Phosphorothioate BCR-ABL Antisense Oligonucleotides Induce Cell Death, but Fail to Reduce Cellular Bcr-Ab1 Protein Levels

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INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by an accumulation of myeloid cells and their progenitors. In over 90% of CML patients a characteristic Philadelphia chromosome (Ph') is found. The onset of the disease is thought to occur in the hematopoietic stem cell because the Ph' chromosome is present in most hematopoietic cell types. The Ph' chromosome is the outcome of a reciprocal translocation t(9;22) between the long arms of chromosomes 9 and 22 (1). The chromosomal regions involved in this translocation harbor the c-abl proto-oncogene located on chromosome 9, and the BCR gene on chromosome 22. As a result of the translocation a bcr-abl fusion gene is formed. This fusion gene encodes for a 210 kDa protein with enhanced tyrosine kinase activity (2–4). Only two possible bcr-abl mRNA breakpoints were found to inhibit cell growth of CML patient cells and cell lines, but doubt exists about their specificity. In order to test the specificity, phosphorothioate and 3'phosphorothioate capped antisense BCR-ABL oligonucleotides of different length were used. Stability, cellular uptake of oligonucleotides and effect on cell growth were studied in two CML cell lines, BV173 and LAMA-84. Phosphorothioate antisense BCR-ABL oligonucleotides were most stable, showed the highest uptake and induced cell death in BV173 but not in LAMA-84 cells. We selected the most effective antisense oligonucleotide for further analysis. The BV173 and LAMA-84 cell lines do not express the normal c-abl protein, therefore we used a c-abl specific monoclonal antibody for the detection of p210(bcr-abl) expression by flow cytometry. Dead cells found after treatment were gated out of analysis. Although BCR-ABL antisense oligonucleotides can induce apoptosis, no reduction of p210(bcr-abl) levels could be detected in living cells after treatment with antisense oligonucleotides. We conclude that antisense mediated inhibition of translation of mRNA into p210(bcr-abl) is not the mechanism responsible for the induction of apoptosis in cell line BV173.

Keywords: antisense; p210(bcr-abl); phosphorothioate; apoptosis; CML

MATERIALS AND METHODS

Cell Lines

Human leukemic cell lines BV173 (CML lymphoid) (18), K562 (CML erythroleukemia) (19), LAMA-84 (CML myeloid) (20), TOM-1 (ALL lymphoid), were cultured in RPMI 1640 medium (Flow Laboratories, Irvine, UK) supplemented with 10% heat inactivated (56°C, 30 min) fetal calf serum (Hyclone, Logan, UT, USA), 2 mM l-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin (Cellcet®; ICN Flow, Irvine, UK) in a humidified incubator at 37°C with 5% CO2 in air.

Oligodeoxynucleotides

Oligodeoxynucleotides were synthesized on an Applied Biosystems DNA synthesizer model 391 EP (Applied Biosystems, Foster City, CA, USA). The DNA sequences and their lengths are shown in Table 1.
Table 1  Location, Sequence and Constitution of Oligonucleotides Used

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<th>Name</th>
<th>Sequence (5' — 3')</th>
<th>Location</th>
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<td>abl</td>
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Positions correspond to: abl, human c-abl mRNA genbank accession no. X16416; bcr, human BCR mRNA genbank accession no. Y00661; abl 1b, human c-abl mRNA with exon 1b genbank accession no. M17310; b2m1, human beta-2-microglobulin gene exon 1 genbank accession no. M17986; b2m2/3, human beta-2 microglobulin gene exon 2 and 3 genbank accession no. M17987.

