COMMUNICATION

ChemComm
www.rsc.org/chemcomm

Psoralen-conjugated oligonucleotide with hairpin structure as a novel photo-sensitive antisense molecule

Asako Yamayoshi, Reiko Iwase, Tetsuji Yamaoka and Akira Murakami*

Department of Polymer Science and Engineering, Kyoto Institute of Technology, Matsugasaki, Kyoto 606-8585, Japan. E-mail: akiram@ipc.kit.ac.jp; Fax: +81-75-724-7814; Tel: +81-75-724-7814

Received (in Cambridge, UK) 27th February 2003, Accepted 16th April 2003
First published as an Advance Article on the web 13th May 2003

Hairpin type psoralen-cojugated oligonucleotides cross-linked with RNA when they hybridized with a perfectly complementary RNA.

The antisense strategy has received considerable attention for its potential application to the regulation of the gene expression in a sequence specific manner.[1] In our previous study, we designed a photo-cross-linking reagent, the psoralen derivative (4,5′,8-trimethylpsoralen), conjugated oligo(nucleoside phosphorothioate)s (Ps–S–Oligo), and examined its regulatory effects on cervical carcinoma cells.[2] The psoralen derivatives have the ability to covalently cross-link with pyrimidine bases, favorably with thymine and uracil, upon UV irradiation (320–400 nm)[3] and have been conjugated with oligonucleotides.[4] Ps–S–Oligo drastically inhibited the cellular proliferation of the cancer cells in a sequence specific manner only upon UV irradiation (IC_{50} = 16 nM) without any drug delivery systems.[2] Our results showed that these oligonucleotides have significant potential to regulate gene expression.

One of the drawbacks of this strategy lies in the possible uncertainty in recognizing RNA. In our previous study,[2] we observed sequence independent regulatory effects on the cellular proliferation at higher concentration. These results suggested that the sequence specificity of the linear type psoralen-conjugated oligonucleotides is not always displayed. To avoid this possibility, we developed a new class of photo-sensitive antisense oligonucleotides. The oligonucleotide forms the hairpin structure with psoralen at the 5′-terminus (Fig. 1). The hairpin structure has been applied to confer additional functions to the oligonucleotides.[5] Agrawal *et al.*[6] adopted the hairpin structure as their antisense molecules to acquire nuclease resistance. The molecular beacon, which displays FRET upon hybridization to its complementary nucleic acid, is based on the hairpin structure.[7] We adopted the hairpin structure for the psoralen-conjugated oligonucleotides for improving their sequence specificity. The synthesized oligodeoxyribonucleotides are shown in Fig. 2. Our designed oligonucleotides consist of 21-nucleotides with a 10-nucleotide-loop sequence. The loop region as well as the 6 nucleotides of the 5′-arm of the stem region are complementary to the target-RNA. The rest of the sequence is not complementary to the target-RNA, but it is necessary to facilitate the hairpin formation. The hybrid formation of this molecule with RNA will proceed *via* four steps. The first step is the hybrid formation between the loop region and the RNA. The second is the unwinding of the stem region, and the third is the additional hybrid formation of the 5′-arm sequence with the RNA. Finally, the psoralen favorably intercalates between bases of the RNA. The initial contact of the loop region of this molecule with the RNA sequence is the key step, which is expected to be primarily responsible for the sequence specificity. If there are any mismatch pairings, the hybrid cannot be stable under the physiological condition. Furthermore, the 5′-arm region, which has a psoralen at the end, does not participate in the hybrid stabilization during the first step. Therefore, the photo-cross-linking reaction between this oligonucleotide and the target-RNA is expected to occur only when the loop region of the oligonucleotide encounters its perfectly complementary target-RNA. To evaluate the selective binding of the hairpin type psoralen-conjugated oligonucleotides to the RNA, two control hairpin oligonucleotides were synthesized. One was composed of 2 base-mismatch bases at the loop and the other was the fully scrambled sequence at the loop. To the 5′-end of these three oligomers, 4′-{[N-(aminomethyl)amino]methyl}-4,5′,8-trimethylpsoralen was introduced.[2]

For the sequence specific cross-linking reaction, it is essential that these oligonucleotides form a certain hairpin structure.

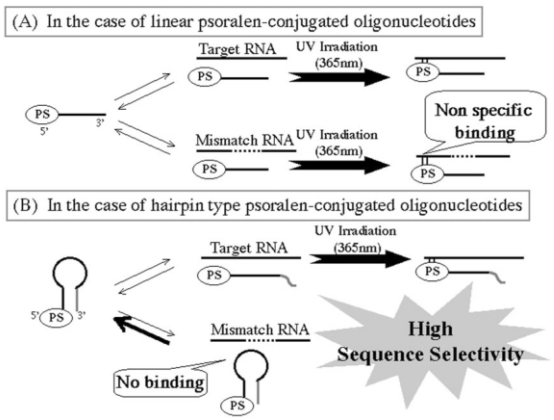

Fig. 1 Schematic overview of specific binding reaction of the hairpin type psoralen-conjugated oligonucleotide and the target-RNA.

