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Properties of Nicked and Circular Dumbbell RNA/DNA Chimeric Oligonucleotides Containing Antisense Phosphodiester Oligodeoxynucleotides

Hidefumi Yamakawa,[a] Takayuki Abe,[a] Takeshi Saito,[b] Kazuyuki Takai,[a] Naoki Yamamoto[c] and Hiroshi Takaku[a,*]

[a]*Department of Industrial Chemistry, Chiba Institute of Technology, Tsudanuma, Narashino, Chiba, 275 Japan*
[b]*Health Science Laboratories, Yamanouchi Pharmaceutical Co. Ltd., Nihonbashi-Honcho, Chuo-ku, Tokyo 103, Japan*
[c]*Department of Microbiology, Tokyo Medical and Dental University School of Medicine, Yushima, Bunkyo-ku, Tokyo 113, Japan*

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Abstract—We have designed a new class of oligonucleotides, 'dumbbell RNA/DNA chimeric phosphodiester oligonucleotides', consisting of a sense RNA sequence and its complementary antisense DNA sequence, with two hairpin loop structures. The reaction of the Nicked (NDRDON) and Circular (CDRNON) dumbbell DNA/RNA chimeric oligonucleotides with RNase H gave the corresponding antisense phosphodiester oligodeoxynucleotide together with the sense RNA cleavage products. The liberated antisense phosphodiester oligodeoxynucleotide was bound to the target 45 mer RNA, which gave 45 mer RNA cleavage products by treatment with RNase H. The circular dumbbell RNA/DNA chimeric oligonucleotide showed more nuclease resistance than the linear antisense phosphodiester oligodeoxynucleotide (anti-ODN) and the nicked dumbbell RNA/DNA chimeric oligodeoxynucleotide. The circularization, achieved by joining the 3′ and the 5′ ends of RNA/DNA chimeric oligonucleotides containing two hairpin loop structures, increases the oligonucleotide uptake into cells, as compared with the nicked dumbbell RNA/DNA chimeric oligonucleotide and the linear antisense phosphodiester oligodeoxynucleotides. When the circular dumbbell RNA/DNA chimeric oligonucleotide is directly delivered into retrovirus infected cells, its antisense phosphodiester oligodeoxynucleotide function appears.

Introduction

Unmodified (phosphodiester) antisense oligonucleotides have been reported to have an inhibitory effect against HIV-1.[1,2] Unmodified oligonucleotides, in particular, have limited survival in vitro and in vivo. These oligonucleotides are degraded by nucleases present in serum and in cells.[3,4] Antisense oligonucleotides with phosphorothioate backbones exhibit several advantages over the other forms, including relatively high nuclease resistance and the capacity to induce the degradation of the target sequence by RNase H.[5,6] However, phosphorothioate oligodeoxynucleotides hybridize more weakly with the complementary nucleic acids than the unmodified oligodeoxynucleotides and are eventually degraded, primarily from the 3′ end. Phosphorothioate oligodeoxynucleotides have also been shown to block the proliferation of HIV-1 in acutely infected cells in a non-sequence specific manner,[7] probably by the inhibition of reverse transcriptase,[8,9] and/or the viral entry process.[10,11] Another problem in the use of antisense phosphorothioate oligodeoxynucleotide is their inefficient cellular uptake. One approach to these problems has been the development of modified antisense oligonucleotides with P=O groups in the internucleotidic bonds. In addition, the degradation of phosphodiester oligodeoxynucleotides can be slowed considerably by blocking 3′ or 3′ and 5′ ends of the chain, because the primary degrading enzymes present in cells are of the

Key words: Circular dumbbell RNA/DNA chimeric oligonucleotide; antisense phosphodiester oligonucleotide; melting temperature; RNase H; exonuclease resistance; retrovirus infected-cells.
*Corresponding author: Tel: +81-474-78-0407; Fax: +81-474-71-8764; E-mail: hiroshi@miida.ca.it-chiba.ac.jp

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3′-exonuclease type.[12,13] Several stabilization methods for the phosphodiester oligonucleotides have been proposed, such as the incorporation of various chemical substitutents at the 3′-hydroxyl groups,[4,12,13] the circularization of the oligonucleoties by joining the 3′ and the 5′ ends,[14–16] and the formation of a hairpin loop structure at the 3′ end.[17–21]

In this paper, we describe the design of a new class of oligonucleotides, 'dumbbell RNA/DNA chimeric phosphodiester oligonucleotides', consisting of a sense RNA sequence and its complementary antisense DNA sequence, with two hairpin loop structures. These oligonucleotides have increased nuclease resistance and cellular uptake. Of particular interest, the antisense phosphodiester oligodeoxynucleotide is liberated by RNase H treatment of the dumbbell RNA/DNA chimeric oligonucleotides. That is to say, when the circular dumbbell RNA/DNA chimeric oligonucleotide is directly delivered into retrovirus infected cells, its antisense phosphodiester oligodeoxynucleotide function appears.

