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Oligonucleotide N3′→P5′ phosphoramidates as antisense agents

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ABSTRACT

Uniformly modified oligonucleotide N3′→P5′ phosphoramidates, where every 3′-oxygen is replaced by a 3′-amino group, were synthesized. These compounds have very high affinity to single-stranded RNAs and thus have potential utility as antisense agents. As was shown in this study, the oligonucleotide phosphoramidates are resistant to digestion with snake venom phosphodiesterase, to nuclease activity in a HeLa cell nuclear extract, or to nuclease activity in 50% human plasma, where no significant hydrolysis was observed after 8 h. These compounds were used in various *in vitro* cellular systems as antisense compounds addressed to different targeted regions of c-*myb*, c-*myc* and *bcr-abl* mRNAs. C-myb antisense phosphoramidates at 5 μM caused sequence and dose-dependent inhibition of HL-60 cell proliferation and a 75% reduction in c-*myb* protein and RNA levels, as determined by Western blot and RT–PCR analysis. Analogous results were observed for anti-*c-myc* phosphoramidates, where a complete cytostatic effect for HL-60 cells was observed at 1 μM concentration for fully complementary, but not for mismatched compounds, which were indistinguishable from untreated controls. This was correlated with a 93% reduction in c-*myc* protein level. Moreover, colony formation by the primary CML cells was also inhibited 75–95% and up to 99% by anti-*c-myc* and anti-*bcr-abl* phosphoramidate oligonucleotides, respectively, in a sequence- and dose-dependent manner within a 0.5 nM–5 μM dose range. At these concentrations the colony-forming ability of normal bone marrow cells was not affected. The presented *in vitro* data indicate that oligonucleotide N3′→P5′ phosphoramidates could be used as specific and efficient antisense agents.

INTRODUCTION

Synthetic oligonucleotides may become a new generation of rationally designed therapeutic agents, offering potentially general applicability and high target selectivity in action. Currently oligonucleotide phosphorothioates are the leading candidates for the first generation of antisense compounds and several of them are in phase I/II clinical trials (1–3). Unfortunately, phosphorothioates possess properties that may not always be suitable for antisense and/or antigene agents: mainly, low (relative to natural phosphodiesters) binding affinity to single-stranded (ss) RNA and especially to double-stranded (ds) DNA targets (4,5) and nucleotide sequence independent binding to a variety of intra- and extracellular proteins in mono- or multimeric forms (6–8). Thus, the search for better oligonucleotide analogues continues and new classes of compounds have been introduced. Among these are 2′-modified oligomers (9–11), chimeric methylphosphonate-phosphodiesters (12), 5-propynyl-pyrimidine containing compounds (13), phosphodithioates (14) and peptide nucleic acids (15).

Recently, a new type of oligonucleotide analogue, uniformly modified N3′→P5′ phosphoramidates, where a 3′-amino group was substituted for the 3′-oxygen of the 2′-deoxyribose ring, was synthesized (16). Physico-chemical studies of these compounds demonstrated that they form very stable duplexes with complementary ssRNA, as well as triplexes with dsDNA polypurine targets, and that they are resistant to nuclease catalyzed hydrolysis (16–18). These features of the oligonucleotide N3′→P5′ phosphoramidates prompted us to evaluate these compounds as potential antisense agents in different *in vitro* cell culture systems well characterized with other oligonucleotide analogs, using *bcr-abl*, c-*myc* and c-*myb* proteins as model targets. These three systems were chosen and studied to demonstrate the generality of antisense activity of the oligonucleotide phosphoramidates not in one but in several *in vitro* cell cultures. Results presented here demonstrate that N3′→P5′ phosphoramidates do act as sequence-specific and efficient antisense agents in the model systems studied.

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MATERIALS AND METHODS

General methods

Oligonucleotide N3′→P5′ phosphoramidates were synthesized on an ABI 394 automated synthesizer according to the described procedure (18). Compounds were analyzed and purified by ion exchange (IE) HPLC, on a Dionex DX300 chromatograph using Pharmacia MonoQ 5/5 or 10/10 columns, desalted by gel filtration on Pharmacia NAP-5 or NAP-10 columns and lyophilized *in vacuo* from sterile water according to ref. 18. Sequences of oligonucleotides synthesized and used in this study are presented in Table 1.

Enzymatic digestion experiments

Oligonucleotide **14** (Table 1) 0.2 OD units with phosphodiester or N3′→P5′ phosphoramidate internucleoside linkages was treated with 0.02 U snake venom phosphodiesterase and 0.8 U alkaline phosphatase (both from Sigma) in 0.2 ml 20 mM Tris–HCl buffer, pH 8.9. Reaction mixtures were analyzed by reversed phase (RP) HPLC on a Hypersil ODS 4.6 × 200 mm column using a 0.5%/min gradient of acetonitrile in 0.1 M triethylammonium acetate (TEAA) buffer, pH 7.0; time points were 0, 10 and 50 min, 2.5, 4.5 and 22 h for the phosphoramidate and only 0 and 10 min for the phosphodiester oligomers, since the latter compound was completely digested by that time.

