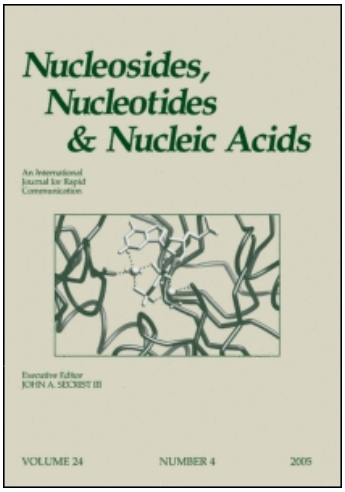

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Approaches to Enhance the Binding Affinity and Nuclease Stability of Triplex Forming Oligonucleotides

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BIOPHYSICS/BIOCHEMISTRY

APPROACHES TO ENHANCE THE BINDING AFFINITY AND NUCLEASE STABILITY OF TRIPLEX FORMING OLIGONUCLEOTIDES

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ABSTRACT: The ability of simple azole nucleosides to promote antiparallel triplex formation at non-homopurine duplex targets was explored. It was also shown that spermidine-cholesterol conjugation is effective in enhancing cellular uptake and stability of oligonucleotides.

Antiparallel triple helix formation involves the third strand oligonucleotide (TFO) binding to the purine strand of a duplex target in an antiparallel orientation with respect to the purine strand. The base triplets are formed as a result of reverse-Hoogsteen G•GC and T•AT (or A•AT) hydrogen bonding.[1] Ideally, the duplex portion of the triplex consists of a homopurine-homopyrimidine strand. However, the triplex stability is considerably reduced by the presence of one or more pyrimidines (*inversion sites*) in the purine-rich strand of the duplex.[2] This prevents triplex formation at mixed sequence duplex targets. Here we describe approaches to enhance the binding affinity of TFOs directed to bind to duplex targets containing CG or TA inversion sites. The stability and cellular uptake problems of TFOs have also been addressed.

We have recently shown[3] that T improves binding at CG inversion sites and the results obtained from T analogs such as pyridin-2-one and pyridin-4-one suggests the importance of O^4 in T•CG interaction. This finding provides a starting point in the design of other T analogs with increased affinity and specificity for CG inversions. Although T binds well at CG inversion sites, it does not appear to have sequence specificity. The relative affinity of T for AT and CG base pairs was apparently similar.

An alternate approach to triplex formation at non-homopurine targets was investigated.[4] Simple azole 2'-deoxyribonucleosides were incorporated in place of natural nucleosides in TFOs (Fig. 1). These TFOs were then tested for their ability to

TFO	
1	5'-ggggttgggggttgggggttgggg-3'
2	5'-ggggttgggaggttggaggttgaggg-3'
3	5'-ggggttgggtggttggtggttgtggg-3'
4	5'-ggggttgggcggttggcggttgcggg-3'
5	5'-ggggttgggyggttggyggttgyggg-3'
6	5'-ggggttgggmggttggmggttgmggg-3'
7	5'-ggggttgggrggttggrggttgrggg-3'
8	5'-ggggttgggeggttggeggttgeggg-3'
9	5'-ggggttgggbggttggbggttgbggg-3'
10	5'-ggggttgggnggttggnggttgnggg-3'
11	5'-ggggttgggLggttggLggttgLggg-3'
12	5'-ggggttgggkggttggkggttgkggg-3'
Duplex	
13	3'-ggggaaggggggaaggggggaaggggg-5'
	5'-ccccttccccccttcccccttccccc-3'
14	3'-ggggaagggaggaaggaggaagaggg-5'
	5'-ccccttccctccttcctccttctccc-3'
15	3'-ggggaagggtggaaggtggaagtggg-5'
	5'-ccccttcccaccttccaccttcaccc-3'
16	3'-ggggaagggcggaaggcggaagcggg-5'
	5'-ccccttcccgccttccgccttcgccc-3'

Where, g = 2'-dG; a = 2'-dA; t = thymidine; c = 2'-dC; y = pyrazole 2'-dR; m = imidazole 2'-dR; r = triazole 2'-dR; e = tetrazole 2'-dR; b = benzimidazole 2'-dR ; n = 2'-d-nebularin; L = linker L; K = linker K.

