Antisense Oligonucleotides with Different Backbones

MODIFICATION OF SPLICING PATHWAYS AND EFFICACY OF UPTAKE*

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Gabriela Schmajuk, Halina Sierakowska‡, and Ryszard Kole§
From the Lineberger Comprehensive Cancer Center and Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599

A novel, positive read-out assay that quantifies only sequence-specific nuclear activity of antisense oligonucleotides was used to evaluate morpholino and 2′-O-methyl sugar-phosphate oligonucleotides. The assay is based on modification of the splicing pathway of human β-globin pre-mRNA. In addition, scrape-loading of cells with oligonucleotides allows the separate assessment of intracellular antisense activity of the oligonucleotides and their ability to penetrate the cell membrane barrier.

The results show that, with scrape-loading, the morpholino oligonucleotides were approximately 3-fold more effective in their intrinsic antisense activity than alternating phosphodiester/phosphorothioate 2′-O-methyl-oligoribonucleotides and 6–9- and almost 200-fold more effective than the exclusively phosphorothioate and phosphodiester bonucleotides. The morpholino oligonucleotides were over 20-fold more effective than the phosphorothioate 2′-O-methyl-oligoribonucleotides in free uptake from the culture media. The antisense activity of the morpholino oligonucleotides was detectable not only in monolayer HeLa cells but also in suspension K562 cells. Time course experiments suggest that both the free uptake and efflux of morpholino oligonucleotides are slow.

Antisense oligonucleotides and RNAs show great promise as sequence-specific agents able to down-regulate the expression of targeted genes. In this capacity, extensively reviewed in a recent volume and review articles (1–4), they have advanced not only to clinical trials (5–8) but also to clinical practice (9); they have also proven to be useful as research tools (1). However, the application of antisense technology both in research and in clinical studies presents a number of outstanding issues.

In principle, the most important feature of antisense oligonucleotides is their ability to block mRNA function by sequence-specific hybridization to the RNA. Surprisingly, the oligonucleotides may also exert their effects by binding directly to a number of proteins in a sequence-dependent but not sequence-specific manner, resulting in unpredictable and non-specific effects (10–14). This non-antisense mechanism of binding was shown to be particularly pronounced in commonly used oligodeoxynucleoside phosphorothioates (15); other mechanisms also contribute to sequence-independent oligonucleotide effects (16).

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‡ On leave of absence from the Institute of Biochemistry and Biophysics, Warsaw, Poland.

§ To whom all correspondence and reprint requests should be addressed: Lineberger Comprehensive Cancer Center, University of North Carolina, CB 7295, Chapel Hill, NC 27599. Tel.: 919-966-1143; Fax: 919-966-3015; E-mail: kole@med.unc.edu.

Our ability to select appropriate target sequences within the RNA is still limited (17). Cell-free selection of susceptible sites is not always helpful (18, 19) because in vivo cellular RNAs are always complexed with proteins (20), which may block the sites and/or change the secondary and tertiary structure of the target RNA. Therefore, oligonucleotide targeting is frequently carried out by trial and error, requiring the synthesis of large numbers of compounds (21, 22).

Equally unsatisfactory is our understanding of the intracellular site of action of oligonucleotides. Most of the antisense oligonucleotides are designed against sequences within mRNA which presumably represents a cytoplasmic target. However, in cell culture, free oligonucleotides reaching the cytoplasm are sequestered in endosomal vesicles which severely limits their access to targeted mRNA (reviewed in Ref. 23). In contrast, oligonucleotides delivered by cationic lipids (24, 25) or other cationic agents (26, 27), resulting in marked antisense effects at low concentrations of the oligonucleotides, seem to be directed to the nucleus (25, 28). Rapid nuclear uptake is also observed with oligonucleotides microinjected into the cytoplasm (29, 30). In animal models, organ uptake of free oligonucleotides has been investigated (31, 32), but the data on their intracellular localization are still limited (33, 34).

