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# Inhibition of Leukemia Cell Proliferation using *c-myb* Antisense Oligonucleotides

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**Abstract:** Antisense oligodeoxynucleotides (AS-ODNs) are short DNA sequences synthesized complementary to the mRNA and they inhibit the expression of the target gene by forming a mRNA-DNA duplex. This strategy can be considered to be an alternative therapy for cancers characterized by amplified oncogene activity.

The purpose of this study was to evaluate the inhibitory effect of AS-ODNs on two human leukemia cell lines (HL-60 and K562). Cells were treated with *c-myb* AS-ODNs at two different concentrations and cell proliferation was monitored on the 5$^{th}$ and 7$^{th}$ days of culture. Sense and scrambled ODNs were used as the control.

In both cell lines, AS-ODNs inhibited cell proliferation up to 90%, with inhibition being more important at high ODN concentration. A slight inhibition was also observed with sense ODNs after 5 days of culture; but in contrast with the AS-ODNs, this inhibition was diminished in 7 days. Scrambled ODNs caused a higher inhibition than sense ODNs.

This study shows that AS-ODNs, targeted to an oncogene involved in cell proliferation, inhibit malignant cell proliferation. The inhibition observed with sense or scrambled ODNs is due to non-sequence-specific ODN binding. However, these non-specific effects decrease at longer incubation times whereas the antisense effect persists.

**Key Words:** Gene therapy, antisense oligonucleotides, *c-myb*, leukemia

## Introduction

*c-myb* proto-oncogene is the cellular homolog of the avian myeloblastosis virus transforming gene *v-myb*. Its protein product *c-myb* is a transcription factor involved in the regulation of hematopoietic cell proliferation and differentiation (1,2). *c-myb* is highly expressed in primitive hematopoietic cells, and its expression decreases during maturation and differentiation. *c-myb* expression is also elevated in some primary hematopoietic tumors and in some leukemic cell lines (3,4). It has been shown that enforced expression of *c-myb* can transform preferentially myeloid lineage of hematopoietic cells, *in vitro* and *in vivo* (5). Although many genes have been identified likely to be regulated by *c-myb*, the critical target genes are not known.

Methods of gene therapy in cancer depend on different strategies (6). Some methods target the gene itself (e.g. homologous recombination) and some target the gene's transcriptional product, the mRNA. Antisense oligodeoxynucleotides (AS-ODNs) are short DNA sequences 15-20 bp long, synthesized complementary to the mRNA (7). Since ODNs are subject to degradation by nucleases, they are chemically modified in order to improve their stability. The most widely used one is the phosphorothioate (PS) modification in which one of the non-bridging oxygens of the internucleotide phosphodiester linkages is replaced with sulfur (7). AS-ODNs form a duplex structure with mRNA and inhibit the translation. In addition, the destruction of the mRNA by RNAse H is promoted (8). It has been proposed that AS-ODNs might have an antiproliferative effect through the stepwise release of deoxyribonucleotides under the effect of exonucleases (9). Therefore, targeting an oncogene with AS-ODNs is a way of inhibiting oncogene expression and this can be an alternative strategy to control the growth of cancers with amplified oncogene activity (10).

In this study, HL-60 human acute promyelocytic leukemia cells and K562 human chronic myelogenous leukemia cells were treated with *c-myb* AS-ODNs. Non-treated cells and sense ODN (S-ODN)- or scrambled ODN (Scr-ODN)-treated cells were used as controls. Our aim was not only to evaluate the effect of AS-ODNs on malignant cell proliferation *in vitro*, but also to evaluate the specificity of the antisense effect.

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## Materials and Methods

**Cell Lines and Culture.** HL-60 human acute promyelocytic leukemia cell line and K562 human chronic myelogenous leukemia cell line were obtained from the American Type Culture Collection. Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated bovine calf serum, 2 mM glutamine and antibiotics. Cells were incubated at 37 ºC in a 5% $CO_2$-95% air mixture.

**Oligodeoxynucleotides.** PS-ODNs corresponding to *c-myb* codons 2-7 were prepared on an automated synthesis instrument in the Nucleic Acid Facility of the Department of Chemistry, University of Pennsylvania Cancer Center. The sense sequence was 5'-GCC CGA AGA CCC CGG CAC-3', the antisense sequence was 5'-GTG CCG GGG TCT TCG GGC-3', and the scrambled sequence was 5'-CGC GCG TGT GGC GTC GGT-3'.