Results and Discussion

Nuclease sensitivities of nicked and circular dumbbell RNA/DNA chimeric oligonucleotides

Selective inhibition of viral gene or oncogene expression may be achieved by the introduction into cells of oligonucleotide sequences complementary to viral RNA or DNA. The application of oligonucleotides in vivo however, may be severely hampered by their sensitivity to nucleases that makes them unstable in biological environment. The phosphodiester oligonucleotides are degraded by nucleases present in serum and in cells.[3,4] In order to overcome the this problem, we examined the design of a new class of oligonucleotides, 'dumbbell RNA/DNA chimeric phosphodiester oligonucleotides', consisting of a sense RNA sequence and its complementary antisense DNA sequence, with two hairpin loop structures. The circular dumbbell DNA oligonucleotides have biological relevance as aptamers or decoys for hybridizing proteins such as transcription factors.[22,23]

The nuclease sensitivities of the nicked (NDRDON) and the circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotides with the RNA-DNA base pairs (sense (RNA) and antisense (DNA)) in the double helical stem were studied.

Each nicked and circular dumbbell RNA/DNA chimeric oligonucleotide used in this study has its 3′- and 5′-ends within the base pairs in the stem (sense-RNA or antisense-DNA) (Figure 1). The circularization of the 40 mer RNA/DNA chimeric oligonucleotide was carried out by enzymatic ligation with T4 ligase (Figure 1). For ligation, proof of synthesis can be verified by altered electrophoretic mobility of product (upper bands: unligated material; lower bands:ligated material) (data not shown).

First, the 3′-exonuclease, snake venom phosphodiesterase (SVPD), was used for comparative digestion studies of the nicked (NDRDON) and the circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotides, and the rate of digestion was determined by hyperchromicity (Figure 2A). Control oligonucleotides were prepared for comparison, such as an antisense phosphodiester oligodeoxynucleotide (anti-ODN, 5′-GGAGATGCCTAAGGC-3′) and an antisense phosphorothioate oligodeoxynucleotide (anti-S-ODN). A comparison of the nuclease sensitivities of the antisense phosphodiester oligodcoxynucleotide (anti-ODN) and the nicked (NDRDON) and the circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotides showed that the anti-ODN was digested extensively by SVPD within 43 min, whereas the nicked (NDRDON) and the circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotides were more stable. On the other hand, the circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotide was considerably more stable than the nicked (NDRDON) dumbbell RNA/DNA chimeric oligonucleotide. However, the nuclease resistance of the circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotide was slightly lower than that of the anti-S-ODN. Similarly, the S1 endonuclease activity digested the anti-ODN and the NDRDON to mono-

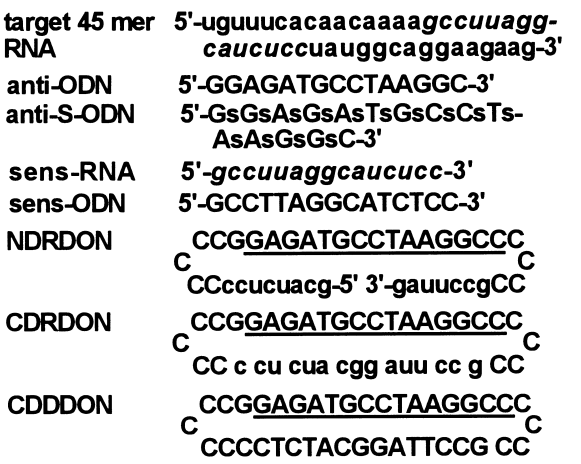

Figure 1. The structures and sequences of the oligonucleotides used in this study and described in the text. Upper case letter: DNA; lower case letter: RNA. 45 mer RNA:RNA sequence of the splice acceptor site of HIV-1 rev gene (5519–5563). The antisense sequences are denoted by underline. Targeted RNA: 5′ to 3′ (italics).

