Current Topics

Biological and Pharmaceutical Aspects of Nucleic Acids Chemistry

2′-*O*,4′-*C*-Ethylene-Bridged Nucleic Acids (ENA™) as Next-Generation Antisense and Antigene Agents

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Novel 2′-*O*,4′-*C*-ethylene nucleosides have been synthesized as building blocks for antisense and antigene oligonucleotides. 2′-*O*,4′-*C*-Ethylene-bridged nucleic acids (ENA™) comprising 2′-*O*,4′-*C*-ethylene nucleosides have considerable affinity to complementary RNA and double-stranded DNA. Incorporation of 2′-*O*,4′-*C*-ethylene nucleosides into oligonucleotides dramatically increase their resistance against exonucleases. In this review, the properties of ENA oligonucleotides and some of their applications as antisense and antigene oligonucleotides are described.

Key words 2′-*O*,4′-*C*-ethylene-bridged nucleic acid; antisense oligonucleotide; antigene; exon-skipping

1. INTRODUCTION

The demand for antisense oligonucleotides (AONs) is increasing due to their use as a tool for gene validation in drug discovery and their potential as a new class of drugs for the treatment of diseases such as cancer, inflammation and viral diseases.[1)] The action mechanisms of AONs involve translation arrest, mRNA degradation mediated by RNase H and splicing arrest as shown in Fig. 1. Phosphorothioate oligodeoxynucleotides (PS ODN, **b** in Fig. 2), which are used as popular antisense molecules, have favorable properties such as nuclease resistance, and are able to be recognized by RNase H.[1,2)] One PS ODN is in the market and others are under clinical testing.[1—3)] However, they have drawbacks in their use, such as low affinity to RNA ($\Delta T_m = -0.5$—1 °C per modification) and nonsequence-specific protein binding, which would be the cause of significant side effects, such as inhibition of the blood clotting cascade, activation of the complement cascade and severe hypotension *in vivo*.[1)] Many researchers have focused on the development of other types of modified oligonucleotides as next-generation AONs.[1,4)] 2′-*O*-Alkyl modifications are known to be of value in enhancing the binding affinity to target RNA and nuclease resistance.[5)] In particular, a 2′-*O*-(2-methoxy)ethyl modification (2′-MOE) showed high RNA affinity ($\Delta T_m = +2$ °C per modification) with high nuclease stability (**c** in Fig. 2). Some oligonucleotide gapmers with four or five 2′-MOE residues at both the 3′ and 5′ ends, which target TNF-α mRNA to inhibit inflammation and PTP1B mRNA to improve diabetes are in the clinical stages.[3,6)] Morpholino oligonucleotides are capable of hybridizing to mRNA with thermal stability (ΔT_m=not more than 1 °C per modification) due to the elimination of anionic repulsion between the phosphodiesters (**d** in Fig. 2).[7)] Thiophosphoramidate oligonucleotides (NPS, **e** in Fig. 2) can