Foster City, CA, USA) according to the manufacturer’s instructions. After deprotection in 25% ammonium hydroxide solution (Applied Biosystems) 8 h at 55°C, oligonucleotides were purified by two ethanol precipitations and dissolved in distilled water. Sequences of the oligonucleotides used are summarized in Table 1, their location is shown in Figure 1. Phosphorothioate (all-PS) and 3'phosphorothioate capped (cap-PS) oligonucleotides were synthesized using tetraethyl thiuram disulfide (TETD; Applied Biosystems) as a sulfurization agent during synthesis. After deprotection and ethanol precipitation the oligonucleotides were purified by HPLC and desalted as described (17). For fluorescein labeling a 6-aminohexanol phosphate linker was attached to the oligonucleotides by the use of Aminohin-2 (Applied Biosystems) in normal synthesis. After HPLC purification, these oligonucleotides were coupled with (5)6-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) (Boehringer Mannheim, Mannheim, Germany) as described (17).

**Figure 1**  Schematic representation of the c-abl and bcr-abl mRNAs and the location of PCR and antisense oligonucleotides used. RNA was reverse transcribed with hexanucleotides. PCR analysis was used to detect the c-abl and bcr-abl mRNA with oligonucleotide BA3 as antisense primer. Oligonucleotide α2A2 was used as a probe for detection of the mRNAs.
Flow Cytometric Analysis of Oligonucleotide Uptake

The cells were determined using a Coulter Epics Elite (Coulter, Hialeah, FL). After various incubation times, cells were washed with phosphate-buffered saline (PBS) and green FLUOS fluorescence was observed using a fluorescence microscope (Coulter). Oligonucleotides were added to the wells at concentrations of 10 nM. After 18 h of FLUOS-labeling of oligonucleotides, cells were washed in a 96-well tissue culture cluster (Coulter), and then plated on a 100 µl volume in a 96-well tissue culture cluster (Coulter). Cells were seeded at a density of 2 x 10^5 cells/ml.

**Figure 2**

Southern blot of a reverse transcriptase PCR analysis of the c-8 and bc1-8 mRNAs in four cell lines. Southern blots were probed internally with an allele-specific radiolabeled oligonucleotide. The probes used are as indicated in Table 1 and Figure 1. T1, T2M, T192, T192x2, and PLK-1.
Inclusion of Cells with Antisense Oligonucleotides

Once a plane in the middle of the cells, the cells was estimated to be 7 pm. Images were obtained of
the PTP/containing segment of the MR-600. The diameter of
the psamplifier tubes of 30%, images were acquired utilizing
a pulsed laser at 90% power of the power.

Fluorescence light was
faint density filter in front of the laser. Fluorescence emission was
totally 10% by introducing a new-

Conical Laser-scanning Microscopy

Conical laser-scanning microscopy was performed on a
confocal laser-scanning microscope equipped with a 60X objective (Pan-Apo, n.a. = 1.4). Excitation
illumination at 488 nm was obtained by detection of the light at a
potential (Fl, eq. 22) was used a control for 69S staining and also used in

Monoclonal Antibodies and Anisera

Sera, 1:50

BCR-ABL Antigen and Protein Expression
Figure 3  
(a) Autoradiogram of separated 32P-labeled unmodified oligonucleotides. 
(b) Autoradiogram of separated 32P-labeled phosphorothioate-capped (PAC) and all phosphorothioate oligonucleotides (PS) labeled oligonucleotides after incubation in RPMI 1640 FCS.

RNA isolation of exponentially growing cells was performed by the guanidium isothiocyanate method and purified over a CCl4 gradient as described (23).

Synthesis of cDNA was performed in a 20 µl volume in a solution containing 1 µg of RNA, 50 ng Tris- HCl (pH 8.3), 7.5 mM KCl, 3 mM MgCl2, 10 mM DTT, 62.5 µM of dTTP, dGTP, dCTP and dATP (Boehringer Mannheim, Germany), 50 µg of random hexanucleotides (RNAse inhibitor RNasin, Promega, Madison, WI, USA) and 100 µM of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Life Technologies, Gaithersburg, MD, USA). The reaction was carried out in a DNA thermal cycle model 480 (Perkin Elmer Cetus, Emeryville, Calif., USA) at 10 min at 20°C, 45 min at 37°C and 10 min at 95°C. Amplification of cDNA was performed by addition of 30 µl of a mixture containing 0.001% gelatine, 50 mM KCl, 10 mM Tris- HCl (pH 8.4), 1.5 mM MgCl2, 62.5 nM of upstream primer, and 6.25 nM of downstream primer in the pres-
Figure 4

(a) Control laser scanning microscopic image of cell line BY173 after 18 h of incubation with FLUOS-labeled DilPS oligomeric lipid.

