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The thermal stability of RNA duplexes containing modified base pairs placed at internal and terminal positions of the oligoribonucleotides

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Abstract

The presence of various modifications within oligomers changes their thermodynamic stability. To get more systematic data, we measured effects of 5- and 6-substituted uridine on thermal stability of $(AUCU^{Mod.}AGAU)_2$ and $(AUCUAGAU^{Mod.})_2$. Collected results lead to the following conclusions: (i) 5-halogenated and 5-alkylated substituents of the uridine affect thermal stability of the RNA duplexes differently. Moreover, the 5-fluorouridine changes stability of the RNA duplexes opposite to remaining 5-halogenouridines; (ii) for oligomers containing 5-chloro, 5-bromo or 5-iodouridine stronger hydrogen bond formed between oxygen-4 of the 5-halogenated uracil and 6-amino group of the adenine is presumably responsible for stabilizing effect; (iii) placing of $A\text{-}U^{5R}$ base pairs closer to the end of the duplex enhance thermal stability relatively to oligomer with central position of this base pair; (iv) the effects of 5-substituents are additive, particularly for substituents which stabilize RNA duplexes; (v) 6-methyluridines (N1 and N3 isomers) as well as 3N-methyluridine present at internal position of $A\text{-}U^{Mod.}$ inhibit duplexes formation; (vi) 6-methyluridines (N1 and N3 isomers) as well as 3N-methyluridine placed as terminal base pairs stabilize the duplexes mostly via 3′-dangling end effect.

Keywords: Modified uridine; Modified oligoribonucleotides; Thermodynamics of RNA duplexes

1. Introduction

The secondary and tertiary structures of the ribonucleic acids (RNA) are important for their functions [1,2]. The RNA, beside four natural nucleotides, contains over 90 differently modified ones [3]. They are particularly often found in the transfer RNA (tRNA) where they can contribute up to 20% of the tRNA nucleotides population. The modifications are the results of the post-transcriptional process in the biosynthesis of the tRNA. The functions of these modifications are not completely understood, however, it has been postulated that they affect the enzyme recognition and the structure of the tRNA.

There is not much systematic information about affects of modifications on thermodynamic stability of RNA duplexes in literature [4–7]. However, there is some more complete data available on the

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influence of 5-alkyl substituents of 2′-deoxyuridine and 2′-deoxycytidine on the thermal stability of DNA duplexes. Sagi et al. reported that the $(dU^{5R}\text{-}dA)_{10}$ substituent with increasing length of *n*-alkyl up to *n*-pentyl gradually enhanced the stability of the DNA duplex [4]. The same effect was observed for 5-alkyl of 2′-deoxycytidine in duplex $(dC^{5R}\text{-}dG)_6$. For both DNA duplexes, the *n*-alkyl substituents longer than *n*-hexyl very drastically reduced the thermodynamic stability. Moreover, the presence of the double or triple bond within the 5-substituents very significantly increased stability relatively to the same size of the alkyl substituent. Froehler et al. and recently Barnes et al. reported a very significant increase in stability of the duplexes due to the presence of 5-propynyl groups in 2′-deoxyuridine and 2′-deoxycytidine [5,8–10].

In this paper, we report the effect of seven different substituents (fluoro, chloro, bromo, iodo, methyl, ethyl and *n*-propynl as well as hydrogen) placed in position 5 of the uridine on the thermal stability of the self-complementary RNA duplexes. The formed A-U^{5R} base pairs are placed within $(AUCU^{5R}AGAU)_2$ as internal (in the tandem position) and $(AUCUAGAU^{5R})_2$ as terminal base pairs. The other group tested derivatives include 6-methyluridines (both N1 and N3 isomers) as well as 3N-methyluridine, for which thermodynamic stability was tested for oligomers at the same position within oligoribonucleotides as 5-substituted derivatives.

The purpose of our experiments was to obtain more general information about the influence of the interesting modifications on thermal stability of RNA duplexes and, based on this knowledge, to be able to modulate the thermodynamic stability of RNA by applying different types of modifications.

2. Experimental

2.1. The chemical synthesis of the oligoribonucleotides containing substituted uridines

The 5-modified nucleosides were synthesized in two ways: (i) substitution of uridine at 5-position (chloro, bromo, iodouridine derivatives) [11]; and (ii) condensation of 5-substituted uracil and 1′-*O*-acetyl-2′,3′,5′-tri-*O*-benzoyl-β-D-ribose using silicone Hilbert–Johson method and $SnCl_4$ as a catalyst (fluoro, methyl, ethyl and *n*-propyluridine derivatives) [12]. The 6-methyluridine (N3 isomer) was obtained by condensation of the 6-methyluracil and 1′-*O*-acetyl-2′,3′,5′-tri-*O*-benzoyl-β-D-ribose in the presence of $SnCl_4$ [13]. The thermodynamically more stable N3 isomer was almost exclusive product of the synthesis. The 6-methyluriduine (N1 isomer) was synthesized by condensation of the 6-methylcytosine and 1′-*O*-acetyl-2′,3′,5′-tri-*O*-benzoyl-β-D-ribose in the presence of $SnCl_4$. The product-6-methylcytidine was transformed into 6-methyluridine by chemical deamination of the cytidine derivative. Finally, the 3N-methyluridine was obtained as a product of methylation of the uridine.

