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Note

**Synthesis and Evaluation of Oligonucleotide-Containing 2’-O-\{[(4,5’,8-trimethylpsoralen)-4’-ylmethoxy]ethylaminocarbonyl\}adenosine as Photo-crosslinkable Gene Targeting Tools**

Yu Mikame, a Yui Sakai, a# Ryo Tahara, a# Kinuka Doi, a Tsuyoshi Yamamoto, a
Chikara Dohno, b Takayuki Shibata, c* and Asako Yamayoshi a*

a*Graduate School of Biomedical Sciences, Nagasaki University; 1-14 Bunkyo-machi, Nagasaki, Nagasaki 852-8521, Japan.

bSANKEN (The Institute of Scientific and Industrial Research), Osaka University; 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan

cGraduate School of Health Sciences, Gunma University; 3-39-22 Showa-machi, Maebashi, Gunma 371-8514, Japan.

*Correspondence e-mail: asakoy@nagasaki-u.ac.jp, tshibata@gunma-u.ac.jp

# These authors contributed equally to this study.
Summary
Several psoralen-conjugated oligonucleotides (Ps-Oligos) have been developed as photo-crosslinkable oligonucleotides targeting DNA or RNA. To avoid potential off-target effects, it is important to investigate the selective photo-crosslinking reactivity of Ps-Oligos to DNA or RNA. However, the selectivity of these Ps-Oligos has not been reported in detail thus far. In this study, we evaluated the photo-crosslinking properties of two Ps-Oligos, 5’-Ps-Oligo and a novel Ps-Oligo containing 2’-O-{{(4,5’,8-trimethylpsoralen)-4’-ylmethoxy]ethyaminocarbonyl}adenosine (APs2-Oligo). Notably, 5’-Ps-Oligo preferentially crosslinked with DNA, whereas APs2-Oligo preferentially crosslinked with RNA. These results demonstrate the interesting crosslinking properties of Ps-Oligos, which will provide useful information for the molecular design of novel Ps-Oligos in future studies.

Keywords
photoreactive, oligonucleotide, psoralen, preference, antisense
Introduction

Psoralen derivatives are used as dermal photosensitizing molecules for the treatment of various skin pigmentation disorders.\(^1\)-\(^3\) These compounds are also known as DNA intercalators and can form covalent bonds via the [2+2] cycloaddition reaction with pyrimidine bases upon ultraviolet (UV) irradiation.\(^4\),\(^5\) Therefore, psoralens have been applied as one of DNA intercrosslinking\(^6\),\(^7\) agents in various research fields.\(^8\)-\(^10\) Several research groups have developed psoralen-conjugated oligonucleotides (Ps-Oligos) targeting DNA or RNA. These Ps-Oligos can be used to form crosslinkages in a sequence-specific manner via duplex formation. Some of Ps-Oligos have been used as antisense oligonucleotides and suppressed tumor growth by crosslinking with target mRNA and interfering with mRNA translation.\(^11\)-\(^14\) In contrast, we have developed Ps-Oligos, which can distinguish a point mutation (from a purine base to a pyrimidine base) in DNA in a sequence-specific manner based on the photo-crosslinking strategy.\(^15\),\(^16\) These Ps-Oligos can be used for the detection of oncogenes, such as ras, egfr, myc, and erb-B, which are well-known causes of tumorigenesis.\(^17\),\(^18\) The selective detection method for these oncogenes may provide valuable insight into the development mechanisms of these diseases. Thus, Ps-Oligos are expected to be a genome-targeting technology.

As described above, Ps-Oligos are widely used as photo-crosslinkable oligonucleotides that target DNA and RNA. Therefore, to avoid potential off-target effects, it is essential to investigate the selective photo-crosslinking reactivity of Ps-Oligos to DNA and RNA. However, the selectivity of these Ps-Oligos has not been reported in detail thus far, and information on the relationship between the Ps-Oligo structure and its target selectivity is limited. This study aims to assess the photo-crosslinking property of 5′-Ps-Oligo (Fig. 1A), which possesses a psoralen moiety at its 5′-end, with complementary DNA and RNA. The psoralen moiety of 5′-Ps-Oligo is inserted into the 3′ side of its complementary nucleobase. Subsequently, a novel Ps-Oligo containing 2′-O-\([(4,5′,8\text{-trimethylpsoralen})-4′\text{-ylmethoxy}ethylaminocarbonyl]\text{adenosine (APs2-Oligo, Fig. 1B) is synthesized and its photo-crosslinking property to complementary DNA and RNA is evaluated to further investigate the}
photo-crosslinking property of Ps-Oligos. It is proposed that by introducing psoralen at the 2’-O-position of adenosine, the psoralen moiety of Aps2-Oligo is inserted into the 5’ side of its complementary nucleobase. Thus, Aps2-Oligo may crosslink with the same target thymidine (or uridine) as 5’-Ps-Oligo, which form base pairs with adenosine, in the target gene. Notably, Aps2-Oligo showed significant photo-crosslinking efficiency with RNA compared with DNA, suggesting that the photo-crosslinking selectivity of Ps-Oligo can be controlled by structural modification of Ps-Oligo.