(b) Same as in (a) for the high microscopic image.

(c) Same as in (b) for the high microscopic image.
Figure 5  (a) Cellular accumulation of FLUOS-labeled all-PS oligonucleotides in cell line BV173. (b) The same as (a) for LAMA-84. (c) Cellular accumulation of FLUOS-labeled Cap-PS oligonucleotides compared to the α2A2.26M all-PS oligonucleotide in cell line BV173. (d) The same as (c) for LAMA-84. All-PS oligonucleotides: Y, α2A2.26M; V, α2A2.26M; •, α2A2.16M; O, α2A2.16M. Cap-PS oligonucleotides: ♦, α2A2.18M; ▼, α2A2.18M; A, α2A2.18M; A, α2A2.16M

Electrophoresis and Blotting of PCR Products
After amplification PCR products were separated on a 2% agarose gel. The separated PCR products were transferred to a Hybond™-N+ nylon membrane (Amersham, Buckinghamshire, UK). The oligonucleotide α2A2 was 5' end-labeled with [γ-32P]dATP using T4 kinase (Boehringer Mannheim). Unincorporated radioactivity was removed using a sephadex G-50 spin column. Membranes were screened using the radioactive labeled oligomer probe according to the instructions of the manufacturer. After this membranes were exposed to an X-ray film.

Analysis of Cellular Bcr-Abl Protein levels by Flow Cytometry
For analysis of Bcr-Abl protein levels, cells were washed twice with PBS. After this cells were incubated with 100 µl of FACS™-lysing solution (Becton Dickinson, San José, CA, USA) for fixation and permeabilization for 1 h at room temperature. Alter-
natively cells were fixed with 0.5% paraformaldehyde for 5 min at 4°C followed by permeabilization using 0.1% Triton-X-100 also at 4°C for 5 min. Cells were washed once with PBS and placed in 96-well point bottom wells (Costar) and blocked for non-specific binding with 50 µl of PBS containing 20% pooled human serum for 15 min at 37°C. Hybridoma supernatant (50 µl) and the polyclonal anti Ki-67 antibody were added and incubated for 30 min at 4°C. Cells were washed three times with cold PBS containing 0.5% bovine serum albumin (BSA) (Organon Teknika, Boxtel, The Netherlands). After this, cells were incubated with PBS 20% containing 0.5% bovine serum for 30 min at 4°C, and incubated with the diluted FITC or RPE conjugates for 30 min at 4°C. Cells were washed twice with cold PBS 0.5% BSA and incubated with 20 µg/ml propidium iodine (PI) in PBS for DNA staining at 4°C. Cells were then analyzed by flow cytometry.

For flow cytometric detection of the Bcr-Abl protein, Ki-67 antigen and DNA, a 488 nm 40 mW argon ion laser running at 15 mW was used. Dichroic mirrors of 550 nm and 625 nm and band pass filters of 525/40 nm for green fluorescence and 575/30 nm for red fluorescence and a 610 nm long pass filter for PI fluorescence were used.

RESULTS

Detection of the c-abl and bcr-abl mRNAs in Cell Lines

We tested the mRNA expression of the c-abl and bcr-abl mRNAs in four cell lines by reverse transcriptase PCR (Figure 1). The amount and quality of RNA was checked by a PCR for the beta-2-microglobulin and was approximately the same in the four cell lines (data not shown). Only cell line TOM-1 was positive in a PCR for detection of the e1a2 bcr-abl mRNA (Figure 2). Cell lines K562 and LAMA-84 expressed the b3a2 mRNA and BV173 the b2a2 type mRNA. Cell lines K562 and TOM-1 were both positive in a PCR for the detection of the normal c-abl mRNAs. The c-abl mRNA has two splicing variants (1a and 1b) that are detected with two different PCR reactions. Both types of c-abl mRNA were found in these cell lines. Cell lines LAMA-84 and BV173 showed no product after reverse transcriptase PCR for the normal c-abl mRNAs. We therefore decided to use these cell lines for protein analysis using flow cytometry using a c-abl specific monoclonal antibody.