The substituted uridines were converted into 5′-*O* – dimethoxytrityl - 2′ - *O* - tetrahydropyranyl-5-substituted uridines by analogy to the early published procedures [14]. The last derivatives were transformed into corresponding 3′-*O*-phosphoramidites and used for synthesis of the oligoribonucleotides on a solid support as described earlier [15–17]. The deprotection and purification of the oligoribonucleotides were performed as published earlier [15–18]. The purity of the oligoribonucleotides was analyzed by TLC or HPLC and was found to be higher than 95%. In order to check the base composition several oligoribonucleotides were digested by nuclease P1 and snake venom phosphodiesterase (SVPDE), followed by dephosphorylation with calf intestine phosphatase (CIP). The reaction mixture was analyzed by HPLC on reversed phase column C-8 and was found to contain all expected nucleosides in a proper ratio [19].

2.2. UV melting of the oligoribonucleotides

The oligoribonucleotides were melted in 1.0 M NaCl, 20 mM sodium cacodylate and 0.5 mM Na_2EDTA, pH 7.0. Oligoribonucleotides single strand concentrations were calculated from high temperature (>80 °C) absorbancies and single strand extinction coefficients approximated by a nearest-neighbor model [20,21]. Absorbance vs.

Table 1
Thermodynamic parameters of helix formation by oligoribonucleotides with A-U^{R} at the internal position[a]

RNA duplex	Average of curve fits				T_M^{-1} vs. log C_T plots				
AUCU^{R} AGAU UAGA ^{R}UCUA	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_M[b] (°C)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_M[b] (°C)	$\Delta\Delta G^\circ_{37}$ (kcal/mol)
R=F	57.1±3.6	161.3±11.0	7.1±0.2	44.9	49.6±1.2	137.6±4.1	7.0±0.0	45.2	0.3
R=Cl	61.8±4.6	171.6±13.8	8.6±0.3	52.4	52.9±1.3	144.1±4.2	8.2±0.1	52.6	−0.9
R=Br	58.2±5.7	160.7±18.1	8.4±0.2	52.0	56.4±2.1	155.3±6.5	8.2±0.1	51.8	−0.9
R=I	57.9±4.4	161.1±13.5	7.9±0.3	49.7	51.0±1.7	139.6±5.5	7.7±0.1	50.0	−0.4
R=H	58.6±2.5	165.2±7.9	7.3±0.1	46.1	59.1±0.5	166.9±1.7	7.3±0.0	46.0	0
R=Me	53.8±8.7	149.7±27.8	7.4±0.2	47.3	52.5±2.6	145.8±8.2	7.2±0.1	46.6	0.1
R=Et	54.1±4.9	154.4±15.5	6.2±0.2	39.9	57.9±3.4	167.1±11.2	6.1±0.1	39.6	1.2
R=n-Pr	57.5±3.1	164.5±9.7	6.4±0.1	41.2	54.2±1.5	154.2±4.8	6.4±0.0	41.2	0.9
U^{3NMec}	28.5±10.7	82.1±38.6	3.1±1.2	11.0					
U$^{6Me(N1)d}$	9.8±3.8	14.5±18.3	5.3±1.9	27.1					
U$^{6Me(N3)e}$	58.2±57.0	177.2±192.4	3.3±2.7	24.9					

U^{R}-the uridine derivative with R substituent at position 5.
[a] Solutions are 1 M NaCl, 20 mM sodium cacodylate and 0.5 mM Na_2EDTA, pH 7.
[b] Calculated for 10^{-4} M oligomer concentration.
[c] 3N-methyluridine.
[d] 6-Methyluridine with N1-C1′ glycosidic bond.
[e] 6-Methyluridine with N3-C1′ glycosidic bond.

temperature melting curves were measured at 260 nm with a heating rate of 1 °C/min from 0 to 90 °C on a Gilford 250 spectrometer controlled by a Gilford 2527 theromoprogrammer [22].

3. Results and discussion

To get required information we synthesized and measured the thermodynamic properties of the several octamers-$(AUCU^{5R}AGAU)_2$ and $(AUCUAGAU^{5R})_2$, where R=F, Cl, Br, I, H, Me, Et and n-Pr. The A-U^{5R} base pairs were placed as internal (in tandem position) and terminal base pairs. The other group of the octamers including $(AUCU^{6Me}AGAU)_2$, $(AUCU^{3NMe}AGAU)_2$, $(AUCUAGAU^{6Me})_2$ and $(AUCUAGAU^{3NMe})_2$ contained A-U$^{Mod.}$ base pairs at the internal and terminal position as well.