Results and Discussion

Synthesis of Aps2-Oligo

The preparation of Aps2-Oligo was started with the synthesis of \( N^6 \)-Bz-5’-O-DMTr-2’-O-\{(4,5’,8-trimethylpsoralen)-4’-ylmethoxy\}ethylaminocarbonyl\}adenosine-3’-(2-cyanoethyl-N,N’-diisopropylphosphoramidite) (Chart 1, 1). We decided to introduce psoralen via carbamate linkage, since it is synthetically easier to prepare than the previously reported 2’-O-psoralenylmethoxyethyladenosine.\(^{12}\) First, the alcohols at the 5’ and 3’ positions were simultaneously protected by disiloxane 2 according to a procedure reported in literature to give compound 3.\(^{19}\) Protected compound 3 was treated with carbonyldiimidazole to generate reactive intermediate 4 for the introduction of the psoralen units. Subsequently, intermediate 4 was mixed with 4’-(2-aminoethoxy)methyl- 4,5’,8-trimethylpsoralen 5, which was prepared separately according to a reported procedure.\(^{20}\) This reaction gave compound 6 in 31% yield with a psoralen moiety at the 2’ position via an ethoxyaminocarbonyl linker. Next, the disiloxane group was removed in the presence of TEA-3HF to give diol 7 in 85% yield. The primary alcohol of 7 was capped with a 4,4’-dimethoxytrityl group to obtain compound 8 in 74% yield, and the final treatment of the secondary alcohol of 8 with 2-Cyanoethyl \( N, N, N \)-tetraisopropylphosphordiamidite produced compound 1 in 29% yield. Using the amidite compound 1, the synthesis of psoralen-introduced oligonucleotide (Aps2-Oligo) was performed at Ajinomoto Genedesign (Osaka, Japan) as described in the
supplemental information.

Crosslinking Behavior of 5’-Ps-Oligo and APs2-Oligo

The photo-crosslinking behaviors of 5’-Ps-Oligo and APs2-Oligo were evaluated and the results were compared (Fig. 1). The fidelity in target sequence recognition by oligonucleotides is an important factor that influences photo-crosslinking efficiency, which is attributed to the difference in the thermal stability of the target hybrids. To properly evaluate the photo-crosslinking selectivity of 5’-Ps-Oligo toward DNA or RNA, two sequences were prepared for target DNA and RNA, as shown in Fig. 1. These sequences showed slightly higher $T_m$ value (Supplementary Fig. S5) during duplex formation with APs2-Oligo ($T_m = 69 \, ^\circ C$ for DNA and RNA) (Fig. 1B) than 5’-Ps-Oligo ($T_m = 68 \, ^\circ C$ for DNA and RNA) (Fig. 1A). These differences might be probably attributed to the additional base-pairs of adenosine of APs2-Oligo.

Subsequently, the photo-crosslinking efficiencies of 5’-Ps-Oligo and APs2-Oligo were evaluated using denaturing polyacrylamide gel electrophoresis (denaturing PAGE). UV irradiation was conducted for a maximum of 120 s, and samples were collected at different time steps (0, 1, 5, 10, 20, 30, 60, and 120 s). Consequently, 5’-Ps-Oligo was selectively crosslinked with DNA (Fig. 2A) but not efficiently with RNA (Fig. 2B). The crosslinking efficiency of 5’-Ps-Oligo with DNA was 40% higher than that with RNA. On the other hand, APs2-Oligo crosslinked with both DNA (Fig. 3A) and RNA (Fig. 3B), and interestingly, the crosslinking efficiency of APs2-Oligo with RNA was higher than that with DNA (Fig. 3C).

The Difference in Duplex Structure May Affect Target Preference

To determine whether the RNA selectivity of APs2-Oligo in a crosslinking reaction is influenced by differences in the nucleobase structure (DNA: thymine, RNA: uracil), a photo-crosslinking experiment was conducted using DNA in which the target thymine was replaced with uracil (DNA-U). Interestingly, the crosslinking efficiencies of APs2-Oligo with DNA and DNA-U were nearly the same (Fig. 4).
These results suggest that the preferential crosslinking of APs2-Oligo to RNA is not caused by structural differences in the target nucleobase. Based on these results, we propose that certain structural differences in the duplex (DNA duplex generally forms B-form double helix, while DNA-RNA duplex adopts the A-form for both strands)\textsuperscript{21-23} may affect the target preference of APs2-Oligo during photo-crosslinking. We conducted structural simulations of duplex models for both 5’-Ps-Oligo (Supplementary Fig. S6) and APs2-Oligo (Supplementary Fig. S7). The psoralen moiety of 5’-Ps-Oligo stacks with target thymine in DNA models, while the psoralen of 5’-Ps-Oligo is away from the target uracil in RNA models. This is considered as the reason why the 5’-Ps-Oligo crosslinked selectively to DNA. On the other hand, from the structural simulations, the psoralen moiety of APs2-Oligo stacks with both thymine (in DNA) and uracil (in RNA). These results can explain why APs2-Oligo could crosslinked with both DNA and RNA. Although we don’t have enough and clear evidence to explain the RNA crosslinking preference of APs2-Oligo, APs2-Oligo probably forms a better crosslinking intermediate with the target RNA upon duplex formation, and there is no doubt that APs2-Oligo is more suitable than 5’-Ps-Oligo as a candidate of nucleic acid medicines targeting RNA.