Stability of Oligonucleotides in the Culture Medium

We tested the stability of 5’ labeled oligonucleotides at a concentration of 10 µM in RPMI containing 10% FCS. The autoradiogram (Figure 3) shows that the unmodified phosphodiester oligonucleotide aB2A2.18M is rapidly degraded in the culture medium. An oligonucleotide with the same sequence containing two 3’ phosphorothioate linkages (cap-PS) showed an enhanced stability in the culture medium and after 5 days full length product could still be detected. Although this oligonucleotide is more stable, it is not as stable as the full phosphorothioate modified (all-PS) oligonucleotide aB2A2.26M, that only shows a slight breakdown. The same accounts for the 16-mer cap-PS oligonucleotides (Figure 3b). Minor phosphatase or 5’ exonuclease activity is present in the culture medium since radioactivity seems to disappear from the aB2A2.26M band but no smaller oligonucleotide degradation products can be detected. All-PS oligonucleotides were found to be more stable than cap-PS oligonucleotides. After gel analysis, these oligonucleotides show several breakdown products. Remarkably, the sense cap-PS oligonucleotide B2A2.16M lacks two of these breakdown products (Figure 3b, marked by arrows) that are present in the antisense oligonucleotide aB2A2.16M. The absent bands on the autoradiogram indicate that the B2A2.16M oligonucleotide is in some way protected from degradation, possibly by the formation of a secondary structure or by protein binding. The presence of secondary structure was confirmed by computer analysis (data not shown).

Cellular Levels of FLUOS Labeled BCR-ABL Oligonucleotides

In order to study the cellular accumulation of BCR-ABL oligonucleotides of different constitution and length in cell lines, we incubated BV173 and LAMA-84 cells with 10 µM of FLUOS-labeled sense and antisense B2A2 oligonucleotides. Because it is difficult to strip the oligonucleotide from the cellular membrane (5), we determined the total of cell-associated oligonucleotide. At different time points cellular fluorescence was measured by flow cytometry as described above (17). Confocal lasercanning microscopy showed that cellular fluorescence was indeed present inside the cells and not only at the cell membrane. The all-PS 26 mer oligonucleotides showed a vesicular fluorescence (Figure 4a and b), the cap-PS oligonucleotides showed a more diffuse cellular fluorescence (Figure 4c and d) with less vesicles present. Figure 5a shows...
the oligonucleotide uptake of sense and antisense all-PS oligonucleotides of 26-mer and 16-mer length in cell line BV173. While the uptake of antisense oligonucleotide aB2A.16M reaches a maximum after 24 h of incubation, this is not the case for the 26-mer oligonucleotides. LAMA-84 cells (Figure 5b) accumulate more oligonucleotide than BV173 possibly due to differences in cell size. Unlike BV173 cells the 26-mer oligonucleotides are internalized better than the 16-mer oligonucleotides and the 26-mer sense B2A.26M better than the antisense aB2A.26M. Remarkably the cellular amount of the sense B2A.16M showed no time dependency in both cell lines.

When the cellular amount of 16-mer and 18-mer cap-PS oligonucleotides was compared with that of the all-PS oligonucleotides, they were found to internalize less effectively in BV173 and in LAMA-84 cells (Figure 5c and d). The 18-mer capped oligonucleotides accumulate more efficiently than the 16-mer cap-PS. A very poor uptake of the sense 16-mer cap-PS and all-PS oligonucleotides was observed. Because of this we investigated the cellular amount of the all-PS 16-mer anti-sense oligonucleotides targeted against the B2A2 as well as the B3A2 breakpoint after 18 h of incubation with 10 μM of oligonucleotides (data not shown). No difference in the cellular amount of the aB2A.16M compared to aB3A2.16M in the cell line BV173 and LAMA-84 was observed. This indicates that the sense 16-mer B2A.16M clearly shows different uptake kinetics compared to other oligonucleotides of the same length.