To determine the stability of oligonucleotide phosphoramidates in human plasma or HeLa cell nuclear extract, 0.1 OD units compounds **12** and **13** (Table 1) were each 5′-^{32}P-labeled with [γ-^{32}P]ATP, 2 pmol in 20 µl 10 mM Tris–HCl, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, pH 8.5 buffer by 5 U T4 polynucleotide kinase (Amersham) for 3 h at 37°C. Then ~0.01 OD units labeled compound was incubated in 10 µl 50% human plasma or in 10 µl 50% HeLa nuclear extract at 37°C. Reaction mixtures were analyzed by electrophoresis in denaturing 20% polyacrylamide gel at time points 0, 1, 2, 4 and 8 h for human plasma and 0, 0.5, 1 and 2 h for HeLa nuclear extract.

Experiments to determine the binding affinity of oligonucleotide phosphodiesters and phosphoramidates to nuclear proteins derived from *tax*-activated fibroblasts were performed as reported before (7).

Bcr-abl antisense experiments

All *in vitro* antisense experiments were carried out in triplicate. Standard deviations for cell counts were usually within 10–15%. Averaged results are presented in all figures, and error bars are omitted for clarity.

The experiments were conducted in BV173, HL-60 or K562 cells as described (19). Clonogenic assays with primary CML patient cells and normal bone marrow cells were conducted as follows. Marrow cells were obtained by aspiration from the illiac crest of healthy individuals and CML patients after informed consent. Light density mononuclear cells were separated using a Histopaque-1077 (Sigma) density gradient. Marrow cells from healthy individuals were enriched for hematopoietic progenitors after removing adherent cells and T lymphocytes (A-T-NBMC) as described (20). Cells were incubated with ODNs as described (21). Briefly, 10^5 cells in 0.4 ml of Iscove's modified Dulbecco medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), L-glutamine (Gibco–BRL) and penicillin/streptomycin (Gibco–BRL), were cultured in 24-well plates with the indicated concentrations of phosphorothioate or phosphoramidate oligodeoxynucleotides (see legend to Fig. 5) and 10 U/ml of recombinant human interleukin-3 (IL-3, Genetics Institute). The second and the third dose of ODNs (50% of the first dose) were added after 24 and 48 h of culture. After 120 h in culture, cells were plated in MethodCult H4230 (Stem Cell Technologies) semisolid medium as described (20), and colonies were counted 9–12 days later.

RT–PCR amplification of *bcr-abl* mRNA and hybridization with a specific ^{32}P-labeled 18mer 5′-GAAGGGCTTCTTCCTTAT-3′ probe was carried out as described (19). The *bcr-abl* specific primers were located at 3273–3294 (*bcr* portion) and 431–452 (c-*abl* portion) nucleotide positions for 5′- and 3′-primers, respectively. The amplified fragment was 257 bp long.

C-*myb* and c-*myc* antisense experiments

The HL-60 acute promyelocytic leukemia cell line used for antisense experiments was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Sigma), L-glutamine and penicillin/streptomycin (Gibco-BRL). During the *in vitro* treatment with the ODNs, the cells were cultured in the same complete medium supplemented with 10% heat-inactivated fetal bovine serum.

To assess the *in vitro* dose–response of HL-60 cells to anti-*c-myb* ODNs, exponentially growing cells were seeded in 96-well microtiter plates (Falcon) at an initial concentration of 1×10^4/well/100 µl in complete medium. At the beginning of the culture (on day 0) ODNs were added at the following concentrations: 3, 6 or 12 µM for the phosphorothioates and 0.5, 1, 2 or 5 µM for the 15mer phosphoramidates, Table 1. Number of cells and viability (trypan blue exclusion test) were determined daily until day 6.