We have recently developed an application of antisense oligonucleotides (35, 36) and RNAs (37) in which these agents are used for modification of the splicing pattern of pre-mRNA rather than for down-regulation of gene expression by targeting the mRNA. The modification of splicing is accomplished by blocking the aberrant pre-mRNA splice sites resulting from mutations within intron 2 of human β-globin gene. The two point mutations which will be dealt with in this report are located at positions 654 or 705 of the intron (IVS2–654 and IVS2–705, respectively) (38, 39). In the β-globin pre-mRNA, both mutations create aberrant donor (5′) splice sites and activate a pre-existing cryptic 3′ splice site at nucleotide 579 of the intron. Although the correct splice sites still exist at the ends of the intron, the mutated pre-mRNA is spliced aberrantly, resulting in defective β-globin mRNA that retains a fragment of intron 2 (Fig. 1A). In humans, these mutations lead to β-thalassemia, an inherited blood disorder (40). Blocking of the aberrant splice sites by antisense agents redirects the splicing machinery to the correct splice sites, restoring the proper splicing pathway and, therefore, the correct expression of the damaged gene (Fig. 1 in Ref. 36). This result is not only of clinical interest but also provides a positive read-out assay for evaluating the efficacy of various antisense oligonucleotides.

When antisense oligonucleotides are used to decrease the translation of targeted mRNA either by blocking it or via its RNase H mediated degradation (reviewed in Ref. 41), their effects may be easily overlooked due to the high background of the pre-existing mRNA and/or gene product. Moreover, as pointed out above, the down-regulation may be due to non-
to TRI-Reagent lysis. As will be shown below, the shift of splicing from aberrant to correct also indicates that the oligonucleotide hybridized to the aberrant splice site, a target sequence with a well defined function, and therefore that its effect was via a sequence-specific, antisense mechanism.

In this report we have taken advantage of the splicing modification assay to characterize the effects of various oligonucleotide backbones on sequence-specific antisense activity. The assay allowed us to discern the factors, such as efficiency of uptake and delivery, stability in the extra- and intracellular environment, and the potency of binding to the target sequence, which contribute to the differences in the overall antisense effects of oligonucleotides.

**EXPERIMENTAL PROCEDURES**

**Cells**—HeLa IVS2–654 and IVS2–705 cell lines have been described previously (36, 43). IVS2–705 K562 cells were a gift from L. Gorman. The cell lines were obtained by stable transfection of HeLa or K-562 cells with the IVS2–654 or IVS2–705 thalassemic β-globin genes cloned under the immediate early cytomegalovirus promoter (36). HeLa and K562 cells were grown in minimum essential medium modified for suspension cultures, 5% fetal calf serum, 5% horse serum, 50 μg/ml gentamicin, 200 μg/ml kanamycin, and in Dulbecco’s modified Eagle’s medium, 10% filtered Colorado calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, respectively. For all experiments, cells were plated in six-well plates at 3 × 10^5 cells/well, 24 h before oligonucleotide treatment.

**Oligonucleotide Treatment**—18-mer oligonucleotides targeted to the aberrant 5′ splice sites in the IVS2–654 (ON-654, GCCUUACCUCUAAACCAG) or IVS2–705 (ON-705, CUCUUACCUCAGUACUA) mutants were used in all experiments. Morpholino oligonucleotides (44) were a gift from Antivirals and GeneTools companies. 2′-O-Methyl-oligoribonucleoside phosphorothioates were a gift from Hybriden.

Cellular delivery of the oligonucleotides was either by scrape-loading (45) or by free uptake from the media. For HeLa cells scrape-loading, 24 h after plating the cells the culture medium was replaced with 3 ml of fresh medium at 37 °C containing the desired concentration of oligonucleotide at 654. The cells were immediately scraped with a rubber policeman (WVR Scientific), and the cell suspension was transferred with a 5-ml pipette into an adjacent empty well. After 24 h of incubation (or the times indicated in Fig. 6) at 37 °C, the reattached cells were washed in six-well plates at 3 × 10^5 cells/well, 24 h before oligonucleotide treatment.

**RESULTS**

**Scrape-loading of IVS2–654 HeLa Cells with 2′-O-Methyl-oligoribonucleotides**—We have shown previously that 18-mer 2′-O-methyl-oligoribonucleoside-phosphorothioate (ON-654, P= S) in a complex with LipofectAMINE, a cationic lipid (45), efficiently restored correct splicing of human β-globin IVS2–654 pre-mRNA in stably transfected IVS2–654 HeLa cells expressing the mutated β-globin gene (see “Experimental Procedures” and Ref. 36 for more details).

