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THYMIDINE, 2-THIOTHYMIDINE, AND 2-SELENOTHYMIDINE: COMPARISON OF MISMATCH DISCRIMINATIONS

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Abstract – Oligonucleotides modified using 2-thiothymidine (S^2T) or 2-selenothymidine (Se^2T), which are thymidine analogs bearing either sulfur or selenium atoms, respectively, have been independently shown to suppress the formation of mismatched wobble base pairs with guanine when forming a duplex with single stranded DNA. However, limited data comparing the base discriminations of S^2T- and Se^2T-modified oligonucleotides, especially toward single stranded RNA, are available. Here, we report a comprehensive data set of the base discriminations of S^2T- and Se^2T-modified oligonucleotides toward both single stranded DNA and RNA. Our results demonstrate that S^2T- and Se^2T-modified oligonucleotides have similar base discrimination abilities. An important feature observed in both the S^2T- and Se^2T-modified oligonucleotides is their extremely high base discrimination toward single stranded RNA. Thus, S^2T and Se^2T modifications may be useful for therapeutic oligonucleotides targeting single stranded RNAs.

2-Thiothymidine (S^2T) and 2-selenothymidine (Se^2T) are thymidine analogs bearing sulfur and selenium atoms, respectively, at the 2 position. Such analogs in oligonucleotides are capable of forming proper Watson–Crick base pairs with adenine (**Figure 1a**) but efficiently suppress the formation of the thermodynamically stable mismatched wobble base pairs with guanine (**Figure 1b**), when forming a duplex with single stranded DNA (ssDNA).[1,2] Suppression of base pairing with guanine by the 2-thio and 2-seleno modifications can be explained by a steric bulkiness and/or weak hydrogen-bonding ability of the sulfur and selenium atoms. To date, many 2-thio- or 2-seleno-modified nucleic acids, such as 2-thiouridine,[3] 2'-*O*-methyl-2-thiouridine,[4] LNA-2-thiothymine,[5] LNA-2-thiouracil,[6] thioAmNA-2-thiothymine,[7] and 2-selenouridine,[8] have been synthesized and their base discriminations investigated, all of which were found to show a reduced affinity for guanine. These data suggest that S^2T, Se^2T, and their

analogs have promising potential for DNA microarrays, where mismatch discriminations are crucial to capture target strands with high sequence-specificity. While S^2T and Se^2T base discriminations toward ssDNA have been investigated, to our knowledge, limited data on the base discriminations of the S^2T-modified oligonucleotides toward single stranded RNA (ssRNA) are available,[9] and those of the Se^2T-modified oligonucleotides have not been evaluated so far. Here, we present a comprehensive data of the base discriminations of S^2T- and Se^2T-modified oligonucleotides toward both ssDNA and ssRNA. addition, hydrophobicity data of these modified oligonucleotides, evaluated using reverse-phase HPLC, also reported. These data will be helpful to understand the differences between natural, S^2T-modified, and Se^2T-modified oligonucleotides.

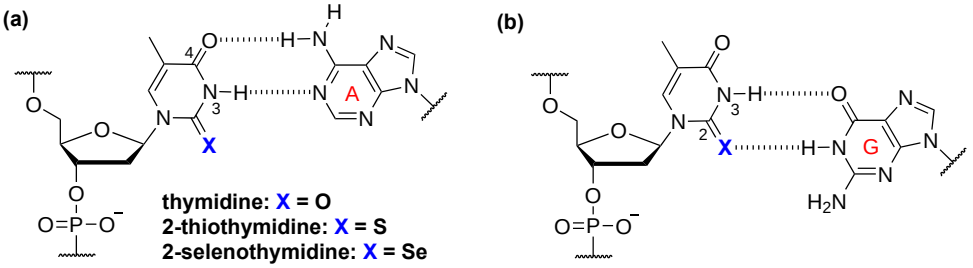

Figure 1. Hydrogen-bonding modes of (a) the Watson–Crick base pair with adenine and (b) the mismatched wobble base pair with guanine

To evaluate the effect of S^2T and Se^2T modifications on hydrophobicity and base discrimination, we designed and synthesized natural, S^2T-, and Se^2T-modified oligonucleotides with a sequence of 5'-d(GCGTT**X**TTTGCT)-3' [**X** = T (**ON1**), S^2T (**ON2**), and Se^2T (**ON3**)]. S^2T-modified **ON2** and Se^2T-modified **ON3** were obtained in high yields using previously reported protocols[10–14] (**Table 1**). **Figure 2** shows the reverse-phase HPLC analytical elution profiles for each of the oligonucleotides. The natural **ON1** peak was observed at 11 to 12 min, whereas peaks for S^2T-modified **ON2** and **ON3** appeared at around 14 min. This result indicated that the oligonucleotides containing S^2T or Se^2T were more hydrophobic than the natural oligonucleotide. Moreover, the elution profile suggested that the S^2T- and Se^2T-modified oligonucleotides were similarly hydrophobic.