3.1. The internal A-U^{5R} base pairs

The thermodynamic data concerning influence of A-U^{5R} base pairs within a model duplex $(AUCU^{5R}AGAU)_2$ on the thermodynamic stability are collected in Table 1. The comparison of the thermodynamic data obtained from fitting of the melting curves and dependence of the melting temperature (T_m) and logarithm concentration of the oligomers indicate the two-state melting transition of the oligoribonucleotides containing 5-alkyluridine. For 5-halogenated oligomers this correlation is weaker. The enthalpy ($\Delta\Delta H^\circ$) and entropy ($\Delta\Delta S^\circ$) parameters calculated by both methods are different by 13–15%, (except oligomers containing 5-bromouridine). However, in the literature that difference ($\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ > 15\%$) is still considered as representative of two-state transition [23].

The tested 5-substituted uridine derivatives can be classified into two categories of the substituents.

The first group, which includes the halogenated derivatives, is affected by different electron withdrawing properties and size of substituents. Both features of substituents are changing in opposite directions which complicates the interpretation of the results. The analysis of the thermodynamic stability of the duplexes containing 5-halogenated uridine demonstrates that 5-fluorouridine destabilizes the duplex ($\Delta\Delta G^\circ_{37} = 0.15$ kcal/mol per modification). However, the most electron withdrawing character of the fluorine atom among all halogens would allow the opposite effect to be

Table 2
Thermodynamic parameters of helix formation by oligoribonucleotides with A-U^{R} at the terminal position[a]

RNA duplex	Average of curve fits				T_M^{-1} vs. log C_T plots					
AUCUAGAU^{R} ^{R}UAGAUCUA	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_M[b] (°C)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_M[b] (°C)	$\Delta\Delta G^\circ_{37}$ (kcal/mol)	$\Delta\Delta G^\circ_{37}{}'$ (kcal/mol)
R=F	52.2±5.1	146.6±15.9	6.7±0.1	43.2	51.9±1.3	145.9±4.2	6.7±0.0	43.2	0.6	1.1
R=Cl	54.9±1.9	153.5±6.2	7.3±0.1	46.5	56.4±1.3	158.3±4.1	7.3±0.0	46.2	0.0	0.5
R=Br	60.5±2.3	169.4±6.9	7.9±0.1	49.0	57.4±1.2	159.8±3.9	7.8±0.0	49.0	−0.5	0
R=I	58.1±2.6	162.1±8.9	7.8±0.2	49.0	71.8±1.4	205.4±4.6	8.1±0.1	47.9	−0.8	−0.3
R=H	58.6±2.5	165.2±7.9	7.3±0.1	46.1	59.1±0.5	166.9±1.7	7.3±0.0	46.0	0	0.4
R=Me	61.2±1.9	171.7±5.9	7.9±0.1	49.1	57.3±0.7	159.5±2.4	7.9±0.1	49.3	−0.6	−0.1
R=Et	59.1±5.6	166.7±18.0	7.4±0.1	46.5	61.9±1.4	175.9±4.6	7.4±0.1	45.9	−0.1	0.4
R=n-Pr	59.7±2.4	168.6±7.8	7.4±0.1	46.2	60.0±2.2	169.8±6.9	7.4±0.1	46.0	−0.1	0.4
U^{3NMec}	49.5±4.9	138.5±16.2	6.6±0.1	42.9	55.7±1.7	158.4±5.5	6.5±0.1	41.9	0.8	1.2
U$^{6Me(N1)d}$	43.3±7.7	121.3±25.8	5.6±0.2	37.0	42.7±2.3	119.1±7.8	5.5±0.1	35.9	1.8	2.3
U$^{6Me(N3)e}$	45.8±5.6	130.6±18.4	5.3±0.1	34.5	47.2±1.5	135.5±5.1	5.2±0.1	33.9	2.1	2.6

U^{R}-the uridine derivative with R substituent at position 5.
[a] Solutions are 1 M NaCl, 20 mM sodium cacodylate and 0.5 mM Na_2EDTA, pH 7.
[b] Calculated for 10^{-4} M oligomer concentration.
[c] 3N-methyluridine.
[d] 6-Methyluridine with N1-C1′ glycosidic bond.
[e] 6-Methyluridine with N3-C1′ glycosidic bond, $\Delta\Delta G^\circ_{37}$c-calculated according to Xia et al. [27].

expected. The presence of the 5-chloro, 5-bromo and 5-iodouridine within oligoribonucleotides increases stability of the RNA duplexes and free energy changes by −0.45, −0.45 and −0.20 kcal/mol (per modification) for chloro, bromo and iodouridine derivatives, respectively (see Table 1).

