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

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The bcr-ab1 oncogene is a fusion gene resulting from a reciprocal translocation which forms the hallmark of chronic myeloid leukemia (CML). Antisense oligonucleotides complementary to the two possible 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-ab1 protein, we therefore used a c-ab1 specific monoclonal antibody for the detection of p210bcraW 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 p210bcraW levels could be detected in living cells after treatment with antisense oligonucleotides. We conclude that antisense mediated inhibition of translation of mRNA into p210bcraW is not the mechanism responsible for the induction of apoptosis in cell line BV173.

Keywords: antisense; p210(bcr-ab1); phosphorothioate; apoptosis; CML

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-ab1 proto-oncogene located on chromosome 9, and the BCR gene on chromosome 22. As a result of the translocation a bcr-ab1 fusion gene is formed. This fusion gene encodes for a 210 kDa protein with enhanced tyrosine kinase activity (2-4). Only two possible bcr-ab1 mRNAs occur. Exon b2 of the bcr (breakpoint cluster region of the BCR gene) can be spliced to c-ab1 exon 2 (b2a2 breakpoint) or exon b3 to the c-ab1 exon 2 (b3a2 breakpoint).

The breakpoint region on the hybrid mRNA molecules is thought to be a unique target for antisense oligonucleotides. Antisense oligonucleotides are short (15-30 nt) synthetic DNA molecules which are actively internalized by cells (5). It is thought that antisense oligonucleotides bind to their complementary mRNA and inhibit translation into protein. This is most likely to occur by a blockade of the ribosomal translation mechanism or by mRNA degradation by a cytoplasmic enzyme RNase H, that recognizes RNA/DNA hybrid molecules (6, 7). Antisense oligonucleotides have been found useful in reducing the expression of a number of genes including the bcr-ab1 gene. Unmodified 18-mer antisense oligonucleotides targeted to the bcr-ab1 mRNA breakpoints reduced the clonogenic capacity of hematopoietic progenitors from the bone marrow of CML patients, but much less from normal hematopoietic progenitor cells (8-11). These 18-mer oligonucleotides inhibited the growth of several CML cell lines as well (12-14). Because these oligonucleotides are rapidly degraded by serum components, more stable 26 and 18-mer phosphorothioate-modified oligonucleotides were tested in cell lines and normal CML cells (15-17). Phosphorothioate 26-mer oligonucleotides directed against the b2a2 breakpoint induced programmed cell death in the CML cell line BV173 expressing a b2a2 type bcr-ab1 mRNA, however B3A2 antisense oligonucleotides were also effective (17). Sense oligonucleotides showed only little effect on cell growth. When tested on cells from CML patients, 26-mer and 18-mer antisense phosphorothioate oligonucleotides showed little specificity for the CML breakpoints (16). Because of this partial sequence specific effect we tested oligonucleotides with different length and chemical constitution. Since the rationale behind the antisense approach is based upon the inhibition of translation we tested the effects of the most effective antisense BCR-ABL oligonucleotides on p210bcraW levels in the CML cell lines. To eliminate annoying effects on this analysis from the presence of dead cells, we chose to do this by flow cytometry in which only the living cells were analyzed. The tested cell lines BV173 and LAMA-84 do not express a normal c-ab1 protein. This enabled us to use a c-ab1 specific monoclonal antibody to measure the p210bcraW expression by flow cytometry in these cells after treatment with BCR-ABL antisense oligonucleotides.

MATERIALS AND METHODS

Cell Lines

Human leukemic cell lines BV173 (CML lymphoid) (18), K562 (CML erythroid blasts) (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 (Cellect®; 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,
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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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 sulphasation 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 Aminohexin-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).
Flow Cytometric Analysis of Oligonucleotide Uptake

The cells were determined using a Coulter Epics Elite (Coulter, Hialeah, FL). The oligonucleotides were added to the cells in concentrations of 10 μM. After various incubations, the cells were washed with PBS and subjected to flow cytometry. The percentage of cells that incorporated the oligonucleotides was determined using a Coulter flow cytometer.

**Figure 2**

Southern blot of a reverse transcriptase-PCR analysis of the c-raf and bcr-raf mRNA in four cell lines. Southern blots were probed with an oligonucleotide labeled with chloroquine-dye-A22. Primers used are as indicated in Table 1 and Figure 1. T. TOM-1.

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K, K562; L, LAMA-84; B, BY173; †, negative control.
incubation of cells with antisense oligonucleotides

In a preliminary experiment, it was observed that incubation of cells with antisense oligonucleotides was ineffectual using the oligonucleotide was investigated using the oligonucleotide was investiga-

To reduce the background fluorescence of the oligonucleotide labeled DNA, the laser beam was set at a 45° slant angle. The laser was focused on a 60x objective (Plan-Neopla, 1.45 NA) and a Nikon Diaphot microscope. The fluorescence was attenuated to about 10% by introducing a neutral density filter. Fluorescence was attenuated to about 10% by introducing a neutral density filter. Fluorescence was attenuated to about 10% by introducing a neutral density filter.

Conical laser scanning microscopy

et al. (22).

of oligonucleotides in the cell according to the method of Sambrook (20) were used to quantify the exact amount of oligonucleotides used.

