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

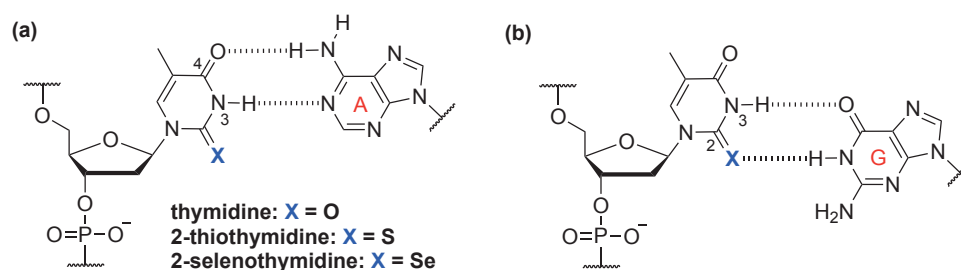
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**Abstract** – Oligonucleotides modified using 2-thiothymidine ( $S^2T$ ) or 2-selenothymidine ( $Se^2T$ ), which are thymidine analogs bearing either sulfur or selenium atoms, respectively, have been independently shown to suppress the formation of 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).<sup>1,2</sup> 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,<sup>3</sup> 2'-*O*-methyl-2-thiouridine,<sup>4</sup> LNA-2-thiothymine,<sup>5</sup> LNA-2-thiouracil,<sup>6</sup> thioAmNA-2-thiothymine,<sup>7</sup> and 2-selenouridine,<sup>8</sup> 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<sup>2</sup>T and Se<sup>2</sup>T base discriminations toward ssDNA have been investigated, to our knowledge, limited data on the base discriminations of the S<sup>2</sup>T-modified oligonucleotides toward single stranded RNA (ssRNA) are available,<sup>9</sup> and those of the Se<sup>2</sup>T-modified oligonucleotides have not been evaluated so far. Here, we present a comprehensive data set of the base discriminations of S<sup>2</sup>T- and Se<sup>2</sup>T-modified oligonucleotides toward both ssDNA and ssRNA. In addition, hydrophobicity data of these modified oligonucleotides, evaluated using reverse-phase HPLC, are also reported. These data will be helpful to understand the differences between natural, S<sup>2</sup>T-modified, and Se<sup>2</sup>T-modified oligonucleotides.



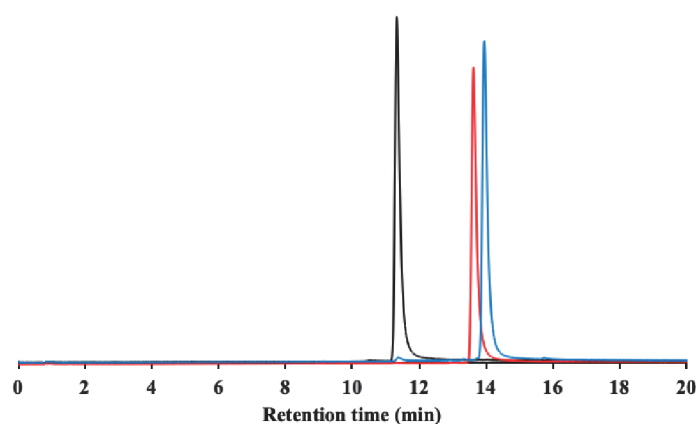
**Figure 1.** Hydrogen-bonding modes of (a) the Watson–Crick base pair with adenine and (b) the mismatched wobble base pair with guanine

To evaluate the effect of S<sup>2</sup>T and Se<sup>2</sup>T modifications on hydrophobicity and base discrimination, we designed and synthesized natural, S<sup>2</sup>T-, and Se<sup>2</sup>T-modified oligonucleotides with a sequence of 5'-d(GCGTTXTTTGCT)-3' [X = T (**ON1**), S<sup>2</sup>T (**ON2**), and Se<sup>2</sup>T (**ON3**)]. S<sup>2</sup>T-modified **ON2** and Se<sup>2</sup>T-modified **ON3** were obtained in high yields using previously reported protocols<sup>10–14</sup> (**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<sup>2</sup>T-modified **ON2** and Se<sup>2</sup>T-modified **ON3** appeared at around 14 min. This result indicated that the oligonucleotides containing S<sup>2</sup>T or Se<sup>2</sup>T were more hydrophobic than the natural oligonucleotide. Moreover, the elution profile suggested that the S<sup>2</sup>T- and Se<sup>2</sup>T-modified oligonucleotides were similarly hydrophobic.