The second group of the oligoribonucleotides includes the 5-alkylated uridines:methyl, ethyl and *n*-propyl derivatives. The presence of the 5-methyl substituent does not affect the thermal stability ($\Delta\Delta G^\circ_{37}$=0.05 kcal/mol) of (AUCUAGAU^{5R})$_2$ relatively to unmodified oligoribonucleotide. However, the presence of the 5-ethyl and 5-*n*-propyluridine within core duplex decrease stability ($\Delta\Delta G^\circ_{37}$) by 0.60 and 0.45 kcal/mol (per modification), respectively (see Table 1).

As discussed so far, RNA duplexes contain two A-U^{5R} base pairs in the center of oligomers (tandem position). To obtain more information on how position of the modification within oligonucleotide affects the thermodynamic parameters, we placed 5-chlorouridine and 5-ethyluridine within (AU^{Cl}CUAGAU)$_2$ and (AU^{Et}CUAGAU)$_2$ with A-U^{5Cl} and A-U^{5Et} at the internal position, but separated by five base pairs. Both duplexes are more stable by 0.5 and 1 kcal/mol relative to (AUCU^{Cl}AGAU)$_2$ (AUCU^{Et}AGAU)$_2$ (Tables 2–4). The reason for this can be less steric hindrance of the 5-substituent of the uridines within (AU^{Cl}CUAGAU)$_2$ and (AU^{Et}CUAGAU)$_2$. The similar phenomenon was observed for single RNA mismatches (U-U and A-A) when they were placed at different positions within the duplex. Moving the single mismatches more to the end of the duplex gradually increases stability of the helix [24].

We also tested the influence of the 6-methyluridines (N1 and N3 isomers) and 3N-methyluridine on the thermodynamic stability of [AUCU$^{6Me(N1)}$AGAU]$_2$, [AUCU$^{6Me(N3)}$AGAU]$_2$ and (AUCU^{3NMe}AGAU)$_2$ (see Fig. 1). The results showed the single stranded character of the melting curves. The obtained results are easy to explain for oligomers containing 3N-methyluridine since 3N-methyl inhibits formation of A-U^{3NMe} base pairs. For 6-methyluridines the results are a surprise, but can be explained by syn conformation of the glycosidic bond of the 6-methyluridines, which also does not allow the hydrogen bonds

Table 3
Thermodynamic parameters of helix formation by oligoribonucleotides with G-U[Ra]

RNA duplex	Average of curve fits				T_M^{-1} vs. log C_T plots				
	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_M[b] (°C)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_M[b] (°C)	$\Delta\Delta G^\circ_{37}$ (kcal/mol)
GUCUAGAU UAGAUCUG	68.7±1.7	196.8±5.3	7.7±0.1	46.3	70.0±1.5	201.0±4.9	7.7±0.1	46.3	0
GUCUAGAU^{F} ^{F}UAGAUCUG	63.5±2.1	182.4±6.7	6.9±0.1	43.2	69.7±1.7	202.4±5.3	6.9±0.1	42.8	0.8
GUCUAGAU^{Cl} ^{Cl}UAGAUCUG	66.9±1.6	191.1±5.1	7.6±0.1	46.2	69.9±0.6	200.9±1.9	7.6±0.1	46.0	0.1
GUCUAGAU^{Br} ^{Br}UAGAUCUG	65.2±5.0	185.6±15.6	7.6±0.2	46.7	66.9±2.0	191.1±6.3	7.7±0.1	46.6	0.0
GUCUAGAU^{I} ^{I}UAGAUCUG	65.2±1.7	185.7±5.7	7.6±0.1	46.6	73.9±3.2	213.1±10.1	7.8±0.1	46.1	−0.1

U^{F}, U^{Cl}, U^{Br}, U^{I}-the uridine derivatives with F, Cl, Br and I at position 5.
[a] Solutions are 1 M NaCl, 20 mM sodium cacodylate and 0.5 mM Na_2EDTA, pH 7.
[b] Calculated for 10^{-4} M oligomer concentration.

between 6-methyluridine and adenosine from a complementary strand to be formed [25].

As mentioned earlier there is little information concerning the effects of 5-substituted nucleotides on thermodynamic stability of helixes in the literature [4–7]. These available data mostly concern DNA duplexes, which exist in B-form and the observed effect can be different than for A-RNA

Table 4
Thermodynamic parameters of helix formation by oligoribonucleotides with A-U[Ra]

