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Circular Antisense Oligonucleotides Inhibit Growth of Chronic Myeloid Leukemia Cells

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Abstract

Background: Antisense represents a conceptually powerful method for regulating gene expression. However, antisense oligonucleotides developed to date manifest two serious limitations—nuclease susceptibility and nonspecific hybridization. Circular oligonucleotides may be superior to conventional linear oligonucleotides in both respects. First, circular agents, having no ends, are exonuclease-resistant. Second, they bind to complementary strands of RNA and DNA with a higher affinity than corresponding linear agents.

Methods and Results: We assessed the activity of circular phosphodiester deoxynucleotides using chronic myeloid cell lines by targeting polypurine sequences. To represent cells having a *bcr3/abl2*–type junction, we used K562 cells. A circle targeting a *bcr* polypurine sequence 385 nucleotides 5′ to the junction decreased the cell number by day 5 with an IC_{50} of 9 μM. To represent cells having a *bcr2/abl2*–type junction, we used BV173 cells. A circle targeting the *bcr-abl* junction itself decreased the cell number by day 7 with an IC_{50} of 8 μM. Control oligonucleotides, whether the same sequence uncircularized or circles with the same nucleotide composition but in scrambled sequence, had little effect. Unlike linear agents, circles were stable when incubated in 10% serum. The amount of bcr-abl protein detected by Western blotting using a specific anti-bcr-abl antibody at 24 hr in antisense-treated BV173 cells was only 10% of that of cells treated with control circles, which demonstrates an antisense mechanism of action.

Conclusions: Circular oligodeoxyribonucleotides (*1*) inhibit the accumulation of CML cells, (*2*) decrease the amount of bcr-abl protein per cell, (*3*) have sequence-selective activity, and (*4*) are more active than linear oligonucleotides containing only the base-pairing region.

Introduction

Energizing the drive to discover the molecular basis of malignant disease has been the conviction that understanding its molecular basis will lead to effective and specific therapeutic agents. The antisense approach targets a nucleotide sequence by the use of an oligonucleotide of complementary sequence. There are two theoretical advantages to targeting the gene's messenger RNA rather than targeting the gene's protein product. One is that there are fewer messenger molecules than protein product molecules. A second is that the structure of the mutation dictates the structure of an agent that can specifically bind it, i.e., the complementary nucleotide sequence.

Chronic myeloid leukemia is a good model for examining the promise of antisense therapy. The primary lesion in most cases, a reciprocal translocation between the ends of the long arms of chromosomes 9 and 22, is well characterized (1). The result is the formation of a hybrid gene on the translocated chromosome 22, *bcr-abl*. This hybrid gene consists of a 5′-portion from the *bcr* gene on chromosome 22, and a 3′-portion from the *abl* gene translocated from chromosome 9. The junction of *bcr* and *abl* thus constitutes a sequence unique to the leukemic cell population.

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An antisense agent that could prevent the multiplication of cells with the hybrid sequence, but spare cells without this sequence, would have the specificity desired in a chemotherapeutic agent.

The antisense approach can be investigated either by gene transfer with the consequent production of an antisense RNA or by the administration of oligonucleotides. The latter approach has been the one generally adopted because it is simpler and permits convenient testing of a large variety of agents. Oligonucleotides of DNA, rather than of RNA, are generally used because of the former's greater stability in biological fluids. Early reports that *bcr-abl* antisense oligonucleotides caused sequence-specific inhibition of the growth of cytomegalovirus (CML) cell lines (2) or of colonies from CML marrow progenitors (3) aroused great interest.

We found that an antisense phosphodiester oligonucleotide directed to the *bcr-abl* junction had no antiproliferative effect on CML cell lines (4). We suspected that it was promptly degraded by nucleases, either in the serum or within the cell. We also tested phosphorothioates, i.e., oligomers in which an oxygen in the naturally occurring phosphodiester linkage is replaced by sulphur to make the linkage relatively nuclease-resistant. This antisense agent was inhibitory, but the effect was not sequence-specific.