The effect of c-*myb* ODNs on c-*myb* expression was studied at both the mRNA and protein level. For mRNA level studies, HL-60 cells were plated in 48-well multiplates (Falcon) at an initial concentration of 2×10^5/ml in complete medium. On the same day, the cells were treated with c-*myb* ODNs at 12 µM for the 24mer phosphorothioates and at 5 µM for the 15mer phosphoramidates. After 48 and 72 h from the beginning of the treatment, samples were collected by centrifugation and washed with PBS. Total RNA was extracted in the presence of 10 µg *Escherichia coli* ribosomal RNA by the acid guanidinium thiocyanate–phenol–chloroform method. The RNA from each sample was then analyzed by RT–PCR. For protein level studies, the cells were plated in 6-well multiplates (Falcon) at 2.5×10^5/ml in complete medium. On the same day, the cells were treated with anti-*c-myb* 24mer phosphorothioates at 15 µM and 15mer phosphoramidate at 2 or 5 µM. After 48 and 72 h from the beginning of the treatment, the samples were collected by centrifugation, washed with ice-cold PBS and solubilized in lysis buffer (10 mM HEPES, pH 7.5; 150 mM NaCl; 1% NP-40; 10% glycerol; 10 µg/ml each leupeptin and aprotinin: 1 mM phenylmethylsulfonyl fluoride; 1 mM $NaVO_4$; 5 mM EDTA). Total lysates were frozen at –80°C, thawed at 37°C, centrifuged at 11 000 r.p.m. at 4°C for 15 min, and postnuclear supernatants were then fractionated by SDS–PAGE.

RT–PCR analysis of c-*myb* mRNA was carried out as described (22).

Table 1. Oligonucleotide N3′→P5′ phosphoramidate sequences and their targets

No.	Oligonucleotide[a]	Target	Type[b]
1	CTTCTTCCTTA	*bcr-abl*	AS
2	**T**T**CTC**TC**TC**TA	*bcr-abl*	MM
3	AACGTTGAGGGGCAT	c-*myc*	AS
4	AACG**AGTT**GGGGCAT	c-*myc*	MM
5	TTTCATTGTTTTCCA	c-*myc*	AS
6	TTTCT**A**T**TG**TTTCCA	c-*myc*	MM
7	GTGCCGGGGTCTTCG	c-*myb*	AS
8	GT**C**C**T**GGGGTC**G**TCG	c-*myb*	MM
9	GTGC**G**GG**T**G**C**CTTCG	c-*myb*	MM
10	TATGCTGTGCCGGGGTCTTCGGGC[c]	c-*myb*	AS
11	GCCCGAAGACCCCGGCACAGCATA[c]	c-*myb*	S
12	CCACCGGGTCCAC	*bcr*	AS
13	TTGGGGTT[d]	–	SCR
14	TTTTTTTTTT	–	SCR
15	TGTGGGATTTTCCCAT[e]	–	SCR
16	ATGGGAAAATCCCACA	–	SCR

[a]Oligonucleotide sequences are 5′ to 3′.
[b]AS, S, MM and SCR are abbreviations for antisense, sense, mismatched and scrambled oligomers, respectively.
[c]These oligonucleotides were used only as phosphorothioates.
[d]This compound binds to gp120 HIV protein, see refs 35 and 36.
[e]Oligomers **15** and **16** are complementary to each other and contain the recognition site for NF-kB transcription factor, see ref. 7.

SDS–PAGE and Western blotting

The protein lysates from each sample were fractionated by 8.5% SDS–PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell). The membrane was saturated in PBS containing 5% non-fat dry-milk/0.5% Tween-20 at room temperature for 2 h and incubated for 12 h at 4°C with a monoclonal anti-mouse c-*myb* antibody (UBI) used at a concentration of 1 µg/ml in TBS (Tris-buffered saline) containing 0.1% gelatin and 0.01% NaN_3. After washing five times with 0.125% Tween-20, 0.125% NP-40 in TBS, the filter was incubated for 1 h at room temperature with sheep anti-mouse IgG conjugated to horseradish peroxidase (Amersham) and then washed again five times as described above. Bound proteins were detected by using the ECL Western blotting detection system (Amersham) according to the manufacturer's instructions. After stripping, the filter was blotted with human heat shock protein (HSP 72/73) monoclonal antibody (Oncogene Science Inc.) as a control for loaded protein amounts.

Antisense experiments in HL-60 cells with c-*myc* as a target and subsequent Western blot analysis were conducted analogously to those for c-*myb*; experiments in primary CML patient cells were done as described above for the anti-*bcr-abl* oligomers.

RESULTS AND DISCUSSION

Hydrolytic stability and protein binding of the oligonucleotide N3′→P5′ phosphoramidates

Oligonucleotide N3′→P5′ phosphoramidates used in this study and their targets are listed in Table 1.