RNA duplex	Average of curve fits				T_M^{-1} vs. log C_T plots				
	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_M[b] (°C)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_M[b] (°C)	$\Delta\Delta G^\circ_{37}$ (kcal/mol)
AUCUAGAU UAGAUCUA	58.6±2.5	165.2±7.9	7.4±0.1	46.1	59.1±0.6	166.9±1.8	7.3±0.1	46.0	0
AUCU^{Cl}AGAU UAGA^{Cl}UCUA	61.8±4.6	171.6±13.8	8.6±0.4	52.4	52.9±1.4	144.1±4.2	8.2±0.1	52.6	−0.9
AUCU^{Et}AGAU UAGA^{Et}UCUA	54.1±5.0	154.4±15.5	6.2±0.2	39.9	57.9±3.4	167.1±11.2	6.1±0.1	39.6	1.2
AU^{Cl}CUAG--AU UA--GAUC^{Cl}UA	66.5±3.1	186.8±10.0	8.6±0.1	51.1	75.0±3.5	213.7±11.0	8.7±0.1	50.3	−1.4
AU^{Et}CUAG--AU UA--GAUC^{Et}UA	62.3±3.8	177.7±12.4	7.2±0.1	44.6	66.3±1.6	190.7±5.0	7.1±0.1	44.0	0.2
AU^{Cl}CU^{Cl}AG--AU UA--GA^{Cl}UC^{Cl}UA	67.4±2.2	186.9±6.8	9.4±0.1	55.3	67.0±2.4	185.8±7.5	9.4±0.1	55.2	−2.1
AU^{Et}CU^{Et}AG--AU UA--GA^{Et}UC^{Et}UA	58.2±2.3	167.6±7.3	6.3±0.1	40.2	59.8±1.5	172.7±4.8	6.2±0.1	40.0	1.1
AU^{Cl}CU^{Et}AG--AU UA--GA^{Et}UC^{Cl}UA	56.4±2.4	158.8±7.6	7.1±0.2	45.2	71.7±3.4	207.6±10.7	7.3±0.1	44.2	0.0
AU^{Et}CU^{Cl}AG--AU UA--GA^{Cl}UC^{Et}UA	64.2±2.8	179.9±8.9	8.5±0.1	51.0	67.2±3.3	189.3±10.4	8.5±0.1	50.7	−1.2

U^{Cl}, U^{Et}-the uridine derivative with Cl and Et at position 5.
[a] Solutions are 1 M NaCl, 20 mM sodium cacodylate and 0.5 mM Na_2EDTA, pH 7.
[b] Calculated for 10^{-4} M oligomer concentration.

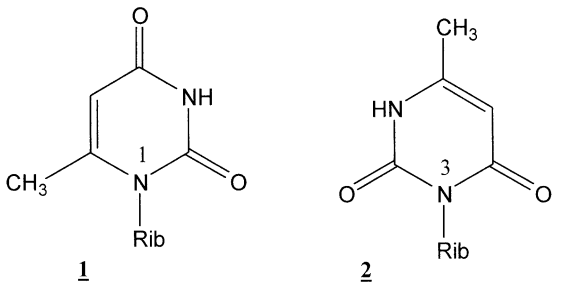

Fig. 1. The structures of 6-methyluridines isomers: N1 (1) and N3 (2).

form. However, comparing our results with those reported by Sahasrabudhe et al. demonstrated that the influence of the 5-fluorouridine on thermal stability of the RNA duplexes is similar [26]. The presence of two internal base pair A-U^{5F} at adjacent positions does not significantly affect the thermal stability of the RNA duplex. A similar small thermodynamic effect was observed when four A-U^{5F} base pairs were placed in the row at the internal position of the oligomer. However, oligoribonucleotides containing at the internal position two A-U^{5F} but separated by two base pairs increases stability by 1.2 kcal/mol. This observation is similar to that presented herein for 5-chloro and 5-ethyl substituted oligoribonucleotides placed at the internal position but separated by several base pairs.

3.2. The terminal A-U^{5R} base pairs

Modified A-U^{5R} base pairs were also placed in the core oligoribonucleotide-$(AUCUAGAU^{5R})_2$ but in the terminal position. The A-U^{5F} destabilizes RNA duplex by 0.30 kcal/mol (per modification), which is twice as big as that observed for the internal A-U^{5F} base pairs (see Table 2). The remaining 5-halogenated uridines placed at the terminal position within $(AUCUAGAU^{5Hal})_2$ stabilize duplex. However, the order of stabilization is opposite than for duplexes with A-U^{5Hal} at internal position. The free energy change is 0.00, −0.25 and −0.40 kcal/mol (per modification) for 5-chloro, bromo and iodouridine within $(AUCUAGAU^{5Hal})_2$, respectively. The terminal position of A-U^{5R} reduces the steric interactions that can be present for A-U^{5R} at the internal position (particularly at tandem position) and, particularly, the electron withdrawing character of the halogen should affect thermal stability of the duplex. Among 5-alkylated uridines, only the 5-methyl substituent stabilizes RNA duplex by 0.30 kcal/mol, whereas the ethyl and *n*-propyl practically do not affect the duplexes stability.