The activity principally responsible for the intracellular degradation of oligonucleotides is a 3′-exonuclease (5). Therefore, we first tried protecting phosphodiester oligonucleotides by capping the ends. We replaced the 5′-hydroxyl group with a dimethoxytrityl group, and the 3′-hydroxyl group with a 3′-amino, 2-hydroxypropyl group. These agents did have a sequence-specific effect (4,6), confirming the idea that the unmodified phosphodiesters were ineffective because of exonuclease degradation. However, the inhibitory effect on proliferation was only modest. Increasing oligonucleotide concentration reduced cell concentration in a sequence-nonspecific manner.

Other investigators have also had mixed results. Some have found sequence nonspecificity (7–9) or at least nonspecificity for the specific breakpoint (10,11). Many studies used phosphorothioates that can have nonspecific effects. Some investigators claim the mechanism is other than an antisense one, i.e., a sequence-dependent mechanism unrelated to the *bcr-abl* sequence (12,13). Others have found no effect on the bcr-abl protein level (14) or direct inhibition of the bcr-abl–associated protein–tyrosine kinase activity (15,16). Problems encountered with antisense inhibition of bcr-abl have been reviewed (17).

We reasoned that, since capped phosphodiesters had shown some promise (4), an ideal antisense agent might have phosphodiester linkages but lack ends, i.e., be a circular phosphodiester. Such a structure confers two advantages. The first is complete resistance to exonuclease activity, and the second is that such circles have the potential to bind much more tightly than linear structures because the cyclic structure limits its conformational freedom (18,19).

Materials and Methods

Cells

K562 cells (20) were obtained from the American Type Culture Collection (Rockville, MD). BV173 cells (21) were generously provided by Dr. Bruno Calabretta of Thomas Jefferson University. Each group was maintained by dilution three times weekly in RPMI 1640-10% heat-inactivated (65°C, 30 min) fetal bovine serum (FBS).

Culture

Cells were first preincubated in RPMI 1640 (Gibco BRL, Grand Island, NY) at the stated oligonucleotide concentration without serum. After 4 hr, heat-treated FBS was added to a final concentration of 10% and oligonucleotide was again added to the same final concentration. Cell concentration was determined by a hemocytometer. Cell counts represent viable cells determined by trypan blue exclusion.

Oligonucleotide Synthesis

Oligodeoxynucleotides were synthesized on a Pharmacia LKB (Piscataway, NJ) or Applied Biosystems 392 (Foster City, CA) instrument using standard phosphoramidite chemistry (22). β-Cyanoethyl phosphoramidite monomers were obtained from Cruachem (Dulles, VA) or Applied Biosystems. Phosphorylation on the 5′-ends of oligomers was carried out with (2-cyanoethoxy)-2-(2′-*O*-4,4′-dimethoxytrityloxyethylsulfonyl)ethoxy-*N*,*N*-diisopropylaminophosphine (23), also purchased from Cruachem. Oligomers were purified by electrophoresis on 20% acrylamide gels in 8 M urea

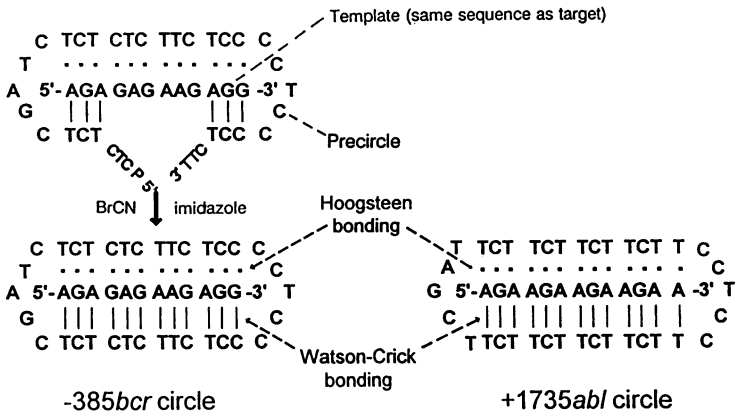

Fig. 1. Structure of circular deoxynucleotides targeting K562 cell *bcr-abl* mRNA 385 nucleotides 5′ and 1735 nucleotides 3′ from the *bcr-abl* junction. Shown also is the method of synthesis of the former, including the target-like template oligonucleotide used to approximate the ends of the linear precursor.

and Tris-borate-EDTA buffer (24). The DNA was isolated from the gels by excision, followed by elution and dialysis, and was quantitated by absorbance at 260 nm using extinction coefficients that were calculated by the nearest neighbor method (25).