In general, uptake of PS-oligonucleotides is better than PS-capped oligonucleotides of the same length. Cellular uptake is length dependent. Sense 16-mers show a considerably reduced and no time dependent uptake.

**Effect of BCR-ABL Antisense Oligonucleotides on Cell Growth of BV173 Cells and LAMA-84 Cells**

Figure 6 shows cell numbers of BV173 after 6 days of treatment with antisense BCR-ABL and control oligos. No effect on cell growth of LAMA-84 cells after treatment with any of the oligonucleotides was observed (data not shown). Cell growth of BV173 was dramatically reduced after addition of the all-PS aB2A.26M. As discussed before, the antisense oligonucleotide targeted against the B3A2 breakpoint aB3A2.26M also inhibited cell growth in these cells (17). The control 26-mer all-PS sense oligonucleotides had little effect on cell growth of BV173. The 16-mer all-PS aB2A2.16M showed growth inhibition, while the antisense oligonucleotide targeted against the B3A2 breakpoint did not. Incubation with both 16-mer sense control oligonucleotides showed a drastic growth inhibition. Of the cap-PS oligonucleotides the aB2A2.18M showed poor inhibition of cell growth BV173 while all other control oligonucleotides of the same length had no effect. Cap-PS 16-mer oligonucleotides did not induce a cell growth reduction of cell line BV173.

We conclude that some of the all-PS oligonucleotides tested are effective in reducing cell growth of BV173 whereas cap-PS are not. The effectiveness depends on the sequence and length of the oligonucleotide. Sense 16-mer oligonucleotides also reduce growth of BV173. The all-PS aB2A2.26M is the most effective antisense oligo.

**BCR-ABL Protein Levels after Antisense Treatment**

In order to determine whether the cytotoxic effect of BCR-ABL antisense oligos was caused by a decrease in Bcr-Abl protein expression we determined p21\textsuperscript{Obc-\textsubscript{cr}} expression using flow cytometry. The effect of antisense on the levels of p21\textsuperscript{Obc-\textsubscript{cr}} was also analyzed in LAMA-84 cells. Because the antisense 26-mer all-PS oligonucleotides were more effective, whereas the 26-mer sense controls were not, we selected these oligonucleotides for protein studies. Measurements of expression of p21\textsuperscript{Obc-\textsubscript{cr}} after antisense treatment were complicated in BV173 because of induction of apoptosis (17). Apoptotic cells could be found after 4 days of incubation with antisense oligonucleotides and were identified by their low DNA staining ability. We analyzed cells stained for the p21\textsuperscript{Obc-\textsubscript{cr}} protein in combination with the DNA stain propidium iodine (PI). Figure 7 shows a dual parameter flow cytometric analysis for p21\textsuperscript{Obc-\textsubscript{cr}} and DNA of BV173 cells after 5 days of treatment with 26-mer all-PS BCR-ABL oligonucleotides. The p21\textsuperscript{Obc-\textsubscript{cr}} accumulates gradually as cells pass through S phase and is highest in the G2/M phase of the cell cycle. Cells with low PI staining, i.e. the apoptotic cells also show a low staining for the p21\textsuperscript{Obc-\textsubscript{cr}}. Because these cells are not viable, the determination of Bcr-Abl protein levels in these cells is not reliable.

After treatment with all 26-mer all-PS oligonucleotides, no decrease of p21\textsuperscript{Obc-\textsubscript{cr}} levels could be found in the cells with normal PI staining. Also no difference in expression during different phases of the cell cycle could be found. To exclude staining artefacts caused by improper fixation we used a control antibody specific for the Ki-67 antigen. Cells that were not properly fixed showed a very low staining for the Ki-67 antigen, while dead cells showed an extremely high fluorescence signal after staining (data not shown). Only cells with a normal signal for the Ki-67 antigen were analyzed. The experiment was repeated at least three times at different time periods after incubation. A decrease in levels of cellular p21\textsuperscript{Obc-\textsubscript{cr}} was never observed.

