *Oncogene* **10**:117–122 (1995).
13. Yaswen, P., Stampfer, M. R., Ghosh, K., and Cohen, J. S. Effects of sequence of thioated oligonucleotides on cultured human mammary epithelial cells. *Antisense Res. Dev.* **3**:67–77 (1993).
14. Watson, P. H., Pon, R. T., and Shiu, R. P. Inhibition of cell adhesion to plastic substratum by phosphorothioate oligonucleotide. *Exp. Cell Res.* **202**:391–397 (1992).
15. Trent, J., Meltzer, P., Rosenblum, M., Harsh, G., Kinzler, K., Mashal, R., Feinberg, A., and Vogelstein, B. Evidence for rearrangement, amplification, and expression of c-*myc* in human glioblastoma. *Proc. Natl. Acad. Sci. USA* **83**:470–473 (1986).
16. Akbasak, A., and Sunar-Akbasak, B. Oncogenes: cause or consequence in the development of glial tumors. *J. Neurol. Sci.* **111**:119–133 (1992).
17. Orian, J. M., Vasilopoulos, K., Yoshida, S., Kaye, A. H., Chow, C. W., and Gonzales, M. F. Overexpression of multiple oncogenes related to histological grade of astrocytic glioma. *Br. J. Cancer* **66**:106–112 (1992).
18. Hirvonen, H. E., Salonen, R., Sandberg, M. M., Vuorio, E., Vastrik, I., Kotilainen, E., and Kalimo, H. Differential expression of myc, max and RB1 genes in human gliomas and glioma cell lines. *Br. J. Cancer* **69**:16–25 (1994).
19. Ghosh, M. K., Ghosh, K., and Cohen, J. S. Phosphorothioate-phosphodiester oligonucleotide co-polymers: assessment for antisense application. *Anticancer Drug Des.* **8**:15–32 (1993).
20. Prins, J., Devries, E. G. E., and Mulder, N. H. The myc family of oncogenes and their presence and importance in small-cell lung carcinoma and other tumour types. *Anticancer Res.* **13**:1373–1385 (1993).
21. Hanson, K. D., Shichiri, M., Follansbee, M. R., and Sedivy, J. M. Effects of c-*myc* expression on cell cycle progression. *Mol. Cell Biol.* **14**:5748–5755 (1994).
22. Milner, A. E., Grand, R. J., Waters, C. M., and Gregory, C. D. Apoptosis in Burkitt lymphoma cells is driven by c-*myc*. *Oncogene* **8**:3385–3391 (1993).
23. Heikkila, R., Schwab, G., Wickstrom, E., Loke, S. L., Pluznik, D. H., Watt, R., and Neckers, L. M. A c-*myc* antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G0 to G1. *Nature (Lond.)* **328**:445–449 (1987).
24. Wickstrom, E. L., Bacon, T. A., Gonzales, A., Freeman, D. L., Lyman, G. H., and Wickstrom, E. Human promyelocytic leukemia HL-60 cell proliferation and c-*myc* protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against c-*myc* mRNA. *Proc. Natl. Acad. Sci. USA* **85**:1028–1032 (1988).
25. Wickstrom, E. L., Bacon, T. A., Gonzales, A., Lyman, G. H., and Wickstrom, E. Anti c-*myc* DNA oligomers increase differentiation and decrease colony formation by HL-60 cells. *In Vitro Cell Dev. Biol.* **25**:297–302 (1989).
26. Holt, J. T., Redner, R. L., and Nienhuis, A. W. An oligomer complementary to c-*myc* mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. *Mol. Cell. Biol.* **8**:963–973 (1988).
27. Shi, Y., Hutchinson, H. G., Hall, D. J., and Zalewski, A. Downregulation of c-*myc* expression by antisense oligonucleotides inhibits proliferation of human smooth muscle cells [see comments]. *Circulation* **88**:1190–1195 (1993).
28. Biro, S., Fu, Y. M., Yu, Z. X., and Epstein, S. E. Inhibitory effects of antisense oligodeoxynucleotides targeting c-*myc* mRNA on smooth muscle cell proliferation and migration. *Proc. Natl. Acad. Sci. USA* **90**:654–658 (1993).
29. Bergan, R., Connell, Y., Fahmy, B., and Neckers, L. Electroporation enhances c-*myc* antisense oligodeoxynucleotide efficacy. *Nucleic Acids Res.* **21**:3567–3573 (1993).
30. Watson, P. H., Pon, R. T., and Shiu, R. P. C. Inhibition of c-*myc* expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-*myc* in the growth of human breast cancer. *Cancer Res.* **51**:3996–4000 (1991).
31. Wyatt, J. R., Vickers, T. A., Roberson, J. L., Buckheit, R. W., Klimkait, T., Debaets, E., Davis, P. W., Rayner, B., Imbach, J. L., and Ecker, D. J. Combinatorially selected guanosine-quartet structure is a potent inhibitor of human immunodeficiency virus envelope-mediated cell fusion. *Proc. Natl. Acad. Sci. USA* **91**:1356–1360 (1994).
32. Uhlmann, E., and Peyman, A. Antisense oligonucleotides: a new therapeutic principle. *Chem. Rev.* **90**:543–583 (1990).
33. Kaddurah-Daouk, R., Greene, J. M., Baldwin, A. S., and Kingston, R. E. Activation and repression of mammalian gene expression by the c-*myc* protein. *Genes Dev.* **1**:347–357 (1987).
34. Prendergast, G. C., and Cole, M. D. Posttranscriptional regulation of cellular gene expression by the c-*myc* oncogene. *Mol. Cell. Biol.* **9**:124–134 (1989).

Send reprint requests to: Dr. Len Neckers, Clinical Pharmacology Branch, National Cancer Institute, National Institutes of Health, Building 10, Room 12N226, Bethesda, MD 20892.