In the experiment shown in Fig. 2A, the ON-654, P=S oligonucleotide was taken up by cells that were scraped off the surface of the culture plate in the presence of increasing concentrations of the oligonucleotide. The scraping leads to a temporary mechanical damage of the cell membranes allowing the oligonucleotides to migrate through the membrane barrier and enter the cells by diffusion. Thus, this method (Ref. 46; see also “Experimental Procedures”) eliminates the need for lipid delivery agents and is particularly useful for direct comparison of oligonucleotides with different backbones since the backbones may affect the efficiency of delivery of oligonucleotides by the lipid carriers. RT-PCR of total RNA from treated cells indicated that the ON-654,P=S corrected IVS2–654 pre-mRNA splicing in a dose-dependent manner (Fig. 2A, lanes 2–7). Quantitation of these results (see “Experimental Procedures”) and calculation of the effective concentration of the oligonucleotide showed 50% correction of splicing (EC_{50}) at 3.1 μM oligonucleotide (Table I).

| Oligonucleotide | IVS2–654 EC_{50} | IVS2–705 EC_{50} |
|-----------------|-----------------|-----------------|
| Scrape-loading  |                |                 |
| ON-654, P=S     | 3.1             | 8.4             |
| ON-654wt, P=S   | 12.0            | 1.7             |
| ON-654, P=O     | 67              | ND              |
| ON-654, P=OP=S  | 0.9             | ND              |
| ON-705, P=S     | ND              | 0.15            |
| ON-705wt, P=S   | NA              | 3.8             |
| ON-654, M       | 0.35            | 5.0             |
| ON-705, M       | NA              | 0.026           |

ND, not determined; NA, oligonucleotide not active.
A phosphodiester version of ON-654 2'-O-methyl oligoribonucleotide (ON-654,P=S) was tested in the same system. It was found to be more effective than the phosphorothioate modification in correcting IVS2–654 pre-mRNA splicing in a cell-free system, a nuclear extract from HeLa cells (35). This was not unexpected since the phosphorothioate modification lowers the Tm values of the oligonucleotide-RNA duplex approximately 0.7 °C per modification (reviewed in Ref. 47). However, despite its higher Tm, the 2'-O-methyl-modified oligonucleotide with a phosphodiester backbone was almost totally ineffective in scrape-loaded HeLa cells (Fig. 2B). Only a trace, if any, of correctly spliced β-globin pre-mRNA could be detected at 3 or 6 μM oligonucleotide (Fig. 2B, lanes 6 and 7, respectively). Its EC50 (67 μM, Table I) indicates that this oligonucleotide is at least 20-fold less efficient than its phosphorothioate counterpart in intracellular correction of pre-mRNA splicing. The phosphodiester derivative was also ineffective when delivered to the cells in oligonucleotide-LipofectAMINE complex.

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\(^3\) Y. Wang and R. Kole, unpublished data.

\(^4\) H. Sierakowska and R. Kole, unpublished data.
Since scrape-loading bypassed the issue of efficiency of oligonucleotide uptake while the $T_m$ values favored the ON-654,P=O oligonucleotide, its ineffectiveness seemed likely to be due to its susceptibility to intracellular nucleases. This interpretation is supported by the observation that an alternating ON-654,P=O/P=S oligonucleotide was much more effective than the phosphodiester or even phosphorothioate versions (Fig. 2C; $EC_{50}=0.9 \mu M$, Table I). Similarly, ON-654,P=O/P=S complexed with LipofectAMINE corrected splicing more efficiently than ON-654,P=S.\(^5\) It appears that the alternating phosphorothioate residues increased the resistance of the oligonucleotide to intracellular nucleases without unduly lowering its hybridization potential.

Scrape-loading of IVS2–654 HeLa Cells with Morpholino Oligonucleotide—Morpholino oligonucleotides have high $T_m$ values and are highly resistant to nucleases. Due to their neutral backbone, they cannot be delivered to cells by cationic lipid carriers but are readily taken up by the cells via scrape-loading (46). Consistently with these properties, the morpholino oligonucleotide, ON-654,M, corrected IVS2–654 pre-mRNA splicing with approximately 9-fold higher efficiency than ON-654,P=S (Table I). The antisense effect of ON-654,M was dose-dependent (Fig. 3, lanes 2–7) and sequence-specific, as shown by the lack of correction of splicing by morpholino oligonucleotides antisense to rabbit $a$-globin mRNA (Fig. 3, lane 8) or to a sequence centered around nucleotide 706 of $\beta$-globin intron 2 (Fig. 3, lane 9).