Linear 5′-phosphorylated circle precursors were synthesized and then hybridized with short complementary DNA templates (Fig. 1). These precircle complexes bring the reactive 3′-hydroxyl and 5′-phosphate ends adjacent to one another, and these ends are ligated using aqueous BrCN/imidazole/Ni^{2+} (26–28). In all cases in this study, the precircle ends were joined at the center position of a Watson-Crick binding domain. We have found that this approach gives higher yields than does ligation within the Hoogsteen domain, although the products are identical. Isolation of the circular product (the principal new product from the reaction) was carried out by gel electrophoresis. The circular products typically migrate on a gel at ~0.9 times the rate of their linear precursors. Circularity was confirmed by resistance to 3′-exonuclease cleavage and by the decrease in electrophoretic mobility upon endonuclease cleavage characteristic of conversion of a circular to a linear structure. An alternative strategy for synthesizing circular oligonucleotides that provides a higher yield, involving one-step circularization from two half-length oligomers (29), was used for some preparations. Staining of oligonucleotides in gels was done with Stains-All (Sigma, St. Louis, MO).

Immunoblotting

BV173 cells (3×10^5) were lysed by vortexing with an equal volume of 2× Laemmli sample buffer (4% SDS, 15% glycerol, 0.1% β-mercaptoethanol, 125 mM Tris-Cl, pH 6.8, and 0.002% bromophenol blue). Samples were heated to 95°C for 90 sec, vortexed, and reheated at 95°C for 90 sec. Entire samples (70 μl) were applied to 7% resolving, 5% stacking, SDS polyacrylamide gels (1.5 mm × 14 cm × 16 cm). Gels were electrophoresed for 30 min at 120 V followed by 54 V for 17.5 hr. Proteins were transferred onto Optitran (Schleicher & Schuell, Keene, NH) in a solution of 0.025 M Tris, 0.192 M glycine, 10% methanol, and 0.1% SDS at 4°C for 2 hr at 24 V using the IDEA Scientific transfer apparatus (Corvallis, OR). Membranes were air dried before immunodetection.

For immunodetection the Western Blot Kit from Kirkegaard & Perry Laboratories (Gaithersburg, MD) was used. The membrane was incubated for 2 hr at room temperature in blocking solution. The membrane was then incubated at room temperature for 2 hr in anti-*abl* pEX5 monoclonal antibody (30) (a generous gift of Dr. Charles Sawyer, UCLA) (1:4000) with rapid agitation. Antibody solution was removed and the membrane was washed for 8 min in wash buffer with mild agitation. Washing was repeated two times. The membrane was then incubated with horseradish peroxidase–conjugated goat anti-mouse IgG (1:10,000) for 1 hr at room temperature with rapid agitation and washed again as above. Chemiluminescence was detected immediately as described by the manufacturer. Film exposure was for 30 min.

Results

There are two types of hybrid message resulting from the two common types of translocation breakpoints. Each type of junction involves exon