Figure 1

bind to several duplex targets to form antiparallel triplexes composed primarily of G•GC and T•AT base triplets. Binding of the azole nucleosides to CG and TA base pairs was compared to binding of either natural nucleosides or abasic linkers to the same target base pairs. The rationale was that the azole nucleosides would be small enough to avoid steric clash with the pyrimidines of duplex, thus providing a potential advantage over natural nucleosides. In addition, it was anticipated that these derivatives would retain stacking interactions with neighbouring bases of the third strand, an added advantage over the use of linkers. The azoles tested include pyrazole, imidazole, 1,2,4-triazole, 1,2,3,4-tetrazole and benzimidazole.

We find that in general, the azole nucleosides do provide a substantial advantage in binding to CG and TA base pairs, relative to natural nucleosides or abasic linkers (Fig. 2). In particular, several of the TFOs containing azoles demonstrated substantial binding to targets containing three TA base pairs, whereas none of the oligonucleotides containing either natural nucleosides or linkers were able to bind. The azoles bound to each of the four base pairs with

	third strand[b]											
duplex[c]	1(g)	2(a)	3(t)	4(c)	5(y)	6(m)	7(r)	8(e)	9(b)	10(n)	11(L)	12(K)
13(gc)	4E9	9E8	2E8	3E8	1E8	2E8	1E8	3E7	5E7	4E7	3E7	2E9
14(at)	4E8	5E9	2E9	4E8	—	—	—	—	1E6	3E8	—	—
15(ta)	—	—	—	—	5E8	3E8	3E8	8E7	9E7	1E8	—	—
16(cg)	—	—	1E9	—	—	2E8	2E7	1E8	—	2E8	—	—

[a] Listed values are apparent association constants for triplexes formed by the indicated combination of oligonucleotide and duplex. Where values are not given (--), little or no triplex was detected, and the apparent association constant is estimated to be less than 10^6. [b] The letter in parentheses represents the nucleoside of interest found in the third strand oligonucleotide. [c] The letters in parentheses indicate the base pair of interest for the indicated duplex.

Figure 2. Apparent Association Constants for TFOs 1-12 Binding to Duplexes 13-16[a]

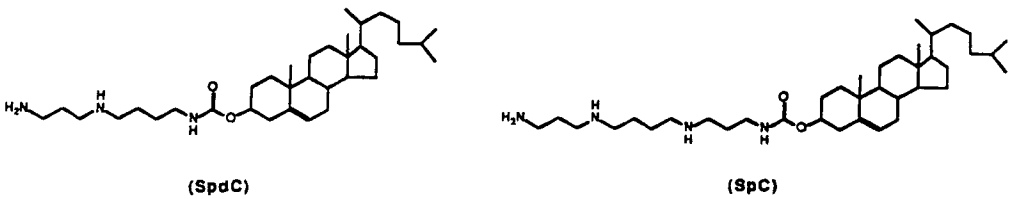

Figure 3

varying degrees of specificity, indicating that base-pair-specific interactions occur for certain triplets. We believe that these compounds will be of significant utility in future attempts to form antiparallel (and perhaps parallel) triplexes at mixed sequence targets.

Two fundamental problems that need to be overcome the biological efficacy of TFOs are uptake and stability. For this purpose, we designed a new class of uptake enhancers (polyamine-lipid conjugates) such as spermine-cholesterol (SpC) or spermidine-cholesterol (SpdC) (Fig. 3).

Since spermine, spermidine and cholesterol are naturally occuring , we anticipated that they might be more easily metabolized by cells, thereby decreasing cellular toxicity. Using a non-radioactive cell proliferation assay, we show that both SpC and SpdC are significantly less toxic than the widely used DOTMA/DOPE. SpdC is the least toxic, having little effect on cell proliferation up to at least 80 μg/ml (Fig. 4 A). Using both fluorescent-tagged and radiolabeled TFOs, we found that SpC and SpdC significantly enhance cellular

