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PAPER

Formation of new base pairs between inosine and 5-methyl-2-thiocytidine derivatives†

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In this paper, we report DNA and 2′-OMe-RNA probes containing 5-methyl-2-thiocytidine (m^5s^2C) residues that can bind selectively and strongly to the corresponding RNA targets containing inosine residues by the significant stacking effect and steric hindrance of the 2-thiocarbonyl group.

Adenosine-to-inosine (A-to-I) RNA editing found in metazoans from worms to humans increases transcriptome diversity[1] and is essential for normal brain function.[2] During A-to-I editing, adenosine deaminase that acts on RNA (ADAR) catalyzes the hydrolytic deamination of adenine bases in double-stranded regions of pre-mRNAs or pri-miRNAs (Fig. 1). In case of mRNA, the translation machinery reads inosine as guanosine, leading to alterations in codons.[3] On the other hand, it was found that the A-to-I RNA editing of pri-miRNAs can modulate the processing and target recognition of miRNAs.[4] Although this editing is generally analyzed by DNA sequencing of cDNAs,[5] probes that can strongly and accurately hybridize with target RNAs containing inosine residues may be powerful tools for the rapid and simultaneous analysis of multiple RNA samples containing inosine residues and for inhibiting mRNAs and miRNAs after A-to-I editing.

The hypoxanthine base of inosine has acceptor and donor hydrogen bonding sites; it can form two hydrogen bonds with a cytosine base (Fig. 2a). It was previously reported by SantaLucia *et al.* that the general trend of base-pair instability is I–C > I–A > I–T ≈ I–G in a DNA duplex.[6] However, the hybridization and base recognition capabilities of probes containing a cytosine base at an inosine recognition site were not high enough to detect or inhibit the corresponding target RNAs because the stability of I–C was much lower than that of G–C, while the difference in stability between I–C and I–A was small. In this study, we designed and synthesized a new nucleobase to increase the hybridization and base recognition capabilities of probes that can bind to complementary targets having an I residue.

Fig. 1 A-to-I RNA editing of pre-mRNA or pri-miRNA catalyzed by ADAR.

In our previous study, it was reported that the introduction of a thiocarbonyl group at the 2-position of the uracil base in single-stranded RNAs increased their hybridization abilities by the strong stacking effect.[7] In addition, it is well known that a methyl group at the 5-position of pyrimidine bases increases the stability of DNA or RNA duplexes.[8] Therefore, we designed 5-methyl-2-thiocytosine (m^5s^2C) derivatives containing thiocarbonyl and methyl groups (Fig. 2b). We synthesized DNA probes 1–4 and RNA probes 1–3 containing m^5s^2C residues with a DNA synthesizer using 2′-deoxy- and 2′-OMe-m^5s^2C phosphoramidite units[9] by the general procedure (Fig. 3). The modified oligonucleotides were isolated by HPLC and characterized by MALDI-TOF mass spectrometry. (Supplementary Information†)

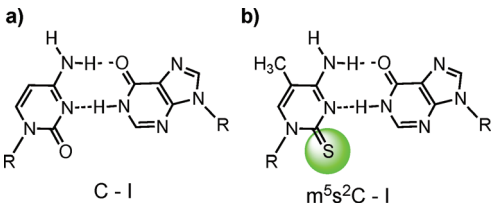

Fig. 2 Structures of a) C–I and b) m^5s^2C–I base pairs.

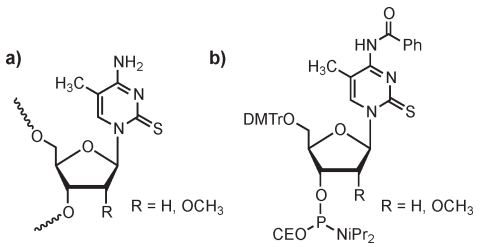

Fig. 3 Structures of a) 2′-deoxy- and 2′-OMe-m^5s^2Cs, b) 2′-deoxy and 2′-OMe-m^5s^2C phosphoramidite units.