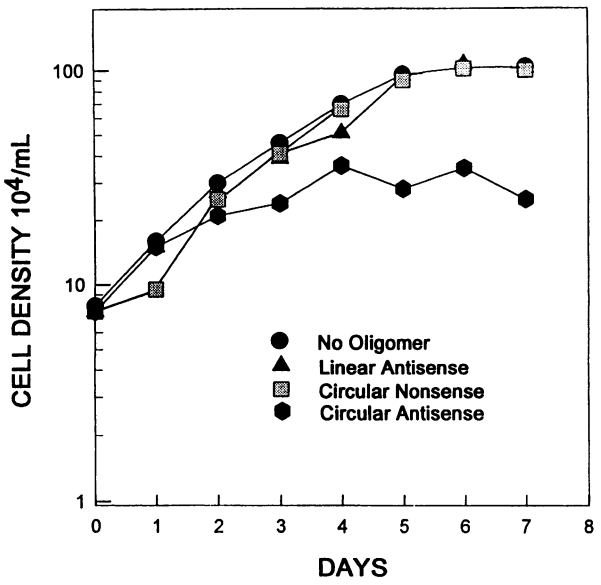

Fig. 2. Effect of oligonucleotides targeting the K562 *bcr-abl* mRNA 385 nucleotides 5′ to the *bcr-abl* junction on cell density over 7 days. The sequence of the 34-nucleotide nonsense circle was 5′CCT TTC CGA CCC TTT CCT CTT TCC CCC CCT TTC T-3′. Oligonucleotide (13 μM) was administered only on day 0.

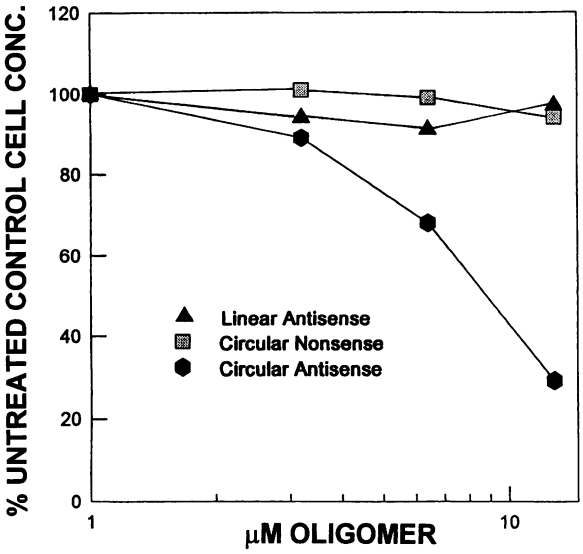

Fig. 3. Effect of various concentrations of oligonucleotide targeting the K562 *bcr-abl* mRNA 385 nucleotides 5′ to the *bcr-abl* junction on K562 cell density at 5 days.

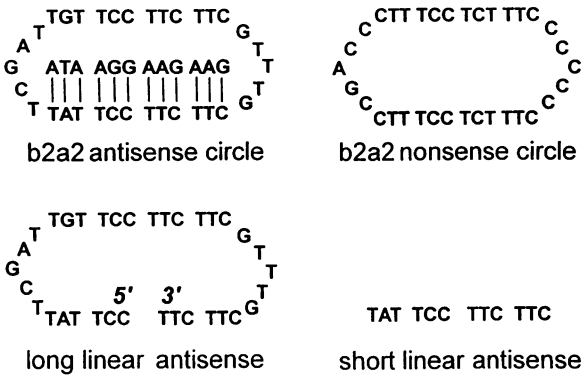

Fig. 4. Structure of antisense circular oligonucleotide targeting the *bcr-abl* junction of BV173 cells and of control oligonucleotides.

2 of *abl*. The *bcr* exon at the junction is either exon 3 (in the b3a2 type) or exon 2 (in the b2a2 type). To represent the b3a2 type we used K562 cells. To represent the b2a2 type, we used BV173 cells.

Studies with K562 Cells

The ideal antisense target is the sequence at the *bcr-abl* junction because it is unique to the CML cell. However, in working with K562 cells, we were forced to choose as a target instead a polypurine sequence (all As and Gs) so that we could also use it as a complementary oligonucleotide to bring together the ends of the oligonucleotide to be circularized (26). (Conventional enzymatic methods using ligases do not efficiently close small circles.) Polypurine sequences as targets are advantageous because they form two types of bonds—not only Watson-Crick bonds but also Hoogsteen bonds. In combination, these two types of bonds facilitate bringing together the two ends as a linear antisense oligonucleotide, permitting their ligation by chemical means in order to form an uninterrupted circle.