Monoclonal Antibodies and Analogs

BCR-ABL Antisense and Protein Expression

 Azerbaijani
Figure 3. (a) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (b) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (c) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (d) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (e) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (f) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (g) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (h) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (i) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (j) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (k) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (l) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (m) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (n) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (o) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (p) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (q) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (r) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (s) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (t) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (u) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (v) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (w) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (x) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (y) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides. (z) Autoradiogram of separated 32P-labeled oligonucleotides (PO) in a polyacrylamide gel. The bands indicate the presence of 32P-labeled oligonucleotides.
(c) Confocal laser-scanning microscopic image of cell line BY173 after 18 h of incubation with FLUOS-labeled αILPS oligonucleotide.

Figure 4.

Figure 4.png
Figure 5  (a) Cellular accumulation of FLUOS-labeled all-PS oligonucleotides in cell line BV173. (b) The same as in (a) for LAMA-84. (c) Cellular accumulation of FLUOS-labeled Cap-PS oligonucleotides compared to the αB2A2.26M all-PS oligonucleotide in cell line BV173. (d) The same as in (c) for LAMA-84. All-PS oligonucleotides: Y, αB2A2.26M; V, B2A2.26M; •, αB2A2.16M; O, B2A2.16M. Cap-PS oligonucleotides: ♦, αB2A2.18M; ◼, B2A2.18M; ▲, αB2A2.16M; Δ, B2A2.16M

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 αA22 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-
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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 αB2A2.18M 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 αB2A2.26M, 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 αB2A2.26M 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 αB2A2.16M. 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 periods cellular fluorescence was measured by flow cytometry as described before (17). Confocal laserscanning 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

![Cell number of cell line BV173 after 6 days of incubation with different BCR-ABL antisense oligonucleotides and controls determined by flow cytometry](Image)
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 αB2A2.16M 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 B2A2.26M better than the antisense αB2A2.26M. Remarkably the cellular amount of the sense B2A2.16M 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 oligonucleotide 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 αB2A2.16M compared to αB3A2.16M in the cell line BV173 and LAMA-84 was observed. This indicates that the sense 16-mer B2A2.16M 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 αB2A2.26M. As discussed before, the antisense oligonucleotide targeted against the B3A2 breakpoint αB3A2.26M 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 αB2A2.16M 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 αB2A2.18M 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 αB2A2.26M 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 p210<sub>bcr-abl</sub> expression using flow cytometry. The effect of antisense on the levels of p210<sub>bcr-abl</sub> was also analyzed in LAMA-84 cells. Because the antisense 26-mer all-PS oligonucleotides were most effective, whereas the 26-mer sense controls were not, we selected these oligonucleotides for protein studies. Measurements of expression of p210<sub>bcr-abl</sub> 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 p210<sub>bcr-abl</sub> protein in combination with the DNA stain propidium iodine (PI). Figure 7 shows a dual parameter flow cytometric analysis for p210<sub>bcr-abl</sub> and DNA of BV173 cells after 5 days of treatment with 26-mer all-PS BCR-ABL oligonucleotides. The p210<sub>bcr-abl</sub> 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 p210<sub>bcr-abl</sub>. 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 p210<sub>bcr-abl</sub> 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 p210<sub>bcr-abl</sub> 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 p210<sub>bcr-abl</sub> decrease. TPA induced cell death in both cell lines. Figure 8a shows flow cytometric analysis of p210<sub>bcr-abl</sub> 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 p210<sub>bcr-abl</sub> 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 p210<sub>bcr-abl</sub>. 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 p210\textsuperscript{bcr-abl} expression by flow cytometry. Therefore, these cell lines provided an excellent model for studies on the p210\textsuperscript{bcr-abl} 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 \textit{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 p210\textsuperscript{bcr-abl} 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 p210\textsuperscript{bcr-abl} levels in LAMA-84 and BV173 cells 3 days after treatment with TPA. These data show that a decrease in p210\textsuperscript{bcr-abl} levels indeed can be detected by the flow cytometric method used. A decrease of p210\textsuperscript{bcr-abl} levels is not necessarily linked to immediate cell death. In cell line BA/F3\textsuperscript{bcr-abl} cell death could already be detected after 12 h of treatment with unmodified BCR-ABL antisense oligonucleotides (12). Considering the half-life of the p210\textsuperscript{bcr-abl}, which is over 24 h (25), and the kinetics of oligonucleotide accumulation it is also not likely that a decrease of p210\textsuperscript{bcr-abl} 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 p210\textsuperscript{bcr-abl}.

First, a slight decrease in p210\textsuperscript{bcr-abl} levels could induce immediate cell death, thereby not allowing detection of cells with low p210\textsuperscript{bcr-abl}. This explanation is not likely, because TPA reduced p210\textsuperscript{bcr-abl} 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 p210\textsuperscript{bcr-abl} 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 p210\textsuperscript{bcr-abl} 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 p210\textsuperscript{bcr-abl} function inhibition is not the only cause of growth inhibition of cell line BV173. More data from carefully controlled antisense
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-
sequence 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 oligonu-
cleotides, 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 \( p21^{bcr-abl} \) decrease. Although
sequence-dependent effects of antisense BCR-ABL oligonu-
cleotides are found in cell line BV173, our experiments on
\( p21^{bcr-abl} \) levels indicate that these are not a result of the pro-
posed antisense mediated inhibition of translation of \( bcr-abl \)
mRNA.

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