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Table 1 T_m values for duplexes containing DNA probes 1–2 and RNA probes 1–3

DNA probe 1-2	5'- d[GCC XGT GAC] -3' X = C (probe 1), m^5s^2C (probe 2)
RNA probe 1-2	5'- MeO[GCC X'GU GAC] -3' X' = C (probe 1), m^5s^2C (probe 2)
RNA probe 3	5'- MeO[GCC m^5s^2C G m^5s^2C GAC] -3'
RNA target 1-3	3'- r[CGG YCA CUG] -5' Y = I (target 1), A (target 2), G (target 3)
RNA target 4	3'- r[CGG ICI CUG] -5'

entry	probes	RNA targets	T_m (°C)[a]	ΔT_m (°C)
1	DNA probe 1	1	40	—
2	DNA probe 1	2	29	−11[b]
3	DNA probe 1	3	50	10[b]
4	DNA probe 2	1	46	—
5	DNA probe 2	2	30	−16[b]
6	DNA probe 2	3	44	−2[b]
7	RNA probe 1	1	59	—
8	RNA probe 1	2	48	−11[b]
9	RNA probe 1	3	67	8
10	RNA probe 2	1	65	—
11	RNA probe 2	2	52	−13[b]
12	RNA probe 2	3	64	−1[b]
13	RNA probe 3	2	36	—
14	RNA probe 3	4	65	29[c]

[a] The T_m values are accurate within ±0.5 °C. T_m measurements were performed in a buffer containing 150 mM sodium phosphate (pH 7.0), 100 mM NaCl, 0.1 mM EDTA, and 2 μM duplex. [b] ΔT_m is the difference in the T_m value between RNA targets 1 and 2–3. [c] ΔT_m is the difference in the T_m value between entry 13 and entry 14.

Table 1 shows the T_m values of duplexes formed between DNA probes 1–2 and RNA targets 1–3 containing a component of sequence chr1:212596363, which is the noncoding region of BM042050.[5] The hybridization affinity of DNA probe 2 having a m^5s^2C residue toward RNA target 1 having an I residue was higher by 6 °C than that of DNA probe 1 having a C residue (entry 4: 46 °C *vs.* entry 1: 40 °C), although the T_m value of the duplex formed between DNA probe 2 and the complementary RNA target 2 having an A residue was similar to that of DNA probe 1 (entry 5: 30 °C *vs.* entry 2: 29 °C). These results demonstrate that the A-to-I discrimination of DNA probe 2 increased by 5 °C compared with that of DNA probe 1 (ΔT_m: 16 °C *vs.* 11 °C). In addition, it was found that the stability of the duplex containing a m^5s^2C–G base pair was lower than that containing a C–G base pair (entry 6: 44 °C *vs.* entry 3: 50 °C) because of the steric hindrance resulting from the thiocarbonyl group (Fig. 4). As a result, we could change the trend in base-pair

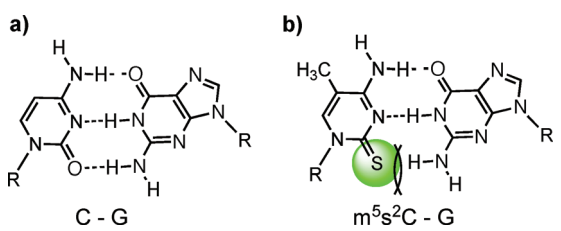

Fig. 4 Structures of a) C–G and b) m^5s^2C–G base pairs.

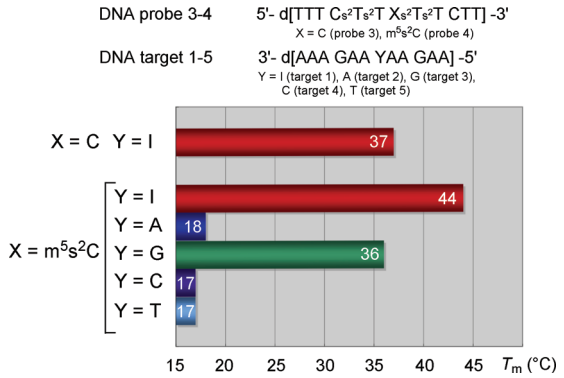

Fig. 5 The T_m values are accurate within ±0.5 °C. T_m values for DNA–DNA duplexes containing DNA probes 3–4. T_m measurements were performed in a buffer containing 150 mM sodium phosphate (pH 7.0), 100 mM NaCl, 0.1 mM EDTA, and 2 μM duplex.

stability from C–G > C–I > C–A to m^5s^2C–I > m^5s^2C–G > m^5s^2C–A.