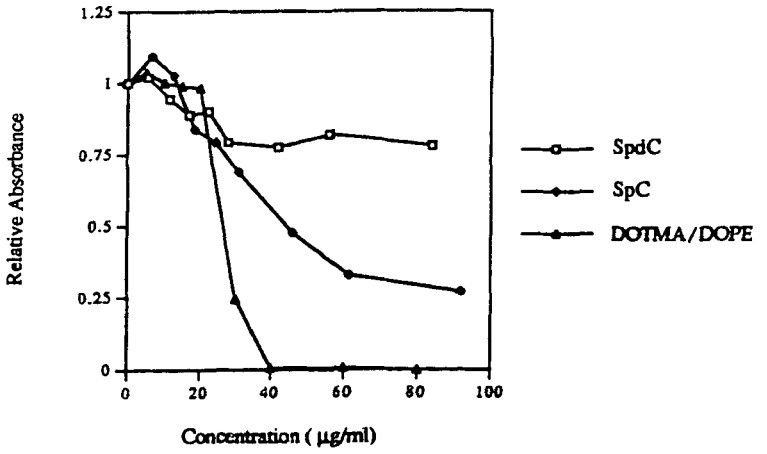

Figure 4A. Effect of cationic lipids on cell proliferation. Vero cells were plated at an initial density of 500 cells per well in 96-well plates. After 20 h, cells were exposed to the indicated concentrations of SpC, SpdC or DOTMA/DOPE for 4 days. Cell proliferation was measured by a non-radioactive cell proliferation assay. The absorbance at 490 nm is directly proportional to the number of living cells. Data points are the average of 4 replicates for each test concentration.

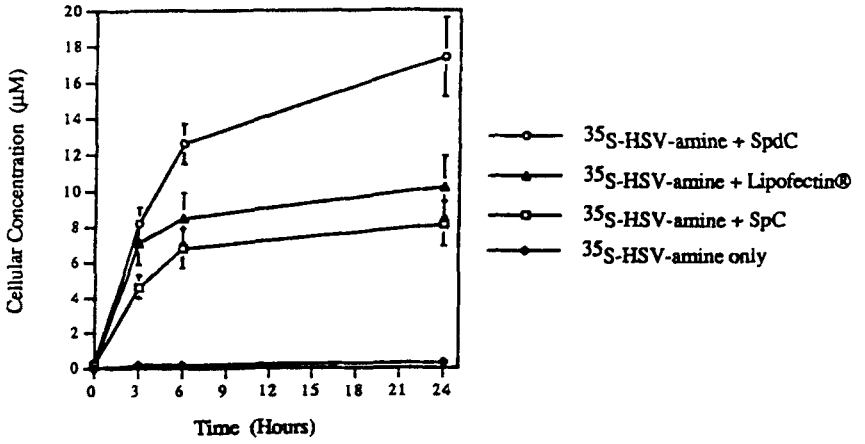

Figure 4B. Enhanced cellular uptake of radiolabeled oligonucleotides in the presence of cationic lipids. Vero cells were treated with 0.1 μM (106 cpm) 35S-labeled oligo 1 in the presence or absence of spermidine-cholesterol, spermine-cholesterol (each at 10 μg/ml), or DOTMA/DOPE (at 20 μg/ml, of which 10 μg/ml is DOTMA). At various time points (0 to 24 hr), the uptake of radiolabeled oligonucleotides was evaluated by scintillation counting. The intracellular oligonucleotide concentration was calculated based on the specific activity of the radiolabeled oligonucleotide and the measured volume of Vero cells. Data points represent a mean of 4 to 6 experiments ± SEM (error bars).

uptake of the oligomers (Fig. 4B). SpC is comparable to DOTMA/DOPE in increasing cellular uptake of oligonucleotides, while SpdC is even more efficient. Both compounds appear to enhance the nuclear localization of fluorescent TFOs within cells.

We examined the intracellular stability of TFOs taken up in the presence of SpdC. At 0, 3, 6 and 24 h after addition of oligonucleotide and cationic lipid, oligonucleotides were isolated from the cells by phenol extraction, ethanol precipitation and subjected to denaturing polyacrylamide gel electrophoresis. The gel data (not shown) show that the internalized oligonucleotides remain stable even after 24 h. G-Rich TFOs with a 3'-propanolamine modification were found to be stable up to 8 h. Thus, SpdC appears to provide added stability for unmodified TFOs that has not been seen before.

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