We chose the long polypurine stretches closest to the b3a2 junction on either side, namely, 385 nucleotides 5′ to the junction in the *bcr* gene and 1735 nucleotides 3′ to the junction in the *abl* gene (Fig. 1). The oligomers were added to the culture only on the day the culture was initiated. Although the 36-nucleotide *abl*-directed circle was inactive, the 34-nucleotide *bcr*-directed circle had activity. At 13 μM, the *bcr* circle reduced the saturating cell number by 68% (note the log scale) (Fig. 2). The controls, including both an uncapped 12-nucleotide linear control containing just the Watson-Crick bonding region and a 34-nucleotide nonsense circle, had no effect.

The effects of the same agents at a variety of concentrations on day 5 are shown in Figure 3. The antisense circles had an antiproliferative ef-

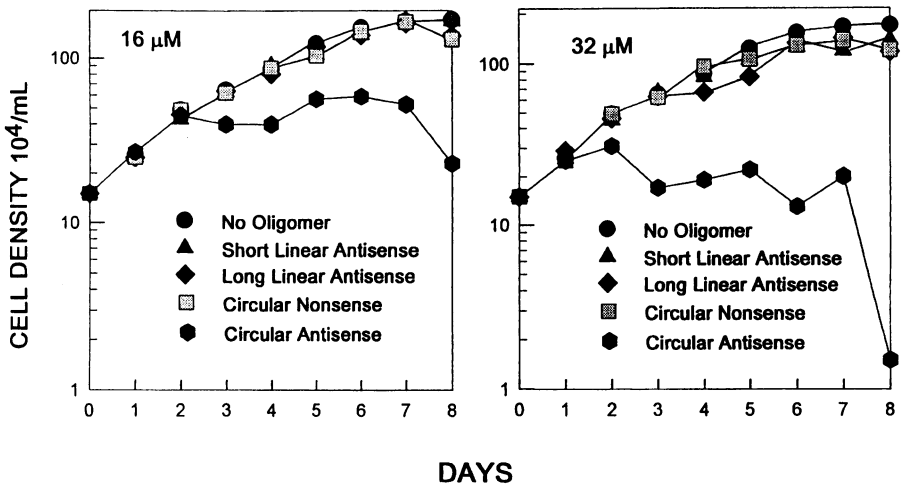

Fig. 5. Effect of oligonucleotides targeting the BV173 *bcr-abl* mRNA junction on cell density over 8 days. The oligonucleotide was added on day 0 at 16 μM (left) or 32 μM (right).

fect, even at 6 μM. The 50% inhibitory concentration (IC_{50}) was about 9 μM. Both the linear antisense and the circular nonsense controls were ineffective. At the same concentrations, neither agent had any effect on the proliferation of HL60 cells, a human leukemic cell line that lacks the *bcr-abl* mutation (data not shown).

Studies with BV173 Cells

To determine whether the circles were equally effective against cells containing the other type of *bcr-abl* junction, b2a2, we used BV173 cells. Fortunately, the b2a2 junction itself is a polypurine sequence. Figure 4 shows the target sequence, the corresponding circular antisense agent, and a circle complementary to a target sequence with the same nucleotide composition but in scrambled sequence. We also used two linear controls, a so-called long linear antisense, which was the entire antisense circle sequence but without circularization, and a short linear antisense corresponding only to the Watson-Crick bonding region.

The results at 16 and 32 μM are shown in Figure 5. At 16 μM, the cells arrested at a density 66% below that of the untreated control. At 32 μM, cells arrested at nearly 90% below the untreated control. The IC_{50} was about 8 μM. Even at this concentration, the control sequences had little effect.

The effects of circular b2a2 antisense at various concentrations are shown in Figure 6. Rather than day 5, as in the case of K562 cells, we chose day 7 because of the slower growth of the BV173 cell line. The antisense circle inhibits growth markedly, even at 3.5 μM. The control sequences (circular nonsense and short and long linear antisense) had virtually no effect. The circularization is clearly important because the same sequence prior to circularization (long linear sequence) had little effect.