nucleotides in 30 and 120 mins, respectively, whereas the circular (CDRDON) dumbbell DNA/RNA chimeric oligonucleotides were more slowly digested (Figure 2B). In particular, the circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotide was found to be more stable to S1 nuclease than the anti-S-ODN. S1 nuclease will attack the single-stranded regions of the dumbbell sequence (interstrand connecting loops), thus allowing to recover the double stranded portion of the oligonucleotide.[22,24] It may be noted that the dumbbell structure can interfere with the endonucleolytic degradation of the loops. On the other hand, the nicked dumbbell RNA/DNA chimeric oligonucleotide is accessible to degradation by S1 nuclease. On the basis of this degradation, the complementary region in the nicked dumbbell RNA/DNA chimeric oligonucleotide is at least partially deshybridized. This result was also supported from the melting temperature values of oligonucleotides (see Table 1).

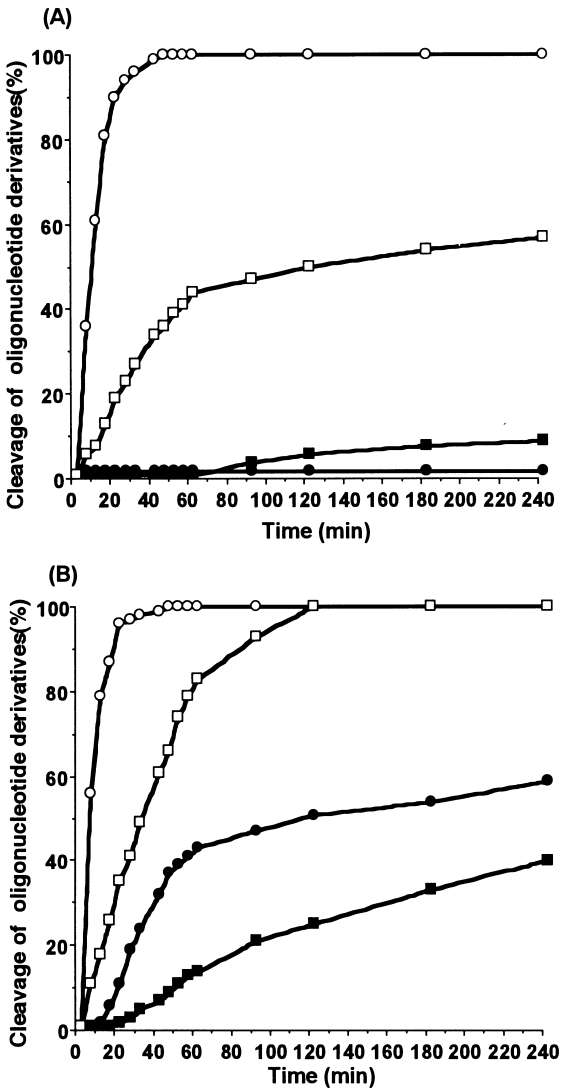

Figure 2. Digestion of the linear antisense oligodeoxynucleotides, and the nicked- and circular dumbbell-RNA/DNA oligonucleotides containing phosphodiester (anti-ODN (○), NDRNOD (□), CDRNON (■)) or phosphorothioate (anti-S-ODN (●)) bonds with snake venom phosphodiesterase (A) and nuclease S1 (B).

Similar results were obtained when the nuclease sensitivities of the anti-ODN, NDRDON, and CDRDON oligonucleotides were studied in fetal bovine serum (Figure 3). Thus, the circular dumbbell RNA/DNA chimeric oligonucleotides containing phosphodiester bonds are more resistant to exo- and endo-nucleases than the linear oligonucleotide (anti-ODN).

The results obtained by incubating the circular dumbbell oligonucleotides in fetal bovine serum provided additional evidence of their relative stability (Figure 3). For comparison, the anti-ODN and the anti-S-ODN were chosen as a control. The CDRDON was stable after 24 h of incubation, whereas the anti-S-ODN was a slightly degraded after 24 h. The NDRDON and the anti-ODN were completely degraded after 12 h. In the fetal bovine serum assay, the circular dumbbell oligonucleotide was converted to an open circular oligonucleotide by the running on denaturing PAGE (20% polyacrylamide containing 8.3 M urea), and it remained stable after 24 h when the circular dumbbell oligonucleotide was converted to the nicked dumbbell oligonucleotide, it was quickly digested. The stability of the oligonucleotides was increased with two hairpin loop structures with RNA–DNA base pairs (sens-RNA and antisense-DNA) in the stem at the 3′- and 5′-ends.