Scrape-loading of IVS2–705 HeLa Cells with ON-705,P=S and ON-705,M Oligonucleotides—IVS2–705 pre-mRNA (Fig. 1A) offers another sequence target for antisense oligonucleotides. The IVS2–705 splice site is more sensitive than the IVS2–654 or -705 splice sites in either IVS2–654 or -705 pre-mRNAs was very inefficient (Fig. 5, A and B, lanes 1–5). Even at the highest concentration of the oligonucleotides, 15 $\mu M$ for ON-654 and 5 $\mu M$ for ON-705, only trace amounts of correctly spliced RNA were detected (Fig. 5, A and B, lanes 5). EC\(_{50}\) values for ON-654,P=S and ON-705,P=S (Table I) indicated that the free uptake of these oligonucleotides reduced their overall antisense activity 70- and over 130-fold, respectively, over scrape-loading.

Although the activity of the morpholino oligonucleotides was also reduced, a dose-dependent correction of splicing by ON-654 and ON-705 morpholino oligonucleotides was clearly detectable under free uptake conditions (Fig. 5, A and B, lanes 7–10, respectively). Overall, in free uptake morpholino oligonucleotides corrected splicing over 20-fold more efficiently than their 2'-O-methyl-phosphorothioate counterparts.

**Time Course of Correction of IVS2–705 Splicing by Morpholino Oligonucleotide in HeLa Cells**—In all of the above experiments, the cells were treated with the oligonucleotides for 24 h (see “Experimental Procedures”). To test the kinetics of correction of splicing, the IVS2–705 HeLa cells were treated with 5 $\mu M$ ON-705 morpholino oligonucleotide either with or without scrape-loading and the RNA was isolated at time intervals indicated in Fig. 6.

In scrape-loaded cells, significant correction of splicing occurred 1 h after treatment (Fig. 6A, lane 2) and within the next 3 h the correction was virtually complete (Fig. 6A, lane 4). Interestingly, although aberrantly spliced $\beta$-globin mRNA was no longer detectable, the correct mRNA continued to accumulate up to 48 h (Fig. 6A, lanes 5–9). The correction efficiency was 100% since no aberrantly spliced $\beta$-globin RNA was observed. Note that, during the experiment, HeLa cells underwent approximately two divisions and their number almost quadrupled. This indicates that despite substantial dilution enough morpholino oligonucleotide remained in the nuclei to

\(^5\) M. Vacek, H. Sierakowska, and R. Kole, unpublished data.
The application of antisense oligonucleotides for modification of splicing not only holds promise as potential treatment for β-thalassemia and other genetic disorders, it also provides a sensitive, relatively easy to quantify assay for antisense activity. Probably its most important feature is that detection of correctly spliced β-globin mRNA proves that the investigated oligonucleotides had been taken up by the cells, migrated to the nucleus, and modified splicing in a sequence-specific manner. Moreover, in conjunction with delivery methods that bypass the membrane barriers, such as scrape-loading, the assay allows for analysis of individual factors contributing to the overall antisense activity of a given oligonucleotide.

Comparison of oligonucleotides with 2′-O-methyl-oligoribonucleoside-phosphodiester, -phosphorothioate, and morpholino backbones showed that the latter were the most effective both when scrape-loaded and when freely taken up from the culture media. This is consistent with the morpholino oligonucleotide stability, high 7tn value of its hybrids (50) and the possibility that the neutral morpholino backbone facilitated oligonucleotide transfer through the cell membrane and the endosomal lipid bilayers. Similar antisense effects were seen in K562 cells (Fig. 7 and data not shown), indicating that the activity and uptake differences were not limited to HeLa cells. However, the fact that the antisense activity in scrape-loaded cells was 30-fold higher for the morpholino backbone and 70–130 for the P=S one than in free uptake (Table I) illustrates that the cellular and endosomal membranes constituted enormous barriers for both types of compounds.