Next, we examined the hybridization and base recognition abilities of RNA probes 1–2 (entry 7–12 of Table 1). The hybridization affinity of RNA probe 2 having a m^5s^2C residue for RNA target 1 having an I residue was much higher than that of DNA probe 2 (entry 10: 65 °C *vs.* entry 4: 46 °C) and RNA probe 1 (entry 10: 65 °C *vs.* entry 7: 59 °C). It was unexpectedly found that the hybridization affinity of RNA probe 2 for RNA target 2 having an A residue increased by 4 °C compared with that of RNA probe 1 (entry 11: 52 °C *vs.* entry 8: 48 °C). Therefore, although the discrimination of RNA probe 2 was higher than that of RNA probe 1 by 2 °C (ΔT_m: 13 °C *vs.* 11 °C), the A-to-I discrimination of RNA probe 2 decreased by 3 °C compared with that of DNA probe 2 (ΔT_m: 13 °C *vs.* 16 °C). Furthermore, it was observed that the difference in the T_m value between the m^5s^2C–G and C–G base pairs was only 3 °C (entry 12: 64 °C *vs.* entry 9: 67 °C) in RNA–RNA duplexes, whereas it was 6 °C in DNA–RNA duplexes (entry 6: 44 °C *vs.* entry 3: 50 °C). These results indicate that although the RNA probe had a higher hybridization affinity, the base recognition of the DNA probe having a m^5s^2C residue was superior to that of the RNA probe with the same residue.

Entries 13–14 of Table 1 show the T_m values of the duplexes formed between RNA probe 3 having two m^5s^2Cs and RNA target 4 having two I residues and RNA target 2. The stability of the duplex containing two m^5s^2C–I base pairs was significantly higher than that containing two m^5s^2C–A base pairs (entry 14: 65 °C *vs.* entry 13: 36 °C). These results indicate that our new probe with m^5s^2C substitutions could detect multiple A-to-I RNA editing. In addition, it was found that the m^5s^2C–I base pair could stabilize the RNA–RNA duplex at the same level as a U–A base pair. This was because the T_m value of the duplex formed between RNA probe 3 and RNA target 2 having two m^5s^2C–I base pairs was similar to that of the duplex formed between RNA probe 2 and the RNA target 1 having a m^5s^2C–I base pair and a U–A base pair (entry 14: 65 °C *vs.* entry 10: 65 °C).

Furthermore, we examined the stability of the m^5s^2C–I base pair in DNA–DNA duplexes (Fig. 5). The s^2T residues were also

introduced into DNA probes 3 and 4 to increase duplex stability by the strong stacking effect. As a result, the hybridization affinity of DNA probe 4 having a m^5s^2C residue toward DNA target 1 having an I residue was higher than that of DNA probe 3 having a C residue by 7 °C (T_m of X–Y = m^5s^2C–I: 44 °C *vs.* X–Y = C–I: 37 °C). Furthermore, it was observed that the stability of DNA duplexes containing m^5s^2C–I base pairs was much higher than those containing the base pairs formed between m^5s^2C and other natural bases. These results indicate that the m^5s^2C–I base pair may be very useful as a new code in DNA nanotechnology.

In summary, we have developed novel probes having m^5s^2C residues that can bind selectively and with high affinity to RNA targets having I residues. The hybridization and base discrimination abilities of these probes were much more substantial than those of previous probes containing a corresponding C residue. These results have encouraged us to explore the rapid detection of RNA samples containing I residues and the inhibition of mRNAs and miRNAs after A-to-I editing using our novel probes. Further studies in this direction are underway.

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