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

| Oligonucleotide sequence <sup>a</sup> |                   | MALDI-TOF MS |                          |                          |
|---------------------------------------|-------------------|--------------|--------------------------|--------------------------|
| ID                                    | <u>X</u>          | Yield (%)    | Calcd [M–H] <sup>–</sup> | Found [M–H] <sup>–</sup> |
| <b>ON2</b>                            | S <sup>2</sup> T  | 72           | 3648.4                   | 3648.6                   |
| <b>ON3</b>                            | Se <sup>2</sup> T | 46           | 3695.3                   | 3695.4                   |

<sup>a</sup>5'-d(GCGTTXTTTGCT)-3'



**Figure 2.** Analytical reverse-phase HPLC elution profiles of natural, S<sup>2</sup>T-modified, and Se<sup>2</sup>T-modified oligonucleotides. Natural, S<sup>2</sup>T- and Se<sup>2</sup>T-modified oligonucleotides are shown in black, red, and blue lines, respectively. The samples were analyzed using a reverse-phase HPLC column (Waters XTerra<sup>®</sup> MS C18 2.5  $\mu$ m, 4.6  $\times$  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,<sup>1,2</sup> S<sup>2</sup>T-modified **ON2** and Se<sup>2</sup>T-modified **ON3** efficiently destabilized the formation of mismatched wobble base pairs with guanine ( $\Delta T_m = -14$  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,<sup>1,2</sup> both S<sup>2</sup>T-modified **ON2** and Se<sup>2</sup>T-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  $\Delta T_m$  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 ( $\underline{Y} = A$ ), S<sup>2</sup>T-modified **ON2** and Se<sup>2</sup>T-modified **ON3** showed similar  $T_m$  values with natural **ON1**. Therefore, the sulfur and selenium atoms at the 2 positions do 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. Interestingly, Se<sup>2</sup>T-modified **ON3** resulted in a lower affinity for guanine than S<sup>2</sup>T-modified **ON2** when forming a duplex with ssRNA. Thus, it seems likely that the greater steric hindrance and/or weaker hydrogen-bonding 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<sup>2</sup>T·U and Se<sup>2</sup>T·U mismatched base pairs

have higher stabilities than the T·U counterpart ( $\Delta T_m = +5$  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.<sup>15</sup> The S<sup>2</sup>T·T mismatched base pair showed the same tendency (**Table 2**). As a consequence of the balance of  $T_m$  values, S<sup>2</sup>T-modified **ON2** and Se<sup>2</sup>T-modified **ON3** had dramatically enhanced overall mismatch discriminations toward ssRNA ( $\Delta T_m$  values of the most stable mismatched duplexes: **ON1**:  $-5$  °C, **ON2**:  $-11$  °C, and **ON3**:  $-11$  °C). S<sup>2</sup>T and Se<sup>2</sup>T were almost equal in mismatch discrimination abilities.

**Table 2.**  $T_m$  Values of duplexes formed between oligonucleotides and ssDNAs<sup>a</sup>

| Oligonucleotide         |                   | $T_m$ ( $\Delta T_m = T_m$ [mismatch] – $T_m$ [match]) (°C) |              |              |              |
|-------------------------|-------------------|---|--------------|--------------|--------------|
| ID                      | <u>X</u>          | <u>Y</u> = A  | <u>Y</u> = G | <u>Y</u> = C | <u>Y</u> = T |
| <b>ON1</b> <sup>b</sup> | T                 | 52  | 41 (–11)     | 37 (–15)     | 38 (–14)     |
| <b>ON2</b>              | S <sup>2</sup> T  | 51  | 37 (–14)     | 31 (–20)     | 40 (–11)     |
| <b>ON3</b>              | Se <sup>2</sup> T | 50  | 35 (–15)     | 30 (–20)     | 38 (–12)     |