Table 1. Melting temperatures of oligonucleotides

Sequences	Tm (°C)[a]
anti-ODN/sens-ODN	40
anti-ODN/sens-RNA	41
anti-S-ODN/sens-ODN	33
anti-S-ODN/sens-RNA	28
NDRNON	47
CDRNON	81
CDDDON	71
anti-ODN/45 mer RNA	55
anti-ODN/sens-ODN/45 mer RNA	41,53
CDRNON/45 mer RNA	80
NDRNON/45 mer RNA	51
CDRNON/45 mer RNA[b]	81
NDRNON/45 mer RNA[b]	52

[a]Values were obtained in 10 mM sodium phosphate buffer and 10 mM NaCl at pH 7.0.
[b]This reaction was carried out in the presence of 5 molar equiv. of the target 45 mer RNA.

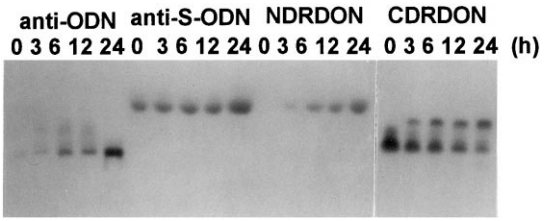

Figure 3. Digestion of the antisense oligodeoxynucleotides and nicked-, and circular-dumbbell RNA/DNA chimeric oligonucleotides in the presence of 10% calf serum at 37 °C for 0–24 h.

Duplex hybridization

The hybridization between antisense-DNA and sense-RNA in the dumbbell RNA/DNA chimeric phosphodiester oligonucleotides is the key to the nuclease resistance in vitro as well as in vivo. We measured the melting temperature of oligodeoxynucleotides with either antisense phosphodiester (anti-ODN) or phosphorothioate (anti-S-ODN) bonds with the complementary sense phosphodiester oligodeoxynucleotide (sens-ODN) and oligoribonucleotide (sens-RNA) (Figure 1). The Tm values of the duplexes of the phosphodiester anti-ODN, with the phosphodiester sens-ODN and the sens-RNA were 4 °C and 41 °C, respectively (Table 1). The Tm values of the duplexes of the phosphorothioate anti-S-ODN with the phosphodiester sens-ODN and the sens-RNA were 33 °C and 28 °C, respectively (Table 1). The antisense oligodeoxynucleotides (anti-S-ODN) containing the phosphorothioate bonds hybridized more weakly with the complementary nucleic acids than the unmodified oligonucleotides.

Next, we measured the melting temperatures of the nicked (NDRDON) and the circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotides. The Tm values of the NDRDON and the CDRDON were 47 °C and 81 °C, respectively. The nicked (NDRDON) and the circular dumbbell (CDRDON) DNA/RNA chimeric oligonucleotides had higher Tms than the DNA–DNA and DNA–RNA duplexes. Furthermore, the Tm of the circular oligonucleotide (CDDDON) with the DNA-DNA base pairs (sense (DNA) and antisense (DNA)) in the double helical stem has an estimated Tm of 71 °C, which is 10 °C less than the Tm of the CDRDON. These results suggest that the stability of the oligonucleotides also increased with the introduction of the two hairpin loop structures to the RNA-DNA base pairs (sense (RNA) and antisense (DNA)) in the double helical stem.