The new expended thermodynamic parameters have been published for the near-neighbor model [27]. The authors improved the parameters for the near-neighbor model and introduced a new parameter for the terminal A-U base pair. After including this parameter in our measurements (Table 2, column marked as $\Delta\Delta G^{\circ}_{37}{}'$) for 5-halogenated oligomers the $\Delta\Delta G^{\circ}_{37}$ value changes in the following order: 0.55, 0.25, 0.00 and −0.15 kcal/mol (per modification) for fluoro, chloro, bromo and iodouridine oligomers, respectively. The same parameters for 5-alkylated oligomers are: −0.05, 0.20 and 0.20 kcal/mol for methyl, ethyl and *n*-propyluridine derivatives, respectively.

The 6-methyluridines (N1 and N3 isomers) and 3N-methyluridine were also placed as terminal base pairs A-$U^{Mod.}$ within $(AUCUAGAU^{Mod.})_2$. The melting profiles of the oligomers showed double stranded character of the transition, however, the thermal stability is lower than for the reference oligomer—$(AUCUAGAU)_2$ (see Table 2). The comparison of the obtained thermodynamic parameters with the effect of 6-methyluridines (N1 and N3 isomers) and 3N-methyluridine as 3′-dangling ends in $(UCUAGAU^{Mod.})_2$ demonstrates that 3′-dangling effects of the terminal A-$U^{Mod.}$ base pairs are mainly responsible for observed thermodynamic changes. For example, for $(AUCUAGAU^{3NMe})_2$ and $(UCUAGAU^{3NMe})_2$ free energy (ΔG°_{37}) are −6.5 and −6.5 kcal/mol, respectively. This supports the assumption that 3N-methyluridine at the terminal A-U^{3NMe} base pair stabilizes the duplex via the 3N-methyluridine 3′-dangling end effect. For 6-methyluridines, we have had similar results, however, in this case a greater stability ($\Delta\Delta G^{\circ}_{37}$ = 0.40 and 0.25 kcal/mol for N1 and N3 isomers, respectively) was obtained relative to the 3′-dangling ends effect only.

3.3. The thermal stability of G-U^{5Hal} within oligoribonucleotides.

The scheme of the hydrogen bond interactions of A-U and wobble G-U base pairs demonstrated that oxygen-4 of the uracil is not involved into G-U base pairing [28,29]. The presence of the electronegative halogens at position 5 of the uracil ring should change the hydrogen bond ability of the closer functional group, i.e. oxygen-4. To test this hypothesis we synthesized and measured the thermodynamic stability of several oligoribonucleotides containing G-U^{5Hal} base pairs. The results are collected in Table 3.

The data demonstrate that the G-U^{5F} base pair again does not fit to other halogen substituents and destabilize RNA duplex $(GUCUAGAU^{5F})_2$ by 0.40 kcal/mol relative to the core duplex containing G-U base pairs. For $(GUCUAGAU^{5F})_2$, the change of the stacking interaction is presumably responsible for destabilization.

For the remaining 5-halogenated oligoribonucleotides with G-U^{5Hal} base pairs, the thermal stability is very similar and does not depend on the nature of the halogen. The stability of the oligoribonucleotides containing the G-U^{5Cl}, G-U^{5Br}, G-U^{5I} are practically the same as for the G-U base pair. Presumably, for these three uridine derivatives, stronger hydrogen bonding between oxygen-4 of the 5-halogenated uridine and 6-amino function of adenosine is responsible for increase of stability of A-U^{5Hal} containing RNA duplexes.

3.4. Additive character of 5-substituted uridines on thermal stability of oligoribonucleotides

To study the effect of two 5-substituted uridines placed in the same oligoribonucleotides we synthesized several oligoribonucleotides and measured their thermodynamic parameters. The results are collected in Table 4. They indicate an additive character of the substituents effect, however, it is not a simple sum of partial effects. For example, $\Delta\Delta G^\circ_{37}$ for $(AUCU^{5Cl}AGAU)_2$ and $(AU^{5Cl}CUAGAU)_2$ is -0.9 and -1.4 kcal/mol, respectively, whereas for the duplex $(AU^{5Cl}CU^{5Cl}AGAU)_2$ containing four A-U^{5Cl} base pairs, the $\Delta\Delta G^\circ_{37}$ are equal to -2.1 kcal/mol. The effect of 5-ethyluridine on duplex stability is different. The $\Delta\Delta G^\circ_{37}$ for $(AUCU^{5Et}AGAU)_2$ and $(AU^{5Et}CUAGAU)_2$ are 1.2 and 0.2 kcal/mol, whereas for $(AU^{5Et}CU^{5Et}AGAU)_2$ the $\Delta\Delta G^\circ_{37}$ is 1.1 kcal/mol. However, in general we can say that an additive character of 5-substituted uridine in duplexes remains the same.