First, stability of oligonucleotide phosphoramidates toward hydrolysis with phosphodiesterases was evaluated. The decathymidilic acid **14** containing natural phosphodiester and N3′→P5′ phosphoramidate linkages were treated with a mixture of snake venom phosphodiesterase and alkaline phosphatase and the course of hydrolysis was monitored by RP–HPLC (see Materials and Methods). Chromatographic analysis showed that the phosphodiester compound was completely hydrolyzed in 10 min and thymidine was formed as the sole product of digestion. In contrast, phosphoramidate **14** (Table 1) was practically intact after 10 min of reaction. After 4.5 h, ~50% of the phosphoramidate still remained intact and mainly the 9mer with a terminal 3′-amino group and thymidine were formed. Complete transformation of the starting 10mer to shorter products, mainly 7-, 8- and 9mers was observed after 22 h (data not shown). Additionally, 5′-^{32}P-labeled phosphoramidates **12** and **13** (Table 1) were completely resistant to 3′-exonuclease hydrolytic activity in 50% human plasma after 8 h of exposure, or in 50% HeLa cell nuclear extract after 2 h, as judged by electrophoretic analysis of the reaction mixture in 20% polyacrylamide gel (data not shown). These experiments demonstrate that oligonucleotide N3′→P5′ phosphoramidates are stable toward hydrolysis with nucleases and can be used for *in vitro* and *in vivo* experiments.

One of the main features of the oligonucleotide phosphorothioates and to some extent of phosphodiesters is non-sequence specific binding to a variety of proteins, which is determined by the nature of their sugar–phosphate backbones (6–8). This binding may lead to unexpected effects of antisense oligonucleotides as well as to a reduction of the effective concentrations of oligonucleotides available to hybridize with the intended mRNA target. We studied the ability of oligonucleotide phosphoramidates to associate with proteins in comparison with isosequential phosphodiesters using a gel shift analysis as described before (7). Nuclear extract from *tax* activated fibroblasts was incubated with 5′-^{32}P-labeled phosphoramidate oligonucleotides **15** and **16** and their phosphodiester counterparts (Table 1) and then the products of nucleic acid–protein interactions were analyzed by gel electrophoresis. As shown in Figure 1, under identical conditions single-stranded phosphodiester oligonucleotides do bind to some nuclear proteins, whereas isosequential phosphoramidates do not.

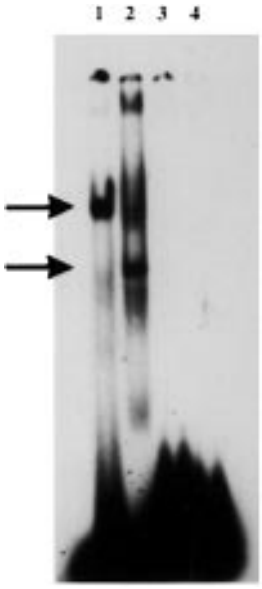

Figure 1. Gel-shift analysis under non-denaturing conditions of the products of oligonucleotide–protein interactions from *tax*-activated fibroblast nuclear extract. Experiments were conducted as described in ref. 7; lanes 1 and 2 correspond to phosphodiesters and lanes 3 and 4 correspond to isosequential phosphoramidates **15** and **16** (Table 1), respectively. Major products of the phosphodiester oligonucleotide–protein interactions are marked by arrows.

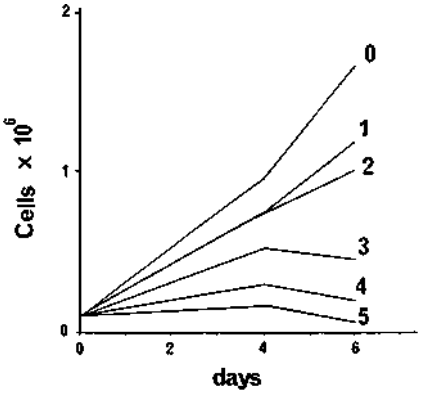

Figure 2. Cellular proliferation curves for BV173 cells treated with oligonucleotide phosphoramidate **1**, Table 1. Curve '0' corresponds to the untreated control cells, and curves '1' through '5' correspond to oligonucleotide concentrations of 1.4, 2.8, 5.6, 11.2 and 22.4 nM, respectively.

Similar results were previously reported (23), where phosphoramidates **3** and **4** (Table 1) but not isosequential phosphorothioates did not seemingly associate with extracellular proteins in U87 human glioblastoma cells. The observed lack of binding to nuclear proteins, which may render oligonucleotide phosphoramidates more available for binding with the mRNA target, could be caused by several important differences between phosphodiester and phosphoramidate compounds: substitution of the 3′-oxygen atom by a 3′-amino group leads to a change in the sugar–phosphate backbone rigidity (18); geometry of the nucleoside sugar rings, where phosphodiester and phosphoramidate linked compounds exist predominantly in S- and N- conformations, respectively (17,24); a different formal negative charge on the phosphate groups resulting in a change in their hydration pattern (18).