Because TPA was known to decrease Bcr-Abl protein expression in K562 cells (24), we analyzed BV173 cells and LAMA-84 cells after 3 days of treatment with TPA as a positive control for p21\textsuperscript{Obc-\textsubscript{cr}} decrease. TPA induced cell death in both cell lines. Figure 8a shows flow cytometric analysis of p21\textsuperscript{Obc-\textsubscript{cr}} expression of BV173 cells with normal Ki-67 staining after 5 days of incubation with oligonucleotides and after 3 days of treatment with TPA. Treatment of TPA resulted in a decrease of p21\textsuperscript{Obc-\textsubscript{cr}} levels in these cells. The same effect of TPA was observed in LAMA-84 (Figure 8b).

**DISCUSSION**

Antisense BCR-ABL oligonucleotides have been shown to reduce the colony formation and cell growth of CML cell lines. The observation that 26-mer and 16-mer phosphorothioates complementary to both types of bcr-abl breakpoint inhibit cell growth of cells from CML patients and CML cell line BV173 independent of breakpoint type (16,17), raised questions about the sequence specificity of these oligonucleotides. To address this issue we used phosphorothioate oligonucleotides and 3' phosphorothioate capped oligonucleotides of different length and tested their ability to inhibit translation of the p21\textsuperscript{Obc-\textsubscript{cr}}. Corresponding sense oligonucleotides were used as controls. Cell lines BV173 and LAMA 84 used in this study express bcr-abl but not c-abl mRNA. The absence of
normal c-abl protein enabled us to use a c-abl specific monoclonal antibody for detection of p210bcrlabl expression by flow cytometry. Therefore, these cell lines provided an excellent model for studies on the p210bcrlabl expression.

Stability, cellular accumulation and localization and effect on cell growth of all-PS and cap-PS BCR-ABL antisense and sense oligonucleotides complementary to both bcr-abl mRNA breakpoints were tested. Phosphorothioate 26 and 16-mer antisense oligonucleotides were most stable, accumulated better in the cells than phosphorothioate capped oligonucleotides and inhibited the growth of BV173 but not of LAMA-84. The 26-mer PS antisense oligonucleotides showed no specificity for either breakpoint while 16-mer antisense oligonucleotides did. Unlike the sense 26-mer PS oligonucleotides, both sense 16-mer PS oligonucleotides showed strong growth inhibition of cell line BV173 as well. Their degradation pattern is different and their cellular accumulation is low and shows no time dependency compared to the corresponding antisense oligonucleotides. It is therefore not likely that a sequence specific cellular mechanism is engaged in cell death induced by these oligonucleotides.

The 26-mer phosphorothioate antisense oligonucleotides were the most cytotoxic. Corresponding control oligonucleotides showed no growth inhibition. We selected these oligonucleotides for determination of their effect on p210bcrlabl levels and to test the rationale of antisense inhibition of translation. To discriminate between the Bcr-Ab1 protein levels in dead and living cells, we used a flow cytometric detection. Staining of DNA with PI and the usage of the Ki-67 monoclonal antibody allowed us to gate out apoptotic cells. We observed that in all-PS antisense 26-mer treated cells the levels of the Bcr-Ab1 protein did not decrease in the remaining non-apoptotic cells. As expected (24), we could detect a decrease of p210bcrlabl levels in LAMA-84 and BV173 cells 3 days after treatment with TPA. These data show that a decrease in p210bcrlabl levels indeed can be detected by the flow cytometric method used. A decrease of p210bcrlabl levels is not necessarily linked to immediate cell death. In cell line BA/F3bcrlabl cell death could already be detected after 12 h of treatment with unmodified BCR-ABL antisense oligonucleotides (12). Considering the half-life of the p210bcrlabl, which is over 24 h (25), and the kinetics of oligonucleotide accumulation it is also not likely that a decrease of p210bcrlabl in this cell line was responsible for the effect.