Oligoribonucleotides containing four A-U^{5R} base pairs demonstrated that the effect of the 5-substituted uridine closer to the end of the helix dominates over the effect of the 5-substituted uridine located in the center of the duplex. The second feature indicates that the 5-substituent which stabilizes the duplex additive character is better. This last conclusion was confirmed by measurement of the thermal stability of two oligoribonucleotides containing one stabilizing (5-chloro) and one destabilizing (5-ethyl) substituent within the oligoribonucleotide both placed at the reverse positions. For example, $\Delta\Delta G^\circ_{37}$ between calculated (as the sum of the effects of the single substitutions) and experimentally measured is 0.20 kcal/mol for $(AU^{5Cl}CU^{5Et}AGAU)_2$ and 0.45 kcal/mol for $(AU^{5Et}CU^{5Cl}AGAU)_2$.

4. Conclusions

The thermodynamic results presented herein for the first time concern several 5-substituted uridines which are placed at internal or terminal base pairs within the same core oligoribonucleotide. As we can see from the results, the thermal stability of duplexes is a function of several factors such the character and size of the substituents as well as their position within the oligoribonucleotide.

The collected results lead to several conclusions. The results demonstrate that comparison of the effects of the 5-halogenated and 5-alkylated uridines present in oligoribonucleotides on thermal stability is difficult. It can be a result of different electronic effect and size as well as hydrophobic properties of the 5-substituents.

Comparing only the effect of the 5-halogen substituents, it is clear that 5-fluorouridine changes the stability of oligomers opposite to 5-chloro, bromo and iodouridine. The experimental data demonstrate that 5-fluorouridine destabilizes the oligoribonucleotides when placed at both the inter-

nal and terminal position of oligomers-$(AUCU^{F}AGAU)_2$ and $(AUCUAGAU^{F})_2$. Other 5-halogened uridines at the same positions stabilize RNA duplexes.

Moreover, the position of A-U^{5R} within oligoribonucleotide affects the thermal stability of the RNA duplexes. The A-U^{5R} base pair placed closer to the end of the helix enhances thermal stability relatively to the duplex with a A-U^{5R} base pair located in the center.

The next conclusion concerns the additivity of the effects of the 5-substituents. The additivity of the effect is observed for stabilizing substituents. However, for destabilizing substituents the trend is similar. Moreover, for the oligomers containing two substituents causing opposite effects, the best additivity is observed when the stabilizing substituent is closer to the end of the duplex than with the reverse location of the 5-substituent.

The presence of the 6-methyluridines (both N1 and N3 isomers) and 3N-methyluridine in the internal position of $(AUCU^{Mod.}AGAU)_2$ prevents duplex formation due to steric hindrance and lacks the ability for hydrogen bond formation as well as syn glycosidic bond orientation. For the same uridine derivatives, but placed in terminal position of $(AUCUAGAU^{Mod.})_2$, the observed thermodynamic effect is mostly due to the 3′-dangling end location of modified uridines. For oligomers modified with 3N-methyluridine, the measured thermodynamic effect is equal to the 3′-dangling end of 3N-methyluridine. However, for 6-methyluridines the 3′-dangling end is presumably enhanced by some hydrogen bond interactions and/or the adenosine 5′-dangling effect.

References

[1] R.F. Gesteland, J.F. Atkins (Eds.), The RNA World, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1993.

[2] J.A. Jaeger, J. SantaLucia, I. Tinoco, Determination of RNA structure and thermodynamics, Ann. Rev. Biochem. 62 (1993) 255–287.

[3] P.A. Limbach, P.F. Crain, J.A. McClosky, Summary: the modified nucleosides of RNA, Nucleic Acids Res. 22 (1994) 2183–2196.

[4] J. Sagi, A. Szamzo, K. Ebinger, et al., Base-modified oligodeoxynucleotides. Effect of 5-alkyl, 5-(1-alkynyl) and 5-(1-alkynyl) substitution of the pyrimidines on duplex stability and hydrophobicity, Tetrahedron Lett. 34 (1993) 2191–2194.

[5] B.C. Froehler, T.J. Wadwani, T.J. Terhorst, S.R. Gerrard, Oligodeoxynucleotides containing C-5 propyne analogs of 2′-deoxyuridine and 2′-deoxycytidine, Tetrahedron Lett. 33 (1992) 5307–5310.

[6] B.C. Froehler, R.J. Jones, X. Cao, T.J. Terhorst, Oligonucleotides derived from 5-(1-propynyl)-2′-*O*-allyl-uridine and 5-(1-propynyl)-2′-*O*-allyl-cytidine: synthesis and RNA duplex formation, Tetrahedron Lett. 34 (1993) 1003–1006.

[7] S.M. Freier, K.H. Altmann, The ups and downs of nucleic acid duplex stability: structure-stability studies on chemically-modified DNA:RNA duplexes, Nucleic Acids Res. 25 (1997) 4429–4443.