**Determination of the Cell Proliferation Rate.** Cells were resuspended in fresh medium containing serum and seeded in 96-well plates at a density of 500 cells per well in a volume of 200 µl. ODNs were prepared in medium without serum and added to cells at two different concentrations: 40 µg/ml (8 µg/well) and 100 µg/ml (20 µg/well). Cells were counted on the 5$^{th}$ and 7$^{th}$ days of culture using a hemacytometer. All experiments were performed at least in quadruplicate. Results are expressed as mean ± SD. A two tailed *t*-test was used for statistical analysis and $P < 0.001$ was considered significant.

## Results

**1. Effect of *c-myb* AS-ODNs on the proliferation of HL-60 cells.** The effect of *c-myb* AS-ODNs and S-ODNs on HL-60 cell growth rate is shown in Figure 1. Cells were counted on the 5$^{th}$ and 7$^{th}$ days of culture, and oligonucleotide-treated cells were compared to non-treated (control) cells. After 5 days of incubation with AS-ODNs, cell growth was inhibited to 88.5% with low dose (40 µg/ml) and 91% with high dose (100 µg/ml) oligonucleotides. For the same period of incubation, the inhibition was 19.8% with low dose S-ODNs and 31.1% with high dose S-ODNs. When the incubation period was prolonged to 7 days, the inhibition of cell proliferation was less prominent but was still over 60% for both low and high doses of AS-ODNs. In fact, no more cell proliferation was observed in control cells after 7 days of culture and S-ODNs had very little effect on cell proliferation: 5.5% at low dose and 2.8% at high dose.

**2. Effect of *c-myb* AS-ODNs on the proliferation of K562 cells.** The effect of *c-myb* AS-ODNs on K562 cell growth rate is shown in Figure 2. The normal growth rate of K562 cells was less than that of HL-60 cells. After 5 days of culture, there were 637.5 ± 14.47 x $10^3$ K562 cells per ml whereas there were 1208.75 ± 102.25 x $10^3$ HL-60 cells per ml of culture medium. On the 5th day of culture there was a 78% inhibition with low dose and 87.4% inhibition with high dose *c-myb* AS-ODNs. Inhibition with S-ODNs for the same period of time was 3.3% for low dose and 26.4% for high dose

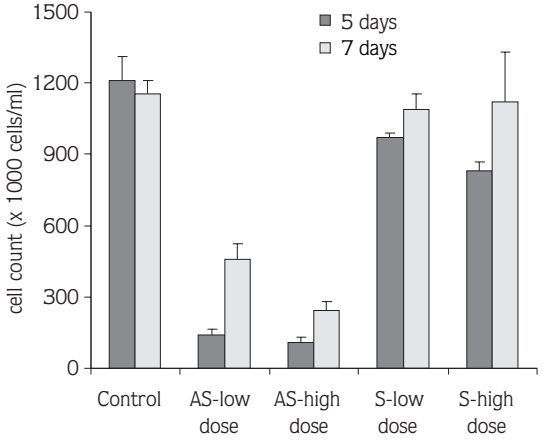


Figure 1. Effect of *c-myb* AS-ODNs on the proliferation of HL-60 cells. The concentration of low dose ODNs is 40 mg/ml and that of high dose ODNs is 100 mg/ml. The results are expressed as mean ± SD of at least quadruple cell counts.

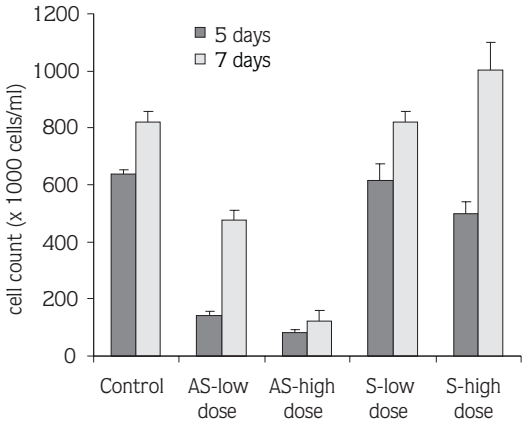


Figure 2. Effect of *c-myb* AS-ODNs on the proliferation of K562 cells. The concentration of low dose ODNs is 40 mg/ml and that of high dose ODNs is 100 mg/ml. The results are expressed as mean ± SD of at least quadruple cell counts.

oligonucleotides. In contrast with HL-60 cells, K562 cells were still proliferating after 7 days of culture. There were 820 ± 36.4 x $10^3$ cells per ml. The inhibition of cell growth was again less prominent with AS-ODNs at 7 days: 41.6% at low dose and 87.9% at high dose. For the same period, there was practically no inhibition with S-ODNs: 0.23% at low dose and 0% at high dose.