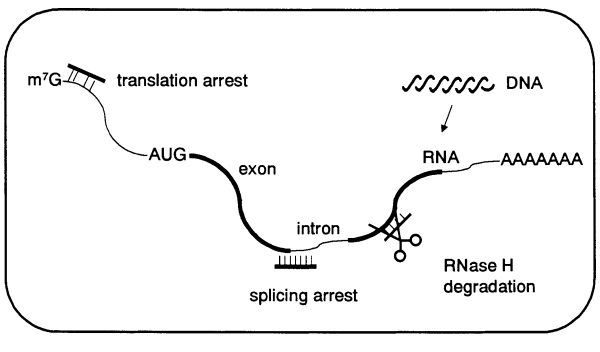

Fig. 1. Proposed Mechanism of Action of AONs

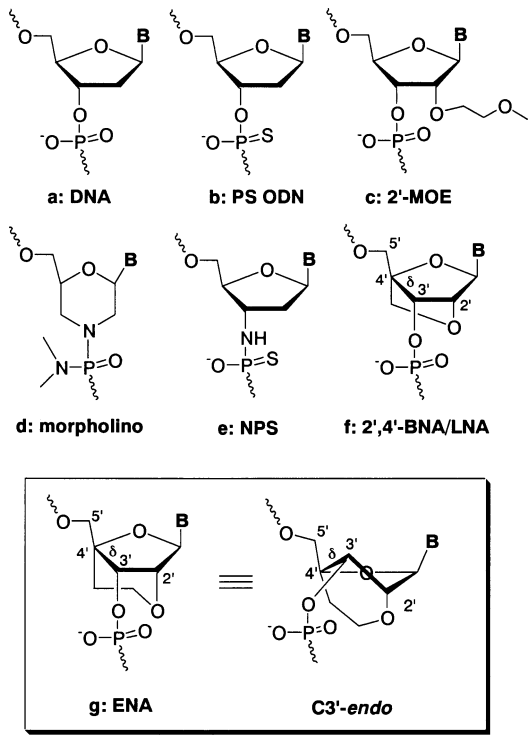

Fig. 2. Structures of DNA and Modified Oligonucleotides (B: Nucleobases)

a, DNA; **b**, phosphorothioate-modified ODN (PS ODN); **c**, 2′-*O*-methoxyethylnucleoside-modified oligonucleotides (2′-MOE); **d**, morpholinonucleoside-modified oligonucleotides (morpholino); **e**, thiophosphoramidate-modified oligonucleotides (NPS); **f**, 2′,4′-bridged nucleic acids/locked nucleic acids (2′,4′-BNA/LNA); **g**, 2′-*O*,4′-*C*-ethylene-bridged nucleic acids (ENA).

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Table 1. Modified Oligonucleotides Used as AONs

Name	Structure[a)]	Target	Company	Stage	Refereneces
PS ODN	**b**	bcl-2, PKCα *etc.*	Genta, ISIS	Ph III	1, 2
2′-MOE	**c**	TNFα, PTP1B *etc.*	ISIS	Ph II	3, 6
Morpholino	**d**	c-myc	AVI	Ph II	7
NPS	**e**	Telomerase	Geron	Pre-clinical	9

a) Their structures are shown in Fig. 2.

hybridize to mRNA with increasing thermal stability (ΔT_m= +2.2—2.6 °C per modification).[8)] Oligonucleotides, such as GRN163, comprising NPS residues have been reported as telomerase inhibitors, which could act as novel antitumor agents.[9)]

Imanishi's group and Wengel's group have independently reported the synthesis of novel 2′-*O*,4′-*C*-methylene nucleosides whose sugar puckering is fixed in the *N*-conformation as in RNA (**f** in Fig. 2), and that oligonucleotides containing these bridged nucleosides (2′,4′-BNA/LNA) showed an unprecedented level of affinity toward their complementary RNA (ΔT_m=+3—8 °C per modification).[10—12)] Recently, we have reported the synthesis of novel 2′-*O*,4′-*C*-ethylene thymidine, which has a less-strained six-membered ring than the five-membered ring of 2′-*O*,4′-*C*-methylene thymidine (**g** in Fig. 2).[13)] The corresponding oligonucleotides with 2′-*O*,4′-*C*-ethylene nucleosides retain a binding affinity to ssDNA and ssRNA as high as 2′,4′-BNA/LNA and show excellent triplex formation with dsDNA.[13—15)] They also exhibit much higher nuclease-resistance than 2′,4′-BNA/LNA.[13—15)] Here, we review the properties of 2′-*O*,4′-*C*-ethylene-bridged nucleic acids (ENA) having 2′-*O*,4′-*C*-ethylene nucleosides and the application of their oligonucleotides for use as antisense and antigene oligonucleotides.

2. CONFORMATIONAL PROPERTIES OF 2′-*O*,4′-*C*-ETHYLENE NUCLEOSIDES

2′-*O*,4′-*C*-Ethylene nucleosides and their corresponding phosphoramidites containing all possible natural bases have been synthesized.[13,14)] The conformational analysis of 2′-*O*,4′-*C*-ethylene nucleosides by ^{1}H-NMR has shown that in all 2′-*O*,4′-*C*-ethylene nucleosides, the coupling constant ($J_{H1'-H2'}$) was 0 Hz, which was identical to that of 2′-*O*,4′-*C*-methylene nucleosides.[14)] This means that the furanose puckering of 2′-*O*,4′-*C*-ethylene nucleosides are all fixed in the C3′-*endo* conformation (Fig. 2, **g**). Similar results have been obtained by X-ray crystal structure analysis of the 2′-*O*,4′-*C*-ethylene nucleosides, which show typical C3′-*endo* conformations.[14,15)] The difference between 2′-*O*,4′-*C*-ethylene nucleosides and 2′-*O*,4′-*C*-methylene nucleosides appear clearly in the δ torsion angle of C5′–C4′–C3′–O3′ (Fig. 2, **f**, **g**), which is one of the defining angles of the ribose conformation.[16)] It has been reported that the mean δ angles of 2′-*O*,4′-*C*-ethylene nucleosides (Fig. 2, **g**) and 2′-*O*,4′-*C*-methylene nucleosides (Fig. 2, **f**) are 77° and 66°, respectively.[15)] From the viewpoint of oligonucleotide structure, this conformation and this difference of the δ angle in each nucleoside unit influences the antisense and triplex formation of oligonucleotides with complementary RNA and dsDNA, respectively, as explained below.

