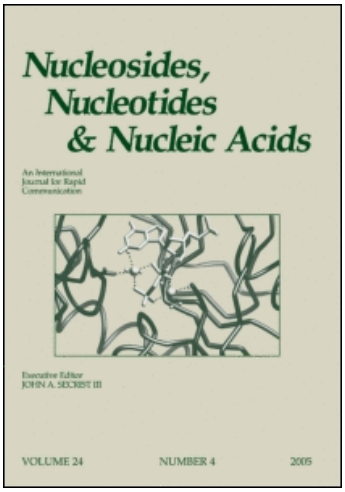

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ENHANCED SPECIFICITY OF MINIMALLY MODIFIED ANTI-C-*MYC* OLIGONUCLEOTIDES

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ABSTRACT: The sequence-specificity of antisense oligonucleotides (ODN) against c-*myc* mRNA was tested by Northern blot analysis. Rat smooth muscle cells were treated with antisense or control ODN against c-*myc* modified by the "minimal protection strategy".[8] At 0.3 μM concentration the ODN show a very specific reduction in c-*myc* mRNA levels. Use of the "minimal protection strategy" minimizes nonspecific effects as observed for all-phosphorothioate ODN containing four consecutive guanine residues.

INTRODUCTION

Balloon angioplasty is a well established therapeutic intervention for ischemic coronary heart disease.[1-3] Although good symptomatic improvement occurs in the majority of cases, the procedure is complicated by restenosis in >30% of cases.[1-3] The principal mechanisms leading to vascular restenosis are smooth muscle cell (SMC) proliferation and extracellular matrix accumulation,[4] which makes the suppression of SMC proliferation a primary goal for the prevention of restenosis. A multitude of factors and mediators contribute to the process of SMC proliferation. The expression of various genes, including immediate-early genes such as c-*myc*, has been identified as crucial to the control of SMC growth *in vitro*.[5] Antisense Oligonucleotides (ODNs) against c-*myc* have been successfully used for the inhibition of SMC proliferation.[4-6] However, the antiproliferative effect of c-*myc* all-phosphorothioate ODNs has also been attributed to a sequence specific non-antisense effect, due to the presence of three or more consecutive guanine residues, which can form a G quartet structure.[7] In the course of our studies on the reduction of these unspecific effects we reported earlier on a new protection strategy ("minimally modified ODNs ") consisting of a combination of end-capping and additional protection of internal pyrimidine positions.[8] This combination minimizes nonspecific effects while retaining nuclease stability. In this paper, we have applied this protection strategy to anti-c-*myc* ODNs and we have studied their effects on c-*myc* expression.

Table 1: Sequence of antisense oligonucleotides. * indicates the position of a phosphorothioate linkage. ODNs were synthesized and purified as described previously.[8] Purity and integrity of all oligonucleotides were found to be greater than 95% by HPLC (Waters GenPak FAX, gradient CH_3CN/H_2O 1:4 (v/v), 0.01M NaH_2PO_4, 0.1M NaCl to CH_3CN/H_2O 1:4 (v/v), 0.01M NaH_2PO_4, 1.5M NaCl). All oligos were analyzed by negative ion electrospray mass spectroscopy (Fisons Bio-Q) which in all cases confirmed the calculated mass.

AS-1	5'- A*A*C*G T*T*G A G G G G*C*A*T -3'	(human, see ref. 4)
AS-2	5'- C*A*C*G T*T*G A G G G G*C*A*T -3'	(rat, see ref. 6)
AS-3	5'- C*G*T*T G*A*G G G G C A*T*C*G -3'	(rat, see ref. 6)
CO-1	5'- A*T*G*C C C*C*T C A A C*G*T*T -3'	(sense)
CO-2	5'- T*A*C*G G G G T*T*G A G*C*A*A -3'	(scrambled)

RESULTS AND DISCUSSION

Table 1 shows the sequences of the oligonucleotides that were used in this work. AS-2 is targeted to the translation initiation site of rat c-*myc* m-RNA (complementary to the first five codons[6]). AS-3 targets a sequence shifted two bases upstream from the AUG initiation codon.[6] AS-1 differs from AS-2 by a single point mutation at the 5'-end and is targeted against human c-*myc* m-RNA.[4] The control oligonucleotides CO-1 and CO-2 are the sense and the scrambled versions of AS-1. The oligonucleotides were "minimally modified", by introduction of phosphorothioate linkages at the 3'- and 5'-end and at internal pyrimidine positions.[8] Each oligonucleotide contains 8 phosphorothioate bonds.