Stability of Circular Oligonucleotides

To directly determine the stability of circles, we incubated circular and uncapped linear oligomers of the same length in 10% FBS at 37°C for 0–72 hr and analyzed the products on a sequencing gel. Uncapped linear agents formed a ladder

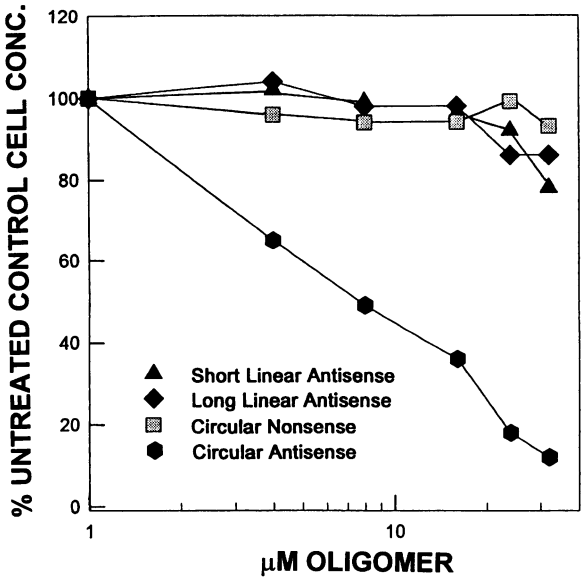

Fig. 6. Effect of various concentrations of oligonucleotide targeting the BV173 *bcr-abl* mRNA junction on BV173 cell density at 7 days.

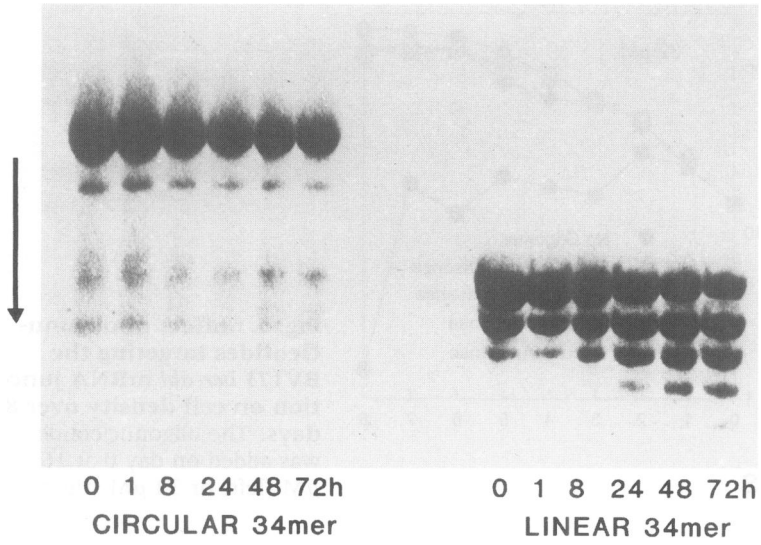

Fig. 7. Stability of deoxynucleotides. Oligomers were incubated in 10% fetal bovine serum at 37°C for 0–72 hr. At various times, the products were electrophoresed on a sequencing gel and stained with Stains-All. (Left) Circular 34mer 385 bcr circles, showing minimal degradation. (Right) Linear 34mer precursor before circularization, showing considerable degradation.

of breakdown products (Fig. 7). No such progressive accumulation of breakdown products was seen in the case of the circular agents, indicating their greater stability.

Effect on bcr-abl Protein

To demonstrate that a given oligonucleotide is acting via an antisense mechanism, the most conclusive evidence is the demonstration that the protein product of the target gene has been reduced in amount. To determine whether our antisense circle reduced the amount of bcr-abl protein in treated cells, we used immunoblotting. BV173 cells were cultured with antisense or nonsense circle for 1, 2, or 3 days. The harvested cells were lysed and the lysate electrophoresed. The electropherogram was exposed to a specific anti-bcr-abl monoclonal antibody. The signal was captured as an image by chemiluminescence and quantitated by densitometry (Fig. 8).

This methodology showed that antisense circles specifically reduced bcr-abl protein content (Fig. 9). At 24 hr, antisense circle–treated cells had <10% of the bcr-abl protein content of nonsense circle–treated cells. This reduction, resulting from oligonucleotide addition only on day 0, had largely disappeared by 72 hr, suggesting the need for repeated treatment as new cells are generated.