3. NUCLEASE RESISTANCE OF ENA OLIGONUCLEOTIDES

It is thought that stable oligonucleotides in plasma might be used in antisense and antigene therapeutics.[1)] Oligonucleotides modified with an ENA residue at the second position from the 3′ end have shown greater stability against exo- and endonucleases than those modified with a 2′,4′-BNA/LNA residue.[13,14)] The stability of an oligonucleotide modified with an ENA residue is identical to that of an oligonucleotide with a PS *R*p diastereomer. An oligonucleotide containing two ENA residues at the 3′ end is more stable than an oligonucleotide with a PS *S*p diastereomer, which is known to be a stable isomer. Oligonucleotides composed of contiguous ENA residues without PS modification show greater nuclease stability than PS ODN. Furthermore, the stability of ENA oligonucleotides in rat plasma has been reported.[17)] ENA gapmers with a PS ODN center still remain even after 24 h. They are more stable than PS ODN and 2′,4′-BNA/LNA oligonucleotides, of which half are degraded in 4 h in rat plasma.

4. DUPLEX FORMATION OF ENA OLIGONUCLEOTIDES WITH RNA AS ANTISENSE MOLECULES

It has been reported that the duplexes of an oligonucleotide containing ENA residues and its complementary RNA have a higher UV melting temperature (T_m) than a natural DNA/DNA and DNA/RNA duplex by +3.5—5 °C per residue modification, which is also the case for duplexes containing 2′,4′-BNA/LNA residues.[13,14)]

Based on the CD spectra, although DNA/DNA duplexes usually show a B conformation, incorporation of some ENA residues into DNA/DNA duplexes changed the conformation from B to an A-like conformation.[14)] In the case of complementary RNA, both the CD spectrum of a duplex containing 2′,4′-BNA/LNA residues and that containing ENA residues indicated an A-like conformation.[14)]

Although a duplex of an ENA oligonucleotide with the complementary RNA is not a substrate for RNase H, a duplex of a DNA–ENA–DNA gapmer-designed oligonucleotide with the complementary RNA is cleaved by RNase H. This cleavage rate is much faster than that of a natural DNA/RNA duplex, possibly due to a higher binding activity of the oligonucleotide containing ENA residues.[17)]

5. TRIPLEX FORMATION OF ENA OLIGONUCLEOTIDES WITH dsDNA AS ANTIGENE MOLECULES

Antigene molecules that inhibit gene expression by binding to dsDNA in a sequence-specific manner and block tran-

scription, are sought for the treatment of various gene-related diseases.[18] As examples of such antigenes, it has been reported that oligopyrimidine nucleotides partially modified with ENA residues can form a triplex with dsDNA at physiological pH.[15] These oligonucleotides form triplexes similarly to those partially modified with 2′,4′-BNA/LNA residues, as determined by UV melting analyses, electromobility shift assays, CD spectral analyses and restriction enzyme inhibition assays.

Moreover, although no triplex is formed with fully modified 2′,4′-BNA/LNA, oligonucleotides fully modified with ENA, which are all in the C3′-*endo* conformation, have high triplex formation ability.[15] This conformation state may explain why the ENA units of these oligonucleotides have torsion angle δ values that are marginally higher than 2′,4′-BNA/LNA by 11°/unit as described above. These results provide useful information for designing fully modified antigene oligonucleotides using modified nucleosides, which are all in the C3′-*endo* conformation.