**Conclusion**

A novel oligonucleotide containing 2’-O-\{[(4,5’,8-trimethyl- psoralen)-4’-ylmethoxy]ethylaminocarbonyl\}adenosine (APs2-Oligo) are developed and the photo-crosslinking behavior of the APs2-Oligo toward target DNA and RNA was evaluated. APs2-Oligo preferentially crosslinked with RNA, whereas previously developed 5’-Ps-Oligo was crosslinked selectively to DNA. Our results suggest that the RNA-selective crosslinking property of APs2-Oligo is not caused by structural differences in the target nucleobase. From the structural simulation analysis, it is proposed that APs2-Oligo forms a more suitable crosslinking intermediate with the target RNA upon duplex formation. This study provides a valuable knowledge for the molecular design of novel Ps-Oligos targeting DNA (5’-Ps-Oligo) or RNA (APs2-Oligo) in the future. Further investigations are ongoing by our research
Experimental

Measurement of UV-Melting Profiles

The UV melting profiles of the 5’-Ps-Oligo and APs2-Oligo duplexes with their target oligonucleotides were measured using a UV spectrophotometer equipped with a programmed thermal controller at a ramp rate of 1.0 °C/min. The sample solutions were prepared in 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl, and the oligonucleotide concentration was set at 1.0 μM. Before the measurements, all the samples were heated at 95 °C for 5 min and slowly cooled from 95 to 25 °C at 1.0 °C/min for annealing.

Photo-crosslinking Reaction

Equimolar mixtures of TAMRA labeled 5’-Ps-Oligo or APs2-Oligo and their complementary oligonucleotides were prepared in a similar manner to the samples prepared for UV-melting profile measurements. After incubation, the reaction mixtures were UV-irradiated for 0–120 s using a UV-LED irradiator (OMRON ZUV-C30H, 365 nm), followed by analysis using denaturing PAGE (20% polyacrylamide/7 M urea/25% formamide). The crosslinking efficiencies were quantified by measuring TAMRA fluorescence.

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Conflict of Interest

The authors declare no conflict of interest.
Supplementary Materials

This article contains supplementary materials.
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**Chart 1.** Synthetic scheme of phosphoramidite 1 prepared from $N^6$-Benzoyladenosine.
Figure 1. Structures, sequences, and Tm values of 5'-Ps-Oligo and APs2-Oligo.

*Measurements were carried out at 260 nm for the equimolar mixture of a psoralen-conjugated oligonucleotide, and its complementary DNA (or RNA) in 0.01 M phosphate buffer (pH 7.0) with 0.1 M NaCl.
Figure 2. Photo-crosslinking behavior of 5’-Ps-Oligo. Analysis of the photo-crosslinking reaction with (A) DNA and (B) RNA was performed using denaturing PAGE (20% polyacrylamide/7 M urea/25% formamide/TBE). \([5’\text{-Ps-Oligo}] = [\text{DNA}] = [\text{RNA}] = 1 \mu\text{M}\) in 10 mM phosphate buffer containing 0.1 M NaCl (pH 7.0). (C) Graph of the photo-crosslinking results.
Figure 3. Photo-crosslinking behavior of APs2-Oligo.

Analysis of the photo-crosslinking reaction with (A) DNA and (B) RNA was performed using denaturing PAGE (20% polyacrylamide/7 M urea/25% formamide/TBE). [APs2-Oligo] = [DNA] = [RNA] = 1 μM in 10 mM phosphate buffer containing 0.1 M NaCl (pH 7.0). (C) Graph of the photo-crosslinking results.
Figure 4. Photo-crosslinking of APs2-Oligo with DNA-U.

(A) Sequences of DNA-U and APs2-Oligo. (B) Analysis of photo-crosslinking reaction with DNA-U was performed using denaturing PAGE (20% polyacrylamide/7 M urea/25% formamide/TBE). \([\text{APs2-Oligo}] = \text{[DNA-U]} = 1 \mu M\) in 10 mM phosphate buffer containing 0.1 M NaCl (pH 7.0). (C) Graph of the photo-crosslinking results.