**3. Effect of Scr-ODNs on the proliferation of HL-60 and K562 cells.** Scr-ODNs also inhibited cell proliferation in both cell lines (Table). The inhibitory effect of Scr-ODNs was close to the effect of AS-ODNs after 5 days of incubation. On the $7^{th}$ day, inhibition was less prominent in HL-60 cells, but in K562 cells the degree of inhibition was approximately the same as that obtained with AS-ODNs.

## Discussion

In this study, we compared the effect of *c-myb* AS-ODNs on the proliferation of two leukemia cell lines, HL-60 and K562, by varying both ODN concentrations and the time of incubation. We also used sense and scrambled sequences to evaluate the specificity of the antisense effect. HL-60 cells have been widely used in antisense research, but very few studies have been done concerning antisense effect on K562 cells, and there is no comparative study. We found that the proliferation of both cell lines was significantly inhibited by *c-myb* AS-ODNs regardless of the ODN concentration. The fact that there were more cells than seeded indicated that there was no direct cytotoxic effect of ODNs and that they in fact had an inhibitory effect on proliferation. The proliferation rate of K562 cells was lower than that of HL-60 cells, which facilitated the observation of time-dependent effects. After 5 days of incubation, the inhibitory effect of high dose AS-ODNs was slightly higher than that of low dose AS-ODNs, but it was not proportional to the dose increase. The difference between the effects of low and high doses was more prominent at 7 days. Apparently, higher doses of AS-ODNs are more effective in inhibiting proliferation at longer incubation times, probably because higher ODN concentrations are necessary to overcome accumulating proliferative signals.

We observed less or no inhibition with S-ODNs, which indicated a more specific inhibitory effect for AS-ODNs. However, Scr-ODNs also showed an inhibitory effect on the proliferation of both cell lines. The interaction of ODNs with other molecules (especially positively charged ones) is a critical problem concerning the specificity of AS-ODNs (11). PS-ODNs show increased avidity in binding to proteins when compared to their phosphodiester counterparts (12). In our study, PS structure should not be the cause of the non-specific effect, because S-ODNs showed no or very little inhibition. It is more likely that Scr-ODNs might be involved in aptamer formation, which means a sequence-specific interaction of ODNs with a protein (13). Aptamer strategy is considered to have potential therapeutic use in some pathologies and this type of interaction can explain the differential effects of S- and Scr-ODNs in our experiments (14).

The presence of a G-quartet gives the oligonucleotide the ability to self-aggregate via the formation of G-G Hoogsteen pairs. The G-quartet motif is thought to be implicated in non-sequence-specific effects although the mechanism is not fully explained (15). The Scr-ODN that we used did not contain any G-quartet whereas the AS-ODN does. Therefore, the inhibitory effect of Scr-ODNs cannot be explained by the presence of a G-quartet. Moreover, G-quartet-containing AS-ODNs have been widely used in antisense studies and even in clinical trials.

Several precautions can be taken in order to improve the specificity of the antisense effect. The length of the oligonucleotide should be in the range of 16-20 nucleotides and the oligonucleotide concentration should

Table. Effect of Scr-ODNs on the proliferation of HL-60 and K562 cells.

| | HL-60 | | K562 | |
|---|---|---|---|---|
| | 5 days | 7 days | 5 days | 7 days |
| Control | 1208,75 ± 102,25 | 1153,75 ± 56,94 | 637,50 ± 14,47 | 820,00 ± 36,44 |
| Scr-ODN low dose | 150,63 ± 57,46 | 696,88 ± 30,69 | 208,75 ± 45,05 | 355,63 ± 11,23 |
| Scr-ODN high dose | 88,75 ± 8,00 | 347,50 ± 39,61 | 88,13 ± 16,14 | 105,63 ± 8,36 |

The results are given as mean ± SD of the cell count x1000/ml of culture medium.

be kept low (not more than 4-5 μM) (16). Our ODNs were 18 nucleotides long and we used them at 6.5 and 13 μM concentration. We delivered the ODNs without a carrier (also called naked) and it is well known that higher concentrations of naked oligonucleotides are required to obtain antisense effect. Unfortunately, non-sequence-specific effects are more commonly produced at such concentrations (16). Using cationic lipids or polyamines as a carrier can facilitate delivery and minimize non-sequence-specific effects by neutralizing negative charges on the oligonucleotides (17-19).

Studies on antisense ODNs has been going on for more than 20 years and the method has faced a number of complex challenges. Several companies have developed antisense drugs that are successfully used in clinical trials, while experiments are going on to improve the methodology (20). Although the development of antisense methods is difficult and expensive, antisense drugs are still promising.

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