Bcr-abl as an antisense target

To evaluate the antisense properties of oligonucleotide phosphoramidates, several *in vitro* model systems were selected. One of them was designed to study inhibition of expression of p210 tyrosine kinase, resulting from the 9/22 chromosomal translocation and associated with chronic myelogenous leukemia (CML) (25). Oligonucleotides **1** and **2** (Table 1) were synthesized to be fully or partially complementary to the junction point of the *bcr*(b2) and *abl*(a2) fragments of the *bcr/abl* mRNA. Thus, BV173 cells containing the b2/a2 translocation and expressing p210 tyrosine kinase were treated with oligonucleotide **1**, and a dose-dependent inhibition of cellular proliferation was observed (Fig. 2). In contrast, proliferation of K562 cells, containing the partially complementary (8/11 bp) b3/a2 junction, or HL-60 cells without any *bcr/abl* translocation, were not affected by this oligonucleotide (data not shown). The study was extended to experiments with primary bone marrow cells taken from CML patients (chronic phase) which contained translocated b2/a2 mRNA. In this clonogenic assay, oligonucleotide phosphoramidate **1** (Table 1) showed good target-specific inhibitory activity against colony formation by CML cells with an IC_{50} value 0.18 μM, but not against normal bone marrow cells, IC_{50} 3.0 μM, thus demonstrating a therapeutic index equal to 17 (Fig. 3C). In addition to the colony formation assay, the antisense effects of oligonucleotides were monitored by RT–PCR analysis of the b2/a2 mRNA level and were normalized to the internal reference β-actin mRNA; oligonucleotide phosphorothioates were also used as reference compounds. As demonstrated in Figure 3A and B, a significant reduction of the mRNA level correlated with the effective inhibition of colony formation observed in cells treated with antisense oligonucleotide **1**, but not with the mismatched control **2**.

The reduction of *bcr-abl* mRNA level observed by RT–PCR analysis cannot be readily explained by RNaseH mediated cleavage of the RNA strand due to the reported inability of this enzyme to cleave the RNA complement in a heteroduplex with phosphoramidate (26). The apparent reduction of RNA level could be due a mechanism other than RNaseH mediated cleavage of the RNA segment involved in the heteroduplex with phosphoramidates that may contribute to the destruction of RNA message. Possibly, formation of the duplexes between phosphoramidate and target RNA leads to change of mRNA folding and/or its further editing (27) and finally degradation. Further discussion of possible processes contributing to apparent mRNA depletion induced by antisense phosphoramidate compounds will also be presented below (see c-*myb* inhibition experiments).

Qualitatively the same effect was observed for phosphorothioate oligomers, but at four times higher doses (see legend to Fig. 3A and B), whereas at equal concentrations phosphorothioates have not shown any effects on mRNA level.

C-*myc* as an antisense target

Oligonucleotide phosphorothioates have been extensively used as antisense compounds for regulation of c-*myc* protein expression in different cellular and *in vivo* systems (28–31). To compare the activity of phosphoramidates with the well-characterized behavior of phosphorothioates, several oligonucleotide phosphoramidates **3–6** (Table 1) were synthesized for antisense inhibition experiments of c-*myc* protein expression. Thus HL-60 cells, expressing c-*myc*,