The effect of oligonucleotides on c-*myc* mRNA expression was tested by Northern blot analysis of total RNA from rat SMCs 5 hours after transfection of the SMCs with oligonucleotides at 0.3 and 3 μM concentration using Lipofectamine (5 μg/mL) for enhanced cellular uptake. The results in Figure 1 and 2 show that the specificity of the oligonucleotides depends strongly on the concentration in which they are applied. At 0.3 μM concentration the oligonucleotides exhibit a very specific behaviour: AS-2, which is directed against the rat sequence suppressses the c-*myc* mRNA expression (in rat cells) by 50%, suggesting that this oligonucleotide acts through an RNase H mechanism. AS-1, which is the human analog of AS-2 having one base-pair mismatch with rat mRNA, is somewhat less effective and leads to a 30% reduction of c-*myc* mRNA. AS-3, which is also directed against the rat sequence, but shifted by two bases relative to AS-2 reduces the c-*myc* mRNA expression only by 5%. This observation is consistent with the results reported by Biro et al.[6] who found that the AS-2 sequence, chemically modified only by two phosphoramidate linkages at the 3'-end, was able to inhibit SMC proliferation in a sequence dependent manner, while the identically modified AS-3 sequence had no effect in the same assay. The c-*myc* mRNA expression was not diminished after transfection with either the sense or the scrambled control-oligonucleotide when used at 0.3 μM

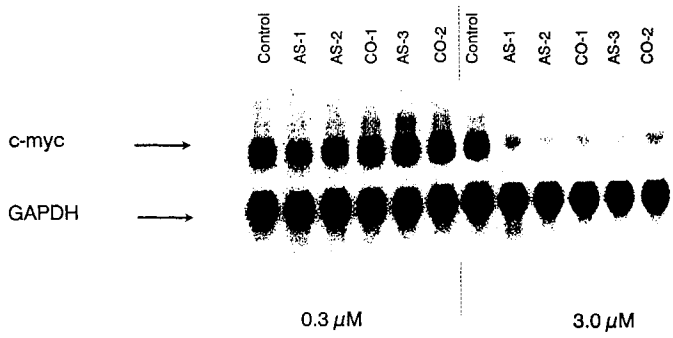

Figure 1: Effect of oligonucleotides on c-*myc* mRNA expression. Rat SMCs were isolated as described.[9] They were seeded at a density of 12,000 cells per cm^2 in medium (basal-Iscove Medium, Seromed), supplemented with 10% fetal bovine serum (Gibco), 160 μM Penicillin G, 62 μM streptomycin and 2 mM L-glutamine. Forty-eight hours later the medium was replaced by serum free medium. On day 5 SMCs were treated with 0.3 or 3 μM oligonucleotide in medium (basal-Iscove, supplemented with 2 mM L-glutamine) for 5h in the presence of Lipofectamine (5 μg/mL). Total RNA was extracted and 20 μg was resolved on a 1.2% agarose gel and transferred to a nylon membrane. The blots were probed with a ^{32}P-radiolabeled c-*myc* cDNA probe and reprobed with a ^{32}P-radiolabeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to confirm equal loading.

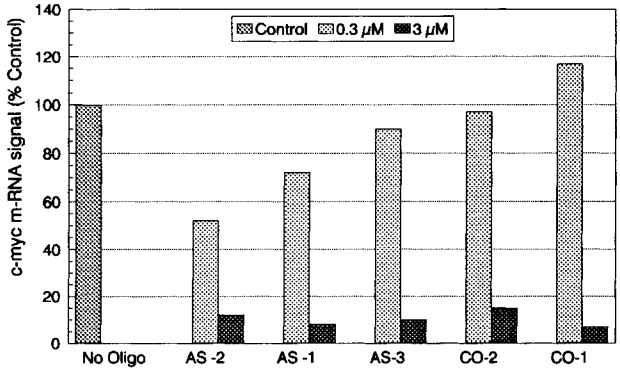

Figure 2: Effect of oligonucleotides on c-*myc* mRNA expression. The c-*myc* mRNA signal is normalized to the GAPDH signal.

concentration. However, at 3μM concentration all oligonucleotides almost completely suppress c-*myc* mRNA expression. It is interesting to note that in all cases the mRNA expression of a control enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was not affected by the oligonucleotides. We hypothesize therefore that the non-sequence-dependent reduction of c-*myc* mRNA levels is not mediated through RNAse H cleavage, but rather by a decrease in the transcription of c-*myc*.

The results obtained here are especially interesting in the light of the analysis carried out by Burgess et al.[7] using all-phosphorothioate (all-PS) antisense oligonucleotides. They attributed the antiproliferative effect of AS-1 (modified as all-PS and tested at 10-60 μM concentration) to the presence of the four consecutive guanine residues, which can form a G quartet structure, since they also found control oligonucleotides containing these structural elements to be equally effective. Here we find that at low oligonucleotide concentrations these unspecific effects can be avoided using the "minimal protection strategy" introduced earlier.[8] Each AS-1, AS-2, AS-3 and CO-2 contain four consecutive guanine residues, but only AS-2 and to a minor extend AS-1 (1 base mismatch) reduce the c-*myc* mRNA expression. AS-3, which has been shown previously to be complementary to a antisense-insensitive target sequence and the scrambled control CO-2 have no effect despite the presence of the four guanosines. Thus, the "minimal protection strategy", consisting of a combination of end-capping and additional protection of internal pyrimidine position, minimizes nonspecific effects while retaining nuclease stability.[8] We have successfully applied this protection strategy not only to antisense oligonucleotides targeted against c-*myc* but also against a variety of other targets.[10-12]

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