Table 1. Isolated yields and MALDI-TOF MS data of the modified oligonucleotides

Oligonucleotide sequence[a]			MALDI-TOF MS	
ID	**X**	Yield (%)	Calcd $[M–H]^-$	Found $[M–H]^-$
ON2	S^2T	72	3648.4	3648.6
ON3	Se^2T	46	3695.3	3695.4

[a]5'-d(GCGTT**X**TTTGCT)-3'

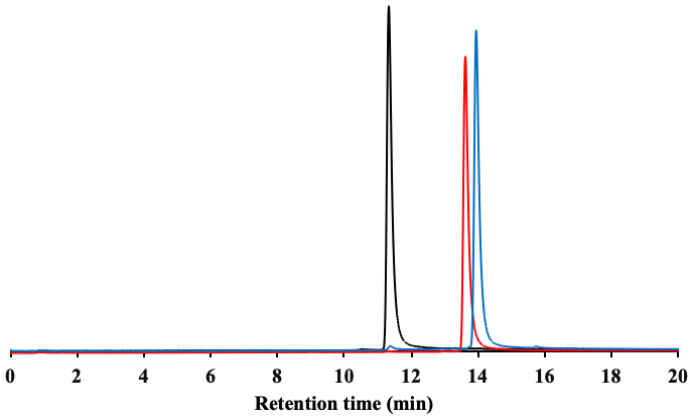

Figure 2. Analytical reverse-phase HPLC elution profiles of natural, S^2T-modified, and Se^2T-modified oligonucleotides. Natural, S^2T- and Se^2T-modified oligonucleotides are shown in black, red, and blue lines, respectively. The samples were analyzed using a reverse-phase HPLC column (Waters XTerra® MS C18 2.5 μm, 4.6 × 50 mm column) and eluted with a linear gradient from 11% to 21% (buffer A: 0.1 M triethylammonium acetate (TEAA); buffer B: a mixture of 0.1 M TEAA and MeCN (1:1); flow rate = 1.0 mL/min).

Table 2 shows melting temperature (T_m) values of **ON1**–**ON3** toward fully matched and one-base-mismatched ssDNAs. Similar to previous reports,[1,2] S^2T-modified **ON2** and Se^2T-modified efficiently destabilized the formation of mismatched wobble base pairs with guanine (ΔT_m = –14 and –15 °C, respectively). Base discrimination patterns of **ON2** and **ON3** were similar, but **ON2** exhibited slightly higher T_m values toward matched and one-base-mismatched ssDNAs than **ON3**. In consonance with previous work,[1,2] both S^2T-modified **ON2** and Se^2T-modified **ON3** greatly improved T·C mismatch discrimination. However, despite their enhanced T·G and T·C mismatch discriminations, overall base discriminations toward ssDNA were not improved from natural **ON1** as the ΔT_m values of their most stable mismatched duplexes were almost the same (**ON1**: –11 °C, **ON2**: –11 °C, and **ON3**: –12 °C).

We next evaluated the duplex forming abilities toward fully matched and one-base-mismatched ssRNAs (**Table 3**). Toward the full complementary strand (**Y** = A), S^2T-modified **ON2** and Se^2T-modified **ON3** showed similar T_m values with natural **ON1**. Therefore, the sulfur and selenium atoms at the 2 positions not negatively affect the formation of a Watson–Crick base pair with adenine. In contrast, both **ON2** and **ON3**, in comparison with **ON1**, exhibited decreased affinities for guanine and cytosine bases. Se^2T-modified **ON3** resulted in a lower affinity for guanine than S^2T-modified **ON2** when forming a duplex with ssRNA. Thus, it seems likely that the greater steric hindrance and/or weaker ability of selenium atoms have even greater impacts on guanine discriminations in the DNA/RNA duplex (A-form duplex). It was also found that S^2T·U and Se^2T·U mismatched base pairs have higher stabilities

than the T·U counterpart (ΔT_m = +5 and +4 °C, respectively). This can be understood because the sulfur atom of 2-thiouridine is known to stabilize the mismatched base pairing with uridine by forming an unusual hydrogen bond in a RNA/RNA duplex.[15] The S^2T·T mismatched base pair showed the same tendency (**Table 2**). As a consequence of the balance of T_m values, S^2T-modified **ON2** and Se^2T-modified **ON3** had dramatically enhanced overall mismatch discriminations toward ssRNA (ΔT_m values of the most stable mismatched duplexes: **ON1**: –5 °C, **ON2**: –11 °C, and **ON3**: –11 °C). S^2T and Se^2T were almost equal in mismatch discrimination abilities.