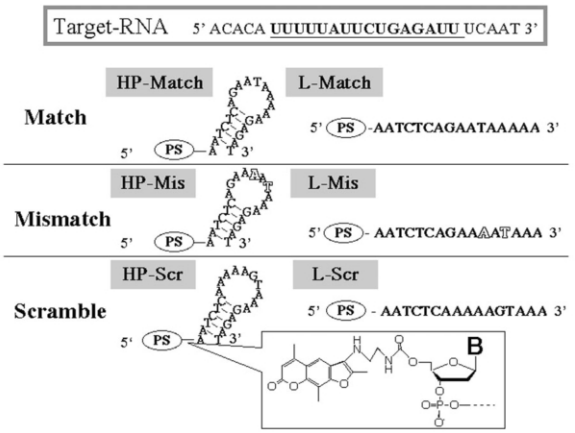

Fig. 2 Chemical structures and sequences of the hairpin type psoralen-conjugated oligonucleotides and the linear one. PS = psoralen.

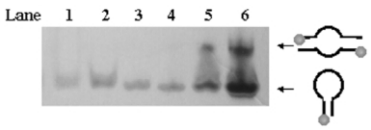

Fig. 3 Analysis of the dependence of the hairpin-duplex population ratio on the concentration of oligonucleotides by non-denaturing PAGE (15% polyacrylamide, 10 mM potassium phosphate, 50 mM NaCl, pH 6.5, 4.5 h, 4 °C). Lane 1, 10 nM; lane 2, 100 nM; lane 3, 500 nM; lane 4, 1 μM; lane 5, 100 μM; lane 6, 500 μM.

DOI: 10.1039/b302291a

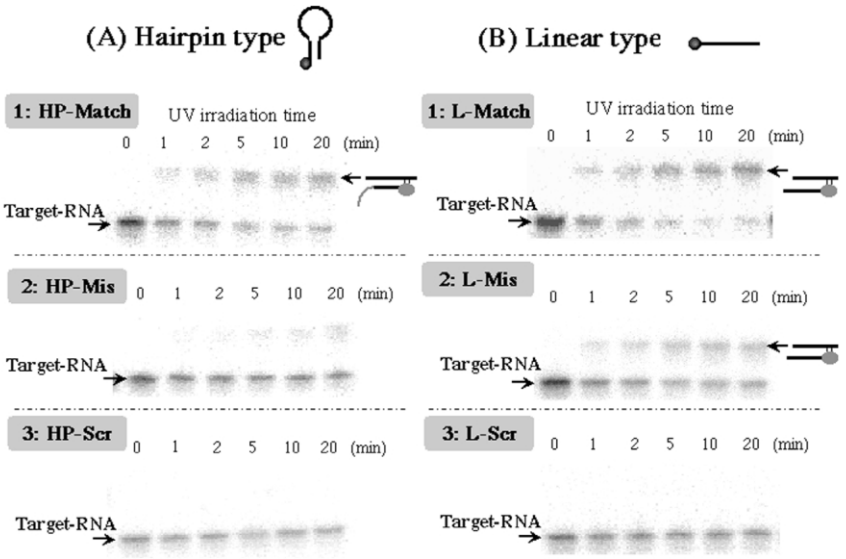

Fig. 4 Analysis of the photo-cross-linking reaction of the psoralen-conjugated oligonucleotides and the target-RNA by denaturing PAGE (20% polyacrylamide, 7 M urea–TBE, 300 V, 1.5 h, r.t.). (A) In the case of the hairpin type psoralen-conjugated oligonucleotides. (B) In the case of the linear psoralen-conjugated oligonucleotides.

There is a possibility that these types of oligonucleotides can form either a hairpin structure or a dimer with a bulged loop flanked by the stem region.[8] Therefore, the concentration dependence of the structural diversity of the HP-match was examined. The HP-match was annealed in the sample buffer (10 mM sodium phosphate, 0.1 M NaCl, 1 mM EDTA, 10% glycerol, pH 6.5) at various concentrations, and then analyzed by non-denaturing PAGE. Visualization of the oligonucleotides on the gels was achieved by silver staining (Fig. 3). A single band was observed in the concentration range from 10 nM to 1 μM, and an additional band with a low mobility clearly appeared at the higher concentration. At 5 μM, the UV-melting profile of the HP-match alone gave a single transition temperature of 44 °C (data not shown). If the HP-match forms a dimer with a bulged loop, the T_m value would be expected to be lower. These results suggest that the HP-match formed the hairpin structure below 5 μM, and the lower mobility band was derived from the dimer.