Finally, we measured the melting temperatures of the oligonucleotides bound with the complementary 45 mer RNA (5′UGUUUCACAACAAAAGCCUUAGGCAUCUCCUAUGGCAGGAAGAAG3′ (5519–5563), the splice acceptor site of HIV-1 rev gene) (Table 1, Figure 1). The Tm values of the duplex between the anti-ODN and the 45 mer RNA was 55 °C. On the other hand, when the double-stranded DNA (anti-ODN/sens-ODN) was mixed with the 45 mer RNA, two transitions were observed: one, at 53 °C, which was typical of the duplex between the anti-ODN and the 45 mer RNA, and one, at 41 °C, which coincided with the double-stranded DNA. The Tms of the duplexes between the nicked dumbbell RNA/DNA chimeric oligonucleotide (NDRDON) and the 45 mer RNA approached the Tm values of the duplex between the anti-ODN and the 45 mer RNA (51 °C). In contrast, the circular (CDRDON) dumbbell RNA/DNA chimeric oligonucleotides did not undergo two transitions (Table 1). Furthermore, when the amount of the 45 mer RNA was increased up to five molar equiv. under the above conditions, the Tms of the duplexes between the nicked dumbbell RNA/DNA chimeric oligonucleotide (NDRDON) and the 45 mer RNA approached the Tm values of the duplex between the anti-ODN and the 45 mer RNA (52 °C). These results suggest that the nicked dumbbell oligonucleotides were only partially bound to the complementary RNA. However, the Tm values of the duplex between the circular dumbbell RNA/DNA chimeric oligonucleotide (CDRDON) and the 45 mer RNA is measured at 81 °C, which is typical of the CDRDON. In particular, the circular dumbbell RNA/DNA chimeric oligonucleotide with the higher Tm value showed more nuclease resistance than the linear antisense phosphodiester oligonucleotide and the nicked RNA/DNA chimeric oligonucleotide (Figure 1 and Table 1).

RNase H treatment of the dumbbell RNA/DNA chimeric oligonucleotides

The RNase H-mediated liberation of antisense phosphodiester oligodeoxynucleotides from the dumbbell RNA/DNA chimeric oligonucleotides was studied. The RNase H activity assay was carried out with the target 45 mer RNA and either the circular (CDRNON) or the nicked (NDRNON) dumbbell RNA/DNA chimeric oligonucleotides in the presence of *E. coli* RNase H for up to 4 h (Figure 4). The antisense phosphodiester oligodeoxynucleotide (anti-ODN) was used for comparison, such as the control oligonucleotide (Figure 4, lanes 3–6). The target 45 mer RNA with the anti-ODN was completely cleaved by *Escherichia coli* RNase H within 4 h (Figure 4, lane 6). On the other hand, the reaction of the NDRDON with RNase H gave the corresponding antisense phosphodiester oligodeoxynucleotide together with the RNA cleavage product (Figure 4, lane 8). The target 45 mer RNA was then added to the above reaction, which produced the characteristically shortened

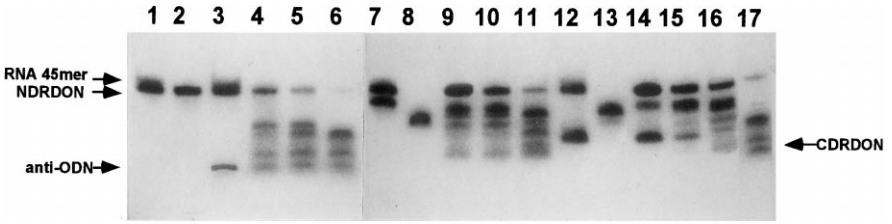

Figure 4. Specific cleavage by RNase H of the target 45 mer RNA in the presence of the dumbbell RNA/DNA chimeric oligonucleotides (NDRDON and CDRDON), and the anti-ODN. lane 1, control: 45 mer RNA; lane 2, control: 45 mer RNA/RNase H/4 h; lane 3, control:anti-ODN/45 mer RNA; lane 4, anti-ODN/45 mer RNA/RNase H/1 h; lane 5, anti-ODN/45 mer RNA/RNase H/2 h; lane 6, anti-ODN/45 mer RNA/RNase H/4 h; lane 7, control: NDRDON/45 mer RNA (faster migrating band: NDRDON, slower migrating band: 45 mer RNA); lane 8, control:NDRDON/RNase H/4 h (liberated anti-ODN from NDRDON); lane 9, NDRDON/45 mer RNA/RNase H/1 h (faster migrating band:liberated anti-ODN, slower migrating band:NDRDON, ladder bands:RNA cleavage product); lane 10, NDRDON/45 mer RNA/RNase 11/2 h; lane 11, NDRDON/45 mer RNA/RNase H/4 h; lane 12, control: CDRDON/45 mer RNA; lane 13, control:CDRDON/RNase H/4 h (liberated anti-ODN from CDRDON); lane 14, CDRDON/45 mer RNA/RNase H/1 h (faster migrating band:CDRDON, intermediate migrating band:liberated anti-ODN, slower migrating band:45 mer RNA); lane 15, CDRDON/45 mer RNA/RNase H/2 h. lane 16, CDRDON/45 mer RNA/RNase H/4 h (faster migrating band:liberated anti-ODN, slower migrating band:45 mer RNA, ladder bands:RNA cleavage product); lane 17, CDRDON/45 mer RNA/RNase H/6 h.