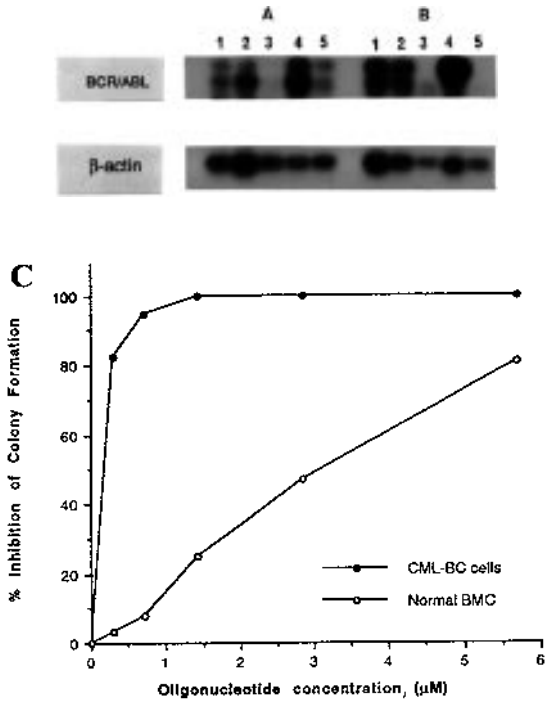

Figure 3. *Bcr-abl* (b2/a2) mRNA level normalized to β-actin mRNA, determined by RT–PCR analyses (see Materials and Methods) after (**A**) 72 h and (**B**) 96 h in primary CML patient cells exposed to phosphorothioate and phosphoramidate oligonucleotides. Starting from day 0, cells were treated with a total of 160 μg/ml (80, 40 and 40 μg/ml on days 0, 1 and 2, respectively) of phosphorothioate oligonucleotides or with 40 μg/ml (20, 10 and 10 μg/ml) of phosphoramidate oligomers. Lanes: (1) untreated cells; (2) cells treated with control mismatched phosphorothioate AAGGGCTTTTGAACTC; (3) cells treated with antisense 16mer phosphorothioate AAGGGCTTCTTCCTTA; (4) cells treated with mismatched phosphoramidate **2**, Table 1; (5) cells treated with antisense phosphoramidate **1**, Table 1. (**C**) Colony formation inhibition curves for a representative experiment using normal bone marrow and CML-BC bone marrow cells treated with oligonucleotide phosphoramidate **1** (Table 1) at the indicated concentrations. The curves show the difference in effect of phosphoramidate **1** on growth of *bcr-abl*-dependent CML-BC cells, IC_{50} 0.18 μM, versus normal bone marrow cells, IC_{50} 3 μM.

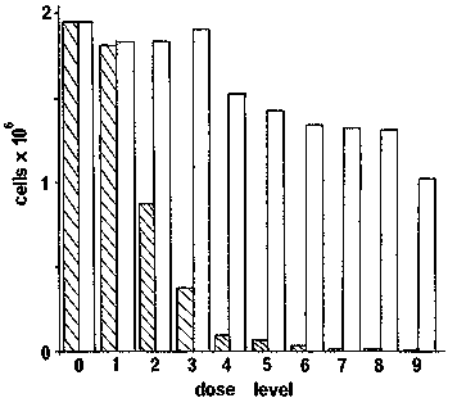

Figure 4. Inhibition of HL-60 cell growth by different concentrations of anti-*c-myc* phosphoramidates **3** and **4** (Table 1) hatched and open bars, respectively. Cells were counted at day 6 post-oligonucleotide treatment. Oligonucleotide concentrations were 0, 0.02, 0.04, 0.08, 0.16, 0.31, 0.62, 1.25, 2.5 and 5 μM for dose levels 0–9, respectively.

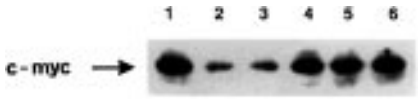

Figure 5. Western blot analysis of c-*myc* protein level reduction after electroporation of U87 glioblastoma cells with c-*myc* antisense oligonucleotides at 20 μM concentration in media. Lanes: (1) untreated cells; (2) antisense phosphodiester oligonucleotide **3** containing three phosphorothioate linkages at the 34-end; (3) antisense phosphoramidate **3**; (4) antisense phosphoramidate **5**; (5) mismatched phosphoramidate **4**; (6) mismatched phosphoramidate **6**. Numbers of oligonucleotides correspond to those in Table 1.

were treated with oligomer **3** complementary to the start site and the following four codons of c-*myc* mRNA, or with compound **5**, complementary to the 1709–1724 nt region of c-*myc* mRNA. Mismatched oligomers **4** and **6** were used as negative controls. A profound dose-dependent inhibition of HL-60 cell proliferation was observed for the antisense oligomers **3** and **5**, but not for the mismatched compounds **4** and **6** (Fig. 4) (data for **5** and **6** not shown). The reductions of c-*myc* protein level in HL-60 cells after treatment with antisense phosphoramidates at 1 μM were in good agreement with the cellular proliferation data, as determined by Western blot analysis (Table 2).

Oligonucleotide phosphorothioates also exhibit sequence specific activity in cellular proliferation assays, correlated with c-*myc* protein Western blot analysis, but at 10 times higher concentrations than phosphoramidates do (Table 2). It is interesting to notice that mismatched phosphoramidate control **4**, containing four contiguous guanosines was not active in the cellular growth assay and did not effect c-*myc* protein level as judged by Western blot analysis (Table 2). Analogous results were also obtained in U87 glioblastoma cells electroporated with the phosphoramidate oligonucleotides. An 81% reduction of c-*myc* protein was observed for the antisense compound **3** applied at 20 μM, addressed against the start codon region, but not for the mismatched control **4** with four contiguous guanosines (Fig. 5). Interestingly, in contrast to HL-60 cells, phosphoramidate **5** addressed to the downstream 1709–1724 nt segment of c-*myc* mRNA was not active in the U87 proliferation assay and did not noticeably change c-*myc* protein level relative to untreated cells (Fig. 5). This may possibly reflect differences in either *c-myc* mRNA folding and consequently accessibility to the antisense oligonucleotide, or in the intensity of ribosomal translation of *c-myc* in different cell lines.