<sup>a</sup>Conditions: 10 mM phosphate buffer (pH 7.2), 100 mM NaCl, and 4  $\mu$ M of each oligonucleotide. The  $T_m$  values are the average of at least three measurements. The oligonucleotide sequence is 5'-d(GCGTTXTTTGCT)-3', and the sequence of ssDNA is 5'-d(AGCAAYAACGC)-3'. <sup>b</sup>Values are taken from reference 16.

**Table 3.**  $T_m$  Values of duplexes formed between oligonucleotides and ssRNAs<sup>a</sup>

| Oligonucleotide         |                   | $T_m$ ( $\Delta T_m = T_m$ [mismatch] – $T_m$ [match]) (°C) |              |              |              |
|-------------------------|-------------------|---|--------------|--------------|--------------|
| ID                      | <u>X</u>          | <u>Y</u> = A  | <u>Y</u> = G | <u>Y</u> = C | <u>Y</u> = U |
| <b>ON1</b> <sup>b</sup> | T                 | 48  | 43 (–5)      | 32 (–16)     | 33 (–15)     |
| <b>ON2</b>              | S <sup>2</sup> T  | 49  | 38 (–11)     | 30 (–19)     | 38 (–11)     |
| <b>ON3</b>              | Se <sup>2</sup> T | 48  | 35 (–13)     | 29 (–19)     | 37 (–11)     |

<sup>a</sup>Conditions: 10 mM phosphate buffer (pH 7.2), 100 mM NaCl, and 4  $\mu$ M of each oligonucleotide. The  $T_m$  values are the average of at least three measurements. The oligonucleotide sequence is 5'-d(GCGTTXTTTGCT)-3', and the sequence of ssRNA is 5'-r(AGCAAYAACGC)-3'. <sup>b</sup>Values are taken from reference 16.

In conclusion, we demonstrated that oligonucleotides containing S<sup>2</sup>T or Se<sup>2</sup>T have a remarkably enhanced base discrimination toward ssRNA rather than ssDNA. The results therefore indicate that S<sup>2</sup>T and Se<sup>2</sup>T 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<sup>®</sup> Autoflex II TOF/TOF). For HPLC analysis, a SHIMADZU system with DGU-20A<sub>3R</sub>,

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<sup>2</sup>T- or Se<sup>2</sup>T-modified oligonucleotides

For the synthesis of oligonucleotides containing S<sup>2</sup>T (**ON2**) or Se<sup>2</sup>T (**ON3**), the phosphoramidite method was carried out using an automated DNA synthesizer. S<sup>2</sup>T phosphoramidite was purchased from Glen Research, and Se<sup>2</sup>T phosphoramidite was prepared according to previous protocols.<sup>2</sup> 10% *tert*-Butyl hydroperoxide was used in each oxidation step (oxidation time = 10 min).<sup>10,11</sup> 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.<sup>10,12,13</sup> 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).<sup>10,14</sup> The resulting oligonucleotides were rapidly purified with NAP<sup>TM</sup>-10 columns (Sephadex<sup>TM</sup> G-25 DNA Grade), and further purified using reverse-phase HPLC (Waters XTerra<sup>®</sup> MS C18 2.5  $\mu$ m, 10  $\times$  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<sup>®</sup> MS C18 2.5  $\mu$ m, 4.6  $\times$  50 mm column), and their compositions were confirmed using MALDI-TOF-MS analysis. In the case of S<sup>2</sup>T-modified **ON2**, the isolated yield was calculated using the previously reported extinction coefficient.<sup>14</sup> In the case of Se<sup>2</sup>T-modified **ON3**, the isolated yield was determined using the following extinction coefficients:  $\epsilon_{260}$  (H<sub>2</sub>O) dG = 12500; dC = 7500; dT = 8500; and Se<sup>2</sup>T = 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  $\mu$ M), target DNA or RNA (4  $\mu$ M), 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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