RNA fragments (Figure 4, lanes 9-11). After 4 h, the target 45 mer RNA was almost completely cleaved (90%) to the shortened RNA fragments (Figure 4, lane 11). Furthermore, when the circular dumbbell RNA/DNA chimeric oligonucleotide (CDRDON) was used in place of the NDRDON, under the same conditions as described above, RNA template cleavage was observed (62%) (Figure 4, lanes 14–16). In the case of the CDRDON, the reaction of the CDRDON with RNase H gave the 100% of the corresponding antisense phosphodiester oligodeoxynucleotide together with the RNA cleavage product within 4 h (Figure 4, lane 13). However, the RNase H cleavage rate for the target 45 mer RNA with the circular dumbbell RNA/DNA chimeric oligonucleotide (CDRDON) is slower than those for the nicked dumbbell RNA/DNA chimeric oligonucleotide (NDRNOD) and the linear antisense oligodeoxynucleotides (anti-ODN and anti-S-ODN). However, after 6 h, the target 45 mer RNA with the circular dumbbell RNA/DNA chimeric oligonucleotide (CDRDON) was completely degraded by *E. coli* RNase H (Figure 4, lane 17). The liberated antisense phosphodiester oligonucleotide is bound to the target mRNA; that is, the inhibition of viral replication occurs in a sequence-specific manner without the inhibition of RNase H activity. In contract the anti-viral activity of the phosphorothioate oligodeoxynucleotide (S-ODN) is due to a direct effect of reverse transcriptase[2,3] and/or the viral entry process.[4,5]

Cellular uptake of dumbbell RNA/DNA chimeric oligonucleotides

Oligonucleotides have been used as antisense inhibitors of gene expression in various culture systems and are considered to be potential therapeutic agents against cancer and viral infections. In order to exert any of these effects, the oligonucleotides must enter into the cytoplasmic and nuclear compartments of the cells. The problem in the use of antisense oligonucleotides is that the cellular uptake of oligonucleotides is inefficient.[24–27] Immunoliposomes have been previously studied as a transport system to deliver both membrane-permeable and impermeable molecules into cells.[28–30] However, in contrast to the use of endogenously transcribed or microinjected antisense oligonucleotides, regulatory activity of exogenous oligonucleotides appears to depend upon the uptake of sufficient amounts by the cells. As shown in Figure 5, the [^{32}P]-labeled CDRNON strongly associated with the MOLT-4 cells, but the [^{32}P]-labeled NDRNON did not. The cell-associated radioactivity was negligible in the MOLT-4 cells treated with the [^{32}P]-labeled anti-ODN. On the other hand, the [^{32}P]-labeled anti-S-ODN also strongly associated with the MOLT-4 cells. The cell-associated radioactivity in the MOLT cells treated with the [^{32}P]-labeled CDRDON increased rapidly and reached a plateau after 60 min of treatment, and the NDRDON and anti-ODN oligomers yielded about 12-fold less cell-associated counts than the CDRDON. A high level of cell-associated anti-SODN was observed. The circularization by resulting from the joining of the 3′ and the 5′ ends of the RNA/DNA chimeric oligonucleotides containing two hairpin loop structures increases the cellular uptake, as compared with the nicked dumbbell RNA/DNA chimeric oligonucleotide (NDRNOD) and the linear antisense phosphodiester oligodeoxynucleotides (anti-ODN).