The *in vitro* experiments with *anti-c-myc* phosphoramidates were extended to primary cells from CML patients (eight patients tested). In a representative experiment a dose-dependent reduction of colony formation in the 0.4 nM–4 μM dose range was observed after treatment of primary cells with antisense oligomers **3** and **5**, but not with mismatched controls **4** and **6**, with oligomer **3** being 10–100 times more active than **5**, indicating a sequence specificity in the action of oligonucleotide phosphoramidates (Fig. 6). In this dose range the growth of normal bone marrow cells was not affected by the mismatched oligomers **4** and **6**, and reduced by 40–50% with antisense oligomer **3** and **5** only at the highest 4 μM concentration (data not shown). The observed high *in vitro* activity of the phosphoramidates is probably due to their high binding affinity to the mRNA targets and low adhesion to proteins. Additionally, one may speculate that an as yet unidentified concentration mechanism might elevate the intracellular concentration of phosphoramidates above extracellular levels and consequently lead to the high apparent antisense activity.

Table 2. Inhibition of c-*myc* protein expression by antisense oligonucleotides in HL-60 cells

Expt	Oligonucleotide	No.[a]	Type and backbone[b]	Concentration (μM)	Relative inhibition (%)[c]
1	AACGTTGAGGGGCAT	**3**	AS, s	10	74
2	**ATGCCCCTCAATGTT**	**17**	S, s	10	41
3	AACGTTGAGGGGCAT	**3**	AS, np	1	70
4	AACG**AGTT**GGGGCAT	**4**	MM, np	1	41
5	TTTCATTGTTTTCCA	**5**	AS, np	1	93
6	TTTC**TA**T**TG**TTTCCA	**6**	MM, np	1	33

[a]Numbers correspond to those from Table 1 and mismatched nucleotides are underlined.
[b]Abbreviations for oligonucleotides type are the same as in Table 1, and s and np correspond to the uniformly modified phosphorothioate and N3′→P5′ phosphoramidate oligonucleotides, respectively;
[c]Amount of the c-*myc* determined densitometrically from Western blot was normalized to amounts of 72/73 heat shock proteins, and for the untreated cells relative inhibition is 0%.

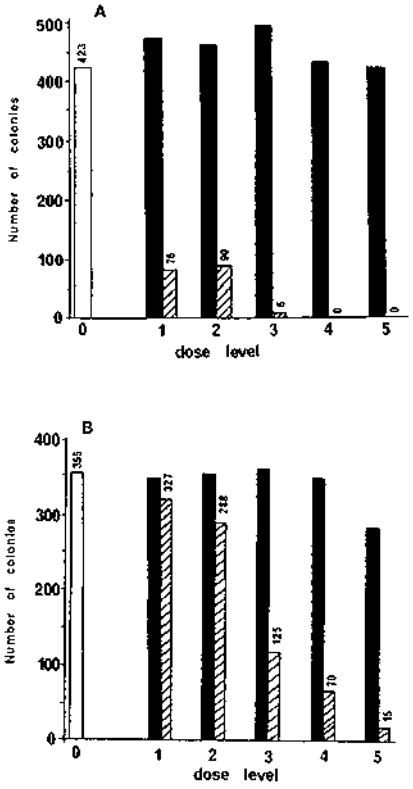

Figure 6. (**A**) Inhibition of colony formation from primary cells of a CML-blast crisis patient by *anti-c-myc* oligonucleotide phosphoramidates. The open bar corresponds to untreated control, and the filled and hatched bars correspond to mismatched oligomer **4** and antisense oligomer **3** (Table 1) respectively. Dose levels are 0.4, 4.0, 40, 400 and 4000 nM for doses 1, 2, 3, 4 and 5, respectively. Numbers above the bars correspond to the numbers of formed and scored colonies. (**B**) Same as in (A) except that the hatched bars correspond to the other antisense compound **5**, Table 1.