[8] T.W. Barnes, D.H. Turner, Long-range cooperativity in molecular recognition of RNA by oligodeoxynucleotides with multiple C5-(1-propynyl) pyrimidines, J. Am. Chem. Soc. 123 (2001) 4107–4118.

[9] T.W. Barnes, D.H. Turner, Long-range cooperativity due to C5-propynylation of oligopirymidines enhances specific recognition by uridine of ribo-adenosine and ribo-guanosine, J. Am. Chem. Soc. 123 (2001) 9186–9187.

[10] T.W. Barnes, D.H. Turner, C5-(1-propynyl)-2′-deoxypyrimidines enhance mismatch penalties of DNA–RNA duplex formation, Biochemistry 40 (2001) 12738–12745.

[11] J.-I. Asakura, M.J. Robins, Cerium (IV)-mediated halogenation at C-5 of uracil derivatives, J. Org. Chem. 55 (1990) 4928–4933.

[12] U. Niedballa, H. Vorbruggen, A general synthesis of N-glycosides II. Synthesis of 6-methyluridine, J. Org. Chem. 39 (1974) 3660–3667.

[13] U. Niedballa, H. Vorbruggen, A general synthesis of N-glycosides. 6. On the mechanism of the stannic chloride catalyzed silyl Hilbert–Johnson reaction, J. Org. Chem. 41 (1976) 2084–2091.

[14] W.T. Markiewicz, E. Biala, R. Kierzek, Application of the tetraisopropyldisiloxane-1,3-diyl group in the chemical synthesis of oligoribonucleotides, Bull. Acad. Polon. Sci. Ser. Chim. 32 (1984) 433–451.

[15] L.J. McBride, M.H. Caruthers, An investigation of several deoxynucleosides phosphoramidites useful for synthesizing deoxyoligonucleotides, Tetrahedron Lett. 24 (1983) 245–249.

[16] R. Kierzek, M.H. Caruthers, C.E. Longfellow, D. Swinton, D.H. Turner, S.M. Freier, Polymer-supported RNA synthesis and its application to test nearest-neighbor model for duplex stability, Biochemistry 25 (1986) 7840–7846.

[17] F. Wincott, A. DiRenzo, C. Shaffer, et al., Synthesis, deprotection, analysis and purification of RNA and ribozymes, Nucleic Acids Res. 23 (1995) 2677–2684.

[18] S.-H. Chou, P. Flynn, B. Reid, Solid-phase synthesis and high-resolution NMR studies of two synthetic double-helical RNA dodecamers: r(CGCGAAUUCGCG)

and r(CGCGUAUACGCG), Biochemistry 28 (1989) 2422–2435.
[19] T. Maniatis, E.F. Fritsch, J. Sambrook (Eds.), Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1993.
[20] P.N. Borer, in: G.D. Fasman (Ed.), 3rd ed., Handbook of Biochemistry and Molecular Biology: Nucleic Acids, I, CRS Press, Cleveland, 1975, p. 589.
[21] E.G. Richards, in: G.D. Fasman (Ed.), 3rd ed., Handbook of Biochemistry and Molecular Biology: Nucleic Acids, I, CRS Press, Cleveland, 1975, p. 597.
[22] M. Petersheim, D.H. Turner, Base-stacking and base-pairing contributions to helix stability: thermodynamics of double-helix formation with CCGG, CCGGp, CCGGAp, ACCGGp, CCGGUp and ACCGGUp, Biochemistry 22 (1983) 256–263.
[23] S.M. Freier, R. Kierzek, J.A. Jeager, et al., Improved free-energy parameters for prediction of RNA duplex stability, Proc. Natl. Acad. Sci. USA 83 (1986) 9373–9377.
[24] R. Kierzek, M.E. Burkard, D.H. Turner, The thermodynamic stability of single mismatches in RNA duplexes, Biochemistry 38 (1999) 14214–14223.
[25] D. Suck, W. Saenger, Molecular and crystal structure of 6-methylurdine. A pyrimidine nucleoside in syn conformation, J. Am. Chem. Soc. 94 (1972) 6520–6526.
[26] P.V. Sahasrahabudhe, R.T. Pon, W.H. Gmeiner, Effects of site-specific substitution of 5-fluorouridine on the stabilities of duplex DNA and RNA, Nucleic Acids Res. 23 (1995) 3916–3921.
[27] T. Xia, J. SantaLucia, M.E. Burkard, et al., Parameters for expended nearest-neighbor model for formation of RNA duplexes with Watson–Crick base pair, Biochemistry 37 (1998) 14719–14735.
[28] L. He, R. Kierzek, J. SantaLucia, E.A. Walter, D.H. Turner, Nearest-neighbor parameters for G-U mismatches, Biochemistry 30 (1991) 11124–11132.
[29] J.D. Robertus, J.E. Ladner, J.T. Finch, et al., Structure of yeast phenylalanine tRNA at 3 A resolution, Nature 250 (1974) 546–555.