The new class of antisense oligonucleotides described here has two hairpin loop structures with RNA/DNA

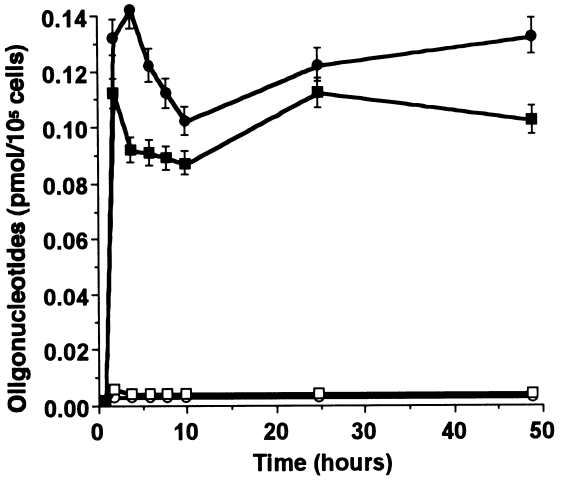

Figure 5. Cellular uptake of the [^{32}P]-labeled-CDRNON and NDRNON. The MOLT-4 cells were incubated with the [^{32}P]-labeled anti-ODN (○), the [^{32}P]-labeled NDRNOD (□), the [^{32}P]-labeled CDRNON (■), and the [^{32}P]-labeled anti-S-ODN (●) at 37 °C for various times. Cell-associated radioactivity was determined as described in the Materials and Methods. Experiments were carried out in triplicate and the data represent average values and standard deviations.

base pairs (sense (RNA) and antisense (DNA)) in the double helical stem. The circular dumbbell RNA/DNA chimeric oligonucleotide with the higher Tm value showed increased nuclease resistance. The circularization, achieved by joining the 3′ and 5′ ends of RNA/DNA chimeric oligonucleotides containing two hairpin loop structures, increases the oligonucleotide uptake into cells, as compared with the nicked dumbbell RNA/DNA chimeric oligonucleotide and the linear antisense phosphodiester oligodeoxynucleotide. The antisense phosphodiester oligodeoxynucleotide is liberated together with the RNA cleavage product by RNase H treatment of the dumbbell RNA/DNA chimeric oligonucleotides. The liberated antisense phosphodiester oligodeoxynucleotide was bound to the target 45 mer RNA, which gave 45 mer RNA cleavage products upon the treatment with RNase H. That is to say, when the circular dumbbell RNA/DNA chimeric oligonucleotide is directly delivered into animal cells or virus infected cells, its antisense phosphodiester oligodcoxynucleotide function appears. The limited toxicity of unmodified phosphodiester oligonucleotide and the sequence specific bound to target mRNA that circular dumbbell RNA/DNA chimeric phosphodiester oligonucleotide can be used with intact cells, and may prevent the retroviral development in culture. We plan to test the dumbbell RNA/DNA chimeric oligonucleotides in cell culture to evaluate their anti-viral activity.

Experimental

Oligonucleotide synthesis

The oligonucleotides were synthesized by means of the phosphoramidite approach using an Applied Biosystems DNA synthesizer, Model 392. The 5′-phosphorylated oligonucleotides were synthesized using a dimethoxytrityl-hexa-ethyloxy-glycol-2-cyanoethyl-*N*,*N*-diisopropyl phosphoramidite as the phosphorylating agent. The oligonucleotide derivatives were purified by polyacrylamide gel electrophoresis or by reverse phase HPLC chromatography. The extinction coefficients of the oligonucleotides were determined by calculating the theoretical extinction coefficients as the sum of the nucleosides and multiplying with the experimentally determined enzymatic hypochromicity. The 5′-phosphorylated nicked dumbbell RNA/DNA chimeric oligonucleotide (0.2 A_{260}) was incubated in 10 µl of 50 mM Tris–HCl, pH 7.5, and 10 mM $MgCl_2$ at 90 °C for 5 min, and then at 15 °C for 2 min. The solution was incubated with 1 µL of 100 mM dATP, 1 µL of 25 µg BSA per mL, and 40 units of T4 DNA ligase at 37 °C for 30 mins. The mixture was extracted with an equal volume of water-saturated chloroform/phenol (1:1, v/v), and was precipitated with ethanol. The circular oligonucleotides were purified by denaturing PAGE (20% polyacrylamide containing 7 M urea). The band corresponding to the desired oligonucleotide was eluted from the gels with water, and was precipitated with ethanol. The identity of each of the ligated dumbbells was verified by phosphodiesterase protection mapping.