We evaluated the ability of the HP-match to photo-cross-link with the RNA at 100 nM. An equimolar mixture of a psoralen-conjugated oligonucleotide and the 5′-^{32}P-labeled target-RNA was incubated in phosphate buffered saline (0.135 M NaCl, 1.25 mM $MgCl_2$, pH 7.4) for 20 min at 37 °C. After incubation, the reaction mixture was UV-irradiated on a transilluminator (FUNAKOSHI FTI-LW, 365 nm, 1.1 mJ cm^{-2} sec^{-1}), and then analyzed by denaturing PAGE. At the stated condition, both HP-Scr and L-Scr did not form any photoproduct with RNA (Fig. 4 (A)-3, (B)-3). As the irradiation time increased, the HP-Match and L-Match cross-linked with the target-RNA (Fig. 4 (A)-1, (B)-1, yields: 71 and 94%, at 20 min, respectively). A similar photo-product was observed in the mixture of the L-Mis and target-RNA (Fig. 4 (B)-2, yield: 44% at 20 min) and was scarcely observed in the mixture of the HP-Mis and target-RNA (Fig. 4 (A)-2, yield: 8% at 20 min). These results suggest that the cross-linking of the mismatch antisense oligonucleotide with RNA was avoided by designing the hairpin type oligonucleotide.

It should be noted that the photo-cross-linking efficiency between the HP-match and target-RNA was lower than the linear type one (Fig. 4 (A)-1 and (B)-1). This result is ascribed to the smaller amount of the hybrid between the HP-match and target-RNA in the system when irradiated. In general, as Kushon *et al.*[9] discussed, the higher order structure including the hairpin structure of the antisense molecules imposes kinetic and thermodynamic penalties during the hybridization with their targets. Our results matched the discussion. Nevertheless, as the specificity in the mismatch discrimination remains the most important factor in the antisense method, it is postulated that the increase in the specificity compensates for the penalties mentioned above.[10]

In this study, we reached the conclusion that the hairpin type psoralen-conjugated oligonucleotides recognize their targets with a higher specificity than the linear type one. Our previous study[2] and the results presented here show that the psoralen-conjugated oligonucleotide can be a prominent next generation nucleic acid drug.

This work was partly supported by the Grant-in-Aid for Scientific Research (No. 14580609, AM, TY, RI), and Grants for Regional Science and Technology Promotion (AM) from the Ministry of Education, Science, Sports and Culture of Japan.

Notes and references

1 (*a*) P. S. Miller, L. T. Braiterman and P. O. P. Ts'o, *Biochemistry*, 1977, **16**, 1988; (*b*) R. J. Klasa, A. M. Gillum, R. E. Klem and S. R. Frankel, *Antisense Nucleic Acid Drug Dev.*, 2002, **12**, 193.
2 A. Murakami, A. Yamayoshi, J. Nishida, R. Iwase, T. Yamaoka and N. Wake, *Eur. J. Pharm. Sci.*, 2001, **13**, 25.
3 G. D. Cimino, H. B. Gamper, S. Issacs and J. E. Heart, *Ann. Rev. Biochem.*, 1985, **54**, 1151.
4 (*a*) B. L. Lee, A. Murakami, K. R. Blake, S-B. Lin and P. S. Miller, *Biochemistry*, 1988, **27**, 3197; (*b*) U. Englisch and D. H. Gauss, *Angew. Chem., Int. Ed. Engl.*, 1991, **30**, 613; (*c*) J. K. Kean and P. S. Miller, *Biochemistry*, 1994, **33**, 9178; (*d*) P. S. Miller, G. Bi, S. A. Kipp, V. Fok and R. K. Delong, *Nucleic Acids Res.*, 1996, **24**, 730; (*e*) L.-X. Wang, D.-Ch. Yang, Y.-Y. Lu, Y. Takagi, K. Taira, T. Li and L.-H. Zhang, *Drug Des. Discovery*, 1995, **13**, 109; (*f*) R. A. Cassidy, N. S. Kondo and P. S. Miller, *Biochemistry*, 2000, **39**, 8683.
5 (*a*) B. Poddevin, S. Meguenni, I. Elias and M. Vasseur, *Antisense Res. Dev.*, 1994, **4**, 147; (*b*) K. Hosono, T. Kuwasaki, T. Inagawa, K. Takai, H. Nakashima, T. Saito, N. Yamamoto and H. Takaku, *Antiviral Chem. Chemother.*, 1996, **7**, 86; (*c*) J. Li and R. M. Wartell, *Biochemistry*, 1998, **37**, 5154.
6 J. Y. Tang, J. Temsamani and S. Agrawal, *Nucleic Acids Res.*, 1993, **21**, 2729.
7 S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1996, **14**, 303.
8 R. Kirchner, M. Vogtherr, S. Limmer and M. Sprinzl, *Antisense Nucleic Acid Drug Dev.*, 1998, **8**, 507.
9 S. A. Kushon, J. P. Jordan, J. L. Seifer, H. Nielsen, P. E. Nielsen and B. A. Armitage, *J. Am. Chem. Soc.*, 2001, **123**, 10805.
10 N. E. Broude, *Trends Biotechnol.*, 2002, **20**, 249.