Discussion

In this report, we demonstrate that circular phosphodiester deoxynucleotides complementary to bcr-abl mRNA impair the accumulation of CML cells in culture. Sequence specificity was demonstrated by the absence of such an effect by a variety of control oligonucleotides. Activity was shown at modest concentrations and was dose-

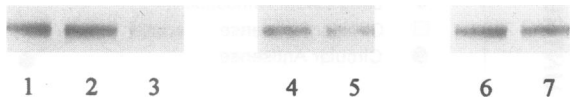

Fig. 8. Immunoblot of BV173 cell lysates using anti-*abl* monoclonal antibody. 1, untreated; 2, nonsense circle–treated, 24 hr; 3, antisense circle–treated, 24 hr; 4, nonsense circle–treated, 48 hr; 5, antisense circle–treated, 48 hr; 6, nonsense circle–treated, 72 hr; 7, antisense circle–treated, 72 hr.

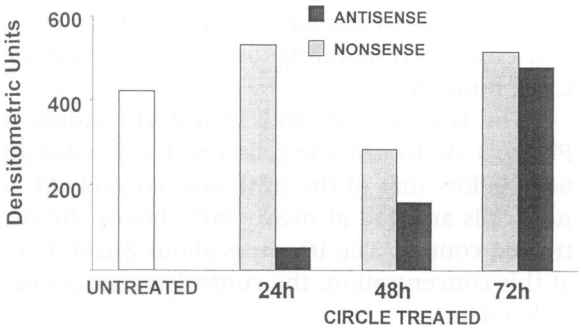

Fig. 9. Effect of circular oligonucleotides on *bcr-abl* content. Following BV173 culture with circular oligonucleotides at 12 μM for 48 hr, cells were harvested and lysed, and membrane-free lysates assayed for bcr-abl protein by immunoblotting, as described in Materials and Methods.

dependent. Stability in serum greatly exceeded that for linear oligonucleotides. Activity was demonstrated against cell lines representing *bcr-abl* junctions of both b3b2 and b2a2 types. Finally, an antisense mechanism was shown by a demonstration of reduction in the gene product.

Strictly speaking, it should be noted that the primary effect of these circles may not be inhibiting cell division. Our studies have shown that linear antisense agents were more effective in reducing the *bcr-abl*–induced impairment in apoptosis than in inhibiting DNA synthesis (6).

Circular oligonucleotides have a number of advantages over linear oligonucleotides as modulators of gene expression. These advantages include marked nuclease resistance, binding affinity, and sequence selectivity. The chemical basis for these properties has been described in detail (31). Circles also have some disadvantages, including the requirement for manual preparation, difficulty in closing the circle for certain oligonucleotide lengths, and the lack of a convenient method of labeling. The last-named disadvantage accounts for our lack of information on the kinetics of cellular uptake. Perhaps the most serious limitation is the limitation of target sequences to polypurine sequences. In the case of CML, this limitation prevented choosing the *bcr3/abl2* junction as a target. Although an alternative target resulted in an equivalent IC_{50}, a penalty might be the unintended targeting of some other mRNA that might share this sequence.

Nearly as important as the circular structure of these agents may be their phosphodiester structure. The substitution of other types of internucleotide linkages appears to contribute to the lack of sequence selectivity evident in the antisense oligonucleotide literature.

Circular oligonucleotides can be made, not only from DNA but also from RNA (28). An advantage of RNA circles over DNA circles for use as antisense molecules is their ability to bind a nucleotide in a target RNA molecule, not only by a Watson-Crick bond but also simultaneously by a Hoogsteen bond, thus forming a triplex structure (32). Because ribonuclease is so ubiquitous, the RNAse-resistant 2'-0-methyl derivative of RNA, which has similar DNA-binding properties, may be preferable to RNA itself (32).

This report demonstrates that one of the main obstacles to antisense therapeutics, the sensitivity of oligonucleotides to nucleases, can be addressed, not by modifying the phosphodiester structure but by circularization while preserving the phosphodiester structure. Although antisense therapy for leukemias may still face obstacles, chemical innovation is far from exhausted.

Acknowledgments

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