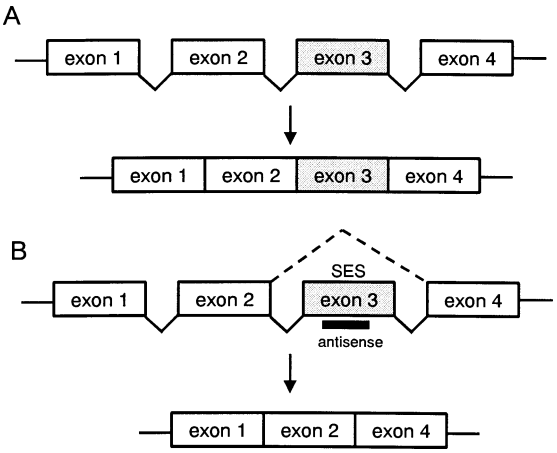

Fig. 3. Mechanism of Exon-Skipping of Pre-mRNA Using Antisense Molecules

(A) Natural splicing reaction; (B) exon-skipping reaction of exon 3 using antisense molecules. SES: splicing enhancer sequences.

6. APPLICATION OF ENA OLIGONUCLEOTIDES AS ANTISENSE MOLECULES

Some examples of applications using ENA oligonucleotides as antisense molecules have been reported.[17,19,20] ENA–DNA–ENA gapmer-designed oligonucleotides that can undergo RNase H-mediated degradation are used as AONs. When ENA-modified AONs against vascular endothelial growth factor (VEGF) mRNA were introduced into human cancer A549 cells in the presence of a cationic polymer, more than 90% inhibition of VEGF mRNA production was observed after RT-PCR analysis.[17] Mismatched ENA AONs did not show any inhibitory activity. These results indicate that ENA AONs act in a sequence-specific manner and could be used as effective antisense drugs.

AONs that specifically target the genes of rat organic anion transporting polypeptide (oatp) subtypes were selected by using antisense *in vitro* selection and a conventional gene alignment program.[19,20] When several ENA residues were incorporated into AONs, the inhibitory activity of these oatp AONs was enhanced. Moreover, these ENA AONs did not lose their oatp-subtype selectivity.

By introducing oligonucleotides, a new technology associated with exon-skipping has been developed whereby RNA may be modified to acquire new properties, as shown in Fig. 3.[21,22] Splicing-related proteins, such as U1 and U2 snRNP, bind to pre-mRNA and play rule in the splicing of pre-mRNA to mature mRNA. These proteins recognize splicing enhancer sequences (SES) to promote a splicing reaction. An AON binding to one of SES in the pre-mRNA could prevent the splicing reaction of the exon. Duchenme muscular dystrophy (DMD) is a rapid and progressive muscle-wasting disease, which is characterized by the absence of dystrophin in the muscle. On the other hand, Becker muscular dystrophy (BMD) is a less-severe form of the disease. Both DMD and BMD are allelic diseases that cause deletion mutations. Although the translational reading frame of the dystrophin gene in DMD is out-of-frame, that in BMD is in-frame.[21] Antisense PS ODN against a particular region of exon 19 of the dystrophin gene have been shown to induce exon-skipping of exon 19 derived from patients, who have an out-of-frame dystrophin gene and to promote the expression of internally deleted dystrophin by correcting the translational reading frame.[23] To make highly active molecules that induce exon skipping, oligonucleotides having the same sequence as the PS ODN but with some stretches of modified backbone, 2′-*O*-methyl RNA with several ENA residues at both the 5′ and 3′ ends, were designed.[24] The 2′-*O*-methyl RNA/ENA chimera induced exon-19 skipping in a dose- and time-dependent manner. The exon-19-skipping activity of the 2′-*O*-methyl RNA/ENA chimera was more than 40 times stronger than that of the corresponding conventional PS ODN.

7. CLOSING REMARKS

AONs are widely applied not only *in vitro* but also *in vivo* to elucidate the mechanisms of target genes related to diseases such as cancer, inflammation and diabetes.[2,3,6] In particular, nuclease-resistant oligonucleotides with ENA residues could have be used as antisense and antigene oligonucleotides because of their high binding affinity to mRNA and dsDNA, respectively, and their nuclease-resistance. As well, ENA oligonucleotides might be used as a tool in functional genomics.

8. NOTE

In Japan, ENA oligonucleotides having 2′-*O*,4′-*C*-ethylene nucleosides are commercially available from Sigma Genosys Japan (http://www.genosys.co.jp). ENA is a trademark of Sankyo Lifetech Co., Ltd.

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