Nuclease stability of nicked and circular dumbbell RNA/DNA chimeric oligonucleotides

The 3′-exonucleolytic activity of snake venom phosphodiesterase (SVPD) was measured by hyperchromicity at 260 nm. The oligonucleotide (0.2 A_{260}) was dissolved in 0.7 ml buffer (10 mM Tris/HCl, pH 8.5, 10 mM $MgCl_2$, and 100 mM NaCl), and was incubated with SVPD at 37 °C in a thermally regulated cell of a UV spectrophotometer, and the A_{260} was recorded against time (Figure 2A). To study the resistance of the oligonucleotides to the endonucleolytic activity of nuclease S1, the oligonucleotide (0.2 A_{260}) was dissolved in 0.7 mL buffer (30 mM CH_3COONa, 280 mM NaCl, 1 mM $ZnSO_4$, pH 4.6), and was incubated with nuclease S1 at 37 °C in a thermally regulated cell of a UV spectrophotometer, and the A_{260} was recorded against time (Figure 2B). To determine the resistance of the oligonucleotides to nucleases in fetal bovine serum, the oligonucleotides (0.2 A_{260}) were incubated with 200 µL of culture medium containing 10% fetal bovine serum for 24 h at 37 °C. Aliquots were taken at 0, 3, 6, 12, and 24 h and were analyzed by PAGE (20% polyacrylamide containing 7 M urea). Densitomeric analysis of gels stained with

silver nitrate was performed on a Millipore BioImage 60 S (Figure 3).

Thermal denaturation profiles

Thermal transitions were recorded at 260 nm using a Shimadzu UV-2200A spectrometer. The insulated cell compartment was warmed from 25 °C to 90 °C, with increments of 1 °C and equilibration for 1 min after attaining each temperature, using a temperature controller, SPR-8 (Shimadzu). Samples were heated in masked, 1 cm path length quartz cuvettes fitted with Teflon stoppers. Each thermal denaturation was performed in 10 mM sodium phosphate buffer (pH 7.0) and 10 mM NaCl, containing 0.5 μM of each strand (sens-RNA-anti-ODN, sens-ODN-anti-ODN, NDRNON, CDRNON, 45 mer-RNA-CDRNON, and 45 mer-RNA-CDRDON). The mixture of duplex and single strands was kept at 90 °C for 10 min, and was then cooled to 4 °C.

RNase H activity

The 45 mer RNA (5′UGUUUCACAACAAAAG-CCUUAGGCAUCUCCUAUGGCAGGAGA - AG3′) (1 pmol) was mixed with oligonucleotides (10 pmol) in 30 μL of 20 mM Tris–HCl (pH 7.5), 10 mm $MgSO_4$, 0.1 mM DTT, and 100 mM KCl. RNasin (40 units) and *E. coli* RNase H (0.5 μL, 0.4 units) were added to the mixture, which was incubated at 37 °C. Aliquots were taken at 0, 1, 2, and 4 h, and were analyzed by PAGE (20% polyacrylarnide containing 8.3 M urea). Densitometric analysis of gels stained with silver nitrate was performed on a Millipore BioImage 60 S (Figure 4).

Cellular uptake of [^{32}P]-labeled nicked and circular dumbbell RNA/DNA chimeric oligonucleotides

The 5′-ends of the nicked dumbbell RNA/DNA chimeric oligonucleotide were labeled with [γ-^{32}P]dATP and T4 polynucleotide kinase in buffer containing 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 10 mM DTT, 0.2 mM spermidine, and 0.2 mM EDTA at 37 °C for 30 mins. The labeled oligonucleotides were purified by denaturing PAGE (20% polyacrylamide containing 7 M urea). The band corresponding to the desired oligonucleotide was eluted from the gels with water, and was precipitated with ethanol. The circularization of the 5′-phosphorylated nicked dumbbell RNA/DNA chimeric oligonucleotide to give the circular dumbbell RNA/DNA chimeric oligonucleotide was carried out as described, and then the oligonucleotide was purified using a Quick spin column (G-25). MOLT-4 cells were diluted to 1×10^5 cells/mL in RPMI-1640 medium containing 10% heat-inactivated FCS and were dispensed in 24 multi-well plates. After a 48 h incubation at 37 °C in a CO_2 incubator, the RPMI-1640 medium was changed. The cells were further incubated with the 3′-end labeled oligomers for the stated periods. After washing the cells four times with chilled phosphate-buffered saline (PBS), the cells were pelleted by centrifugation and were lysed in 0.5 mL of 1% sodium dodecyl sulfate (SDS). The cell associated radioactivity was determined by liquid scintillation counting and is expressed as pmol/10^5 cells.

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