Table 2. T_m Values of duplexes formed between oligonucleotides and ssDNAs[a]

Oligonucleotide		T_m (ΔT_m = T_m [mismatch] – T_m [match]) (°C)			
ID	**X**	**Y** = A	**Y** = G	**Y** = C	**Y** = T
ON1[b]	T	52	41 (–11)	37 (–15)	38 (–14)
ON2	S^2T	51	37 (–14)	31 (–20)	40 (–11)
ON3	Se^2T	50	35 (–15)	30 (–20)	38 (–12)

[a]Conditions: 10 mM phosphate buffer (pH 7.2), 100 mM NaCl, and 4 μM of each oligonucleotide. The T_m values are the average of at least three measurements. The oligonucleotide sequence is 5'-d(GCGTT**X**TTTGCT)-3', and the sequence of ssDNA is 5'-d(AGCAAA**Y**AACGC)-3'. [b]Values are taken from reference 16.

Table 3. T_m Values of duplexes formed between oligonucleotides and ssRNAs[a]

Oligonucleotide		T_m (ΔT_m = T_m [mismatch] – T_m [match]) (°C)			
ID	**X**	**Y** = A	**Y** = G	**Y** = C	**Y** = U
ON1[b]	T	48	43 (–5)	32 (–16)	33 (–15)
ON2	S^2T	49	38 (–11)	30 (–19)	38 (–11)
ON3	Se^2T	48	35 (–13)	29 (–19)	37 (–11)

[a]Conditions: 10 mM phosphate buffer (pH 7.2), 100 mM NaCl, and 4 μM of each oligonucleotide. The T_m values are the average of at least three measurements. The oligonucleotide sequence is 5'-d(GCGTT**X**TTTGCT)-3', and the sequence of ssRNA is 5'-r(AGCAAA**Y**AACGC)-3'. [b]Values are taken from reference 16.

In conclusion, we demonstrated that oligonucleotides containing S^2T or Se^2T have a remarkably enhanced base discrimination toward ssRNA rather than ssDNA. The results therefore indicate that S^2T and Se^2T are valuable modifications for therapeutic oligonucleotides targeting ssRNAs.

EXPERIMENTAL

General experimental procedures

The mass spectra of all oligonucleotides were recorded on a MADLI-TOF mass spectrometer (Bruker Daltonics® Autoflex II TOF/TOF). For HPLC analysis, a SHIMADZU system with DGU-20A_{3R},

LC-20AD, CBM-20A, CTO-20AC, SPD-20A, and FRC-10A, was utilized. UV absorbance measurements were carried out using a SHIMADZU UV-1800 spectrometer.

Synthesis, purification, and characterization of S^2T- or Se^2T-modified oligonucleotides

For the synthesis of oligonucleotides containing S^2T (**ON2**) or Se^2T (**ON3**), the phosphoramidite method was carried out using an automated DNA synthesizer. S^2T phosphoramidite was purchased from Glen Research, and Se^2T phosphoramidite was prepared according to previous protocols.[2] 10% *tert*-Butyl hydroperoxide was used in each oxidation step (oxidation time = 10 min).[10,11] Other synthetic procedures followed the standard phosphoramidite protocol. After the elongation reactions, the columns were initially treated with 0.3 M 1,8-diazabicyclo[5.4.0]undec-7-ene in MeCN (1.0 mL) for 1 h at room temperature.[10,12,13] Cleavage from the solid support and removal of all protecting groups were accomplished using a 28% ammonia solution (1.5 h at room temperature and then 3 h at 55 °C).[10,14] The resulting oligonucleotides were rapidly purified with NAP™-10 columns (Sephadex™ G-25 DNA Grade), and further purified using reverse-phase HPLC (Waters XTerra® MS C18 2.5 μm, 10 × 50 mm column) (buffer A: 0.1 M triethylammonium acetate (TEAA) in water; buffer B: a mixture of 0.1 M TEAA and MeCN (1:1); flow rate = 3.0 mL/min). The purified oligonucleotides were analyzed using reverse-phase HPLC (Waters XTerra® MS C18 2.5 μm, 4.6 × 50 mm column), and their compositions were confirmed using MALDI-TOF-MS analysis. In the case of S^2T-modified **ON2**, the isolated yield was calculated using the previously reported extinction coefficient.[14] In the case of Se^2T-modified **ON3**, the isolated yield was determined using the following extinction coefficients: ε_{260} (H_2O) dG = 12500; dC = 7500; dT = 8500; and Se^2T = 2950.

UV melting experiments

UV experiments were performed on SHIMADZU UV-1650B and SHIMADZU UV-1800 spectrometers equipped with a T_m analysis accessory. The samples, which contained oligonucleotide (4 μM), target DNA or RNA (4 μM), 100 mM NaCl, and 10 mM phosphate buffer (pH 7.2), were annealed at 95 °C and cooled slowly to room temperature. The melting profile was recorded from 5 to 90 °C at a scan rate of 0.5 °C/min with detection at 260 nm. The T_m value was obtained from the temperature at which the formed duplexes were half-dissociated, based on the first derivative of the melting curve.

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