The virtual lack of activity of P=S oligonucleotides in unscraped cells is at variance with several studies that showed free uptake and antisense activity of P=S oligonucleotides both in cell culture and in animal models (reviewed in Ref. 51). This discrepancy may be due to several factors. 1) The splicing modification assay reflects only the nuclear activity of the oligonucleotides. Cytoplasmic antisense effects, if any, would not have been detected. 2) A number of studies showed uptake of oligonucleotides into the cytoplasm without parallel assays of their activity. Those results may not account for the fact that the oligonucleotides become trapped in the endosomes and unavailable for interaction with the target mRNA (23). 3) The oligonucleotide effects in a number of studies were found to be due to non-antisense, not necessarily intracellular mechanisms (11–14).

Likewise, while only minimal or no antisense activity was detected when free morpholino oligonucleotides were used for down-regulation of targeted mRNAs in HeLa cells (52), this report shows clearly detectable antisense activity of these compounds. This difference could be due to a slow rate of uptake of morpholino oligonucleotides and/or the fact that nuclear and not cytoplasmic target were used in this study. Note, however, that morpholino oligonucleotides taken up by the RAW 264.7 macrophage-like cells were found effective in down-regulating tumor necrosis factor-α mRNA (53). Overall, the splicing modification assay provides a more sensitive and stringent test of
true antisense activity of the oligonucleotides.

In oligonucleotides targeted to the IVS2–654 5′ splice site, a change from the A-U base pair in ON-654 to the G-U base pair in ON-654wt (Fig. 2A and Table I) led to an almost 4-fold reduction in efficiency of correction. A mismatch in ON-705wt targeted to IVS2–705 5′ splice site reduced the antisense effects approximately 25-fold (Table I). Such marked changes are not commensurate with the change in the Tm of the duplexes but may be due to competition with splicing factors that bind to the 5′ splice site sequence. A possible candidate is the U1 small nuclear RNA-protein complex, which hybridizes to the 5′ splice site via a 9-nucleotide antisense sequence. This small nuclear RNA-protein complex, presumably due to its protein content, can provide efficient splicing even if the 9-nucleotide sequence includes two mismatches (42, 54). We cannot exclude the possibility that the drop in efficacy of the mismatched oligonucleotides was due to their degradation by single-strand-specific nucleases.

The ON-654wt oligonucleotide hybridized to the IVS2–705 pre-mRNA without a mismatch. Its target site was then located within the fragment of the intron retained in the aberrantly spliced RNA in an exon-like fashion. Interestingly ON-654wt promoted detectable correction of splicing. This result is consistent with the large body of evidence that exons are recognized by the splicing machinery (reviewed in Ref. 55) and suggests the antisense oligonucleotides need not be targeted to the splice sites in order to modify splicing pathways.

This study was focused on correction of splicing of β-globin pre-mRNA in two cell lines, HeLa and K562. Our previous (36) and unpublished data show that correction of antisense oligonucleotides occurs also in 3T3 and Chinese hamster ovary cell lines and not only in mutated β-globin but also in the cystic fibrosis transmembrane conductance regulator gene5 and other model systems with improved read-outs (56). These may provide quantitative information on the uptake and sequence-specific activity of antisense oligonucleotides in a variety of cellular backgrounds. The approach applied here allowed us to identify morpholino oligonucleotides as promising candidates for animal and possibly clinical studies.

Aberrant splicing of β-globin pre-mRNA in β-thalassemia patients may offer an attractive target for morpholino oligonucleotides. Increase in patient β-globin mRNA to 20–30% of the normal level would be of therapeutic significance since carriers of this disorder, with 50% of hemoglobin, are frequently asymptomatic while the status of patients undergoing transfusion therapy, with even lower hemoglobin levels, is markedly improved. Furthermore, β-globin mRNA and protein are very stable while the life span of mature erythrocytes is about 120 days (40). Thus, in principle, relatively infrequent administration of morpholino oligonucleotides may have an extended effect on the in vivo levels of β-globin mRNA and blood hemoglobin. Whether these compounds exhibit favorable pharmacokinetic, pharmacodynamic, and toxicity profiles remains to be established. A recently developed thalassemic mouse (57) provides an excellent model for in vivo testing of the antisense therapies.
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