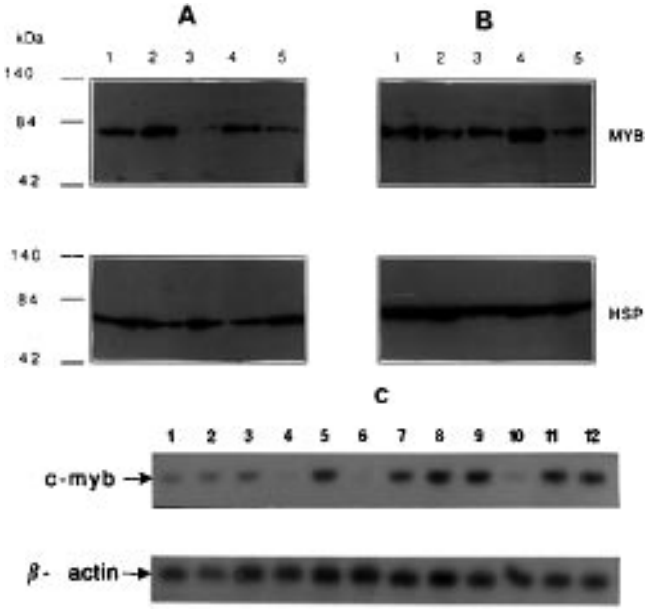

Figure 7. C-*myb* and heat shock protein (HSP) expression levels in HL-60 cells, exposed to oligonucleotides for (**A**) 48 and (**B**) 72 h. Lanes: (1) untreated cells; (2) phosphorothioate sense control **11** at 15 μM; (3) phosphorothioate antisense **10** at 15 μM; (4) phosphoramidate mismatched control **8** at 5 μM; (5) phosphoramidate antisense **7** at 5 μM. All oligonucleotides numbered as in Table 1. (**C**). C-*myb* and β-actin mRNA level determined by RT–PCR analyses (see Materials and Methods) in HL-60 cells treated with oligonucleotide for 48 h (lanes 1–6) and 72 h (lanes 7–12). Lanes: (1,2) untreated cells; (3) phosphorothioate sense control **11** at 15 μM; (4) phosphorothioate antisense **10** at 15 μM; (5) mismatched phosphoramidate **8** at 5 μM; (6) antisense phosphoramidate **7** at 5 μM; lanes 7–12 correspond to lanes 1–6, but mRNA level was determined after 72 h.

C-*myb* as an antisense target

Oligonucleotides **7–11** (Table 1) were synthesized to be either fully or partially complementary to codons 3–7 of c-*myb* mRNA. First, to study effects of oligonucleotide phosphoramidates on c-*myb* expression, HL-60 cells, growth of which is dependent on the expression of c-*myb* protein, were treated with oligomer **7** using **8** and **9** as negative controls. Dose-dependent reduction of cell proliferation within 0.5–5 μM range was observed for the fully complementary phosphoramidate **7**, but not for the mismatched compound **8** which also contains four contiguous guanosines, or for the other mismatched control **9** (data not shown). In concordance with cell growth inhibition, c-*myb* protein level measured at 48 and 72 h was also reduced by ~75% and 70%, respectively (relative to untreated cells), after treatment with antisense but not with mismatched compounds, as was determined by Western blot analysis. The level of the non-targeted heat shock (HP) proteins was not affected by the oligonucleotide treatment (Fig. 7A and B). Isosequential 15mer oligonucleotide phosphorothioates were not active in the cellular

proliferation assays at the concentrations used for the phosphoramidate **7**. Extension of the oligonucleotide phosphorothioate chain length to 24 bases renders it active, but at approximately three times the dose of the 15mer phosphoramidate.

Interestingly, in parallel with c-*myb* protein, the mRNA level was also significantly reduced by the fully complementary antisense oligomer **7**, but not by the mismatched control **8** as determined by RT–PCR analysis 48 h post oligonucleotide treatment (Fig. 7C). However, 72 h after treatment the level of c-*myb* mRNA returned to the original or control level when cells were treated with phosphoramidate **8**, although the protein level was still reduced (Fig. 7C). The origin of these effects is not yet clear, and probably indicate different mechanisms of action of the phosphoramidate and phosphorothioate compounds.

The apparent reduction of c-*myb* mRNA level by oligonucleotide phosphoramidates in HL-60 cells 48 h post treatment is interesting and unexpected in view of the observed lack of RNaseH mediated cleavage of the RNA strand in heteroduplexes formed by phosphoramidates and RNA strands (26). A possible explanation of this phenomenon may be that c-*myb* protein is a positive transcription regulator of its own expression (32). Consequently, reduction of c-*myb* protein level *via* translational arrest by phosphoramidates may lead to a decrease in mRNA level. Additionally, formation of heteroduplexes between phosphoramidate and mRNA might lead to a change of mRNA tertiary structure or its folding accompanied by a reduction of its general stability, as suggested earlier (28,33,34).

CONCLUSIONS

A series of uniformly modified oligonucleotide N3′→P5′ phosphoramidates were synthesized. These compounds are very stable toward the hydrolytic activity of nucleases from snake venom, HeLa cell nuclear extract and human plasma, yet apparently lack non-sequence specific protein binding. Moreover, they act as efficient and sequence-specific antisense agents regulating *bcr-abl*, *c-myc* and c-*myb* protein expression in different *in vitro* cellular systems. The presented data indicate that oligonucleotide N3′→P5′ phosphoramidates have good potential for development as antisense-based therapeutic agents.

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