One could think of several explanations for the inhibition of cell growth found after antisense BCR-ABL oligonucleotide incubation without the expected decrease in levels of p210bcrlabl. First, a slight decrease in p210bcrlabl levels could induce immediate cell death, thereby not allowing detection of cells with low p210bcrlabl. This explanation is not likely, because TPA reduced p210bcrlabl levels in BV173 and LAMA-84 cells, that did not (yet) die of apoptosis. Also LAMA-84-cells, that do not die after BCR-ABL antisense treatment, do not show a protein decrease, indicating that at least in this cell line it is clear that no protein decrease exists after antisense BCR-ABL treatment. Furthermore, because the half-life of the p210bcrlabl is very long it is also not likely that, if even a minor protein decrease exists, it could not be detected.

A second explanation for the observed growth inhibition of BV173 cells is a non-sequence specific toxic effect induced by the phosphorothioate oligonucleotides. Non-sequence specific effects mediated by binding of growth factors, induction of transcription factors or by oligonucleotide degradation products have been described (26–28). Possibly, the all-PS 16-mer sense oligonucleotides used in this study induce cell death by one of these mechanisms. We demonstrated sequence-dependent effects of the antisense 26-mer and 16-mer phosphorothioate oligonucleotides on cell growth of cell line BV173. It is however possible that only the antisense oligonucleotides and not the control oligonucleotides bind a protein or produce toxic degradation products thereby inducing apoptosis. Recently it was found that oligonucleotides containing the sequence GGC can bind to the p210bcrlabl thereby inhibiting its autophosphorylation (29). The antisense oligonucleotides used in this study also contain the sequence GGC while the sense oligonucleotides do not. This therefore can be an explanation for the effect. The oligonucleotide aB3A2.16M does not reduce cell growth of BV173 while aB2A2.16M does. Both oligonucleotides contain the GGC motif. This indicates that the GGC mediated p210bcrlabl function inhibition is not the only cause of growth inhibition of cell line BV173. More data from carefully controlled antisense

Figure 7 Dual parameter flow cytometric analysis of Bcr-Abl protein expression vs. DNA content in BV173 cells after 5 days of incubation with 26-mer phosphorothioate oligonucleotides. Arrow marks apoptotic cells.
Figure 8  (a) Flow cytometric analysis of Bcr-Abl protein expression in BV173 cells by staining with the 8E9 monoclonal antibody after 5 days of treatment with 26-mer phosphorothioate oligonucleotides, and after 3 days with TPA. Cells were gated on scatter and normal Ki-67 expression. ..., CD-2 MoAb negative control; ----, untreated control; -----, oligonucleotide or TPA treated.
Figure 8 (b) The same as in (a) for LAMA-84 cells
experiments are needed to determine to what extent the anti-sense effect is mediated by protein binding.

A third and entirely hypothetical explanation for the growth inhibition of BV173 cells is that the presence of mRNA/oligonucleotide hybrids as such act as a signal for the onset of an apoptotic pathway. Unlike most CML cell lines, BV173 contains a wild type p53 gene (30), that is known to be involved in some mechanisms of apoptosis induction. The p53 protein preferentially binds to free single-stranded DNA ends and promotes renaturation (31). It may therefore have a function in antisense-mediated cytotoxicity. Cell line LAMA-84 contains a mutant p53 gene and could therefore be less sensitive. We are currently testing the involvement of p53 in antisense mediated cytotoxicity using transfection studies. Whatever the direct mechanism of action of antisense oligonucleotides, the cellular background in which the experiments are performed is important. In our hands, BV173 is the most sensitive cell line for a BCR-ABL antisense approach. It could therefore be susceptible to induction of apoptosis by other mechanisms not induced by p210BCR-ABL decrease. Although sequence-dependent effects of antisense BCR-ABL oligonucleotides are found in cell line BV173, our experiments on p210BCR-ABL levels indicate that these are not a result of the proposed antisense mediated inhibition of translation of bcr/abl mRNA.

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