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The influence of various modified nucleotides placed as 3′-dangling end on thermal stability of RNA duplexes

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Abstract

The ribonucleic acids (RNA) form highly folded structures, which behind the helical fragments contain several secondary and tertiary structural motives. All of them have an influence on thermodynamic stability of the RNA. The 5′- and 3′-dangling ends are one of those structural motives, which effect stability of the adjacent helixes. In this paper, we described the influence of 14 different modified nucleotides, placed as 3′-dangling ends, on thermal stability of the RNA duplexes. Collected data demonstrate that: (i) 5-substituents of the uridine have an impact on the 3′-dangling end effect and the largest changes were observed for 5-chloro, bromo and methyl substituents; (ii) position of the methyl group within the uracil residue affect the thermal stability of the duplex; (iii) increasing a size of the heterocycle base placed as the 3′-terminal unpaired nucleotide enhances stabilization of duplexes.

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

1. Introduction

For biological functions the secondary and tertiary structures of the ribonucleic acids (RNA) are very important [1–3]. Various RNA molecules form highly folded structures and only part of molecules exists as double helixes whereas the most of them adopt other structural motives. The most common RNA structural motives are mismatches (single, tandem and terminal), internal loops, bulge loops, dangling ends, hairpins, pseudoknots and mulitibranch loops.

One of the most common structural motive in RNA are 5′- and 3′-dangling ends (or terminal unpaired nucleotides) which are formed when terminal nucleotides do not bind to a complementary strand. The thermodynamic parameters for 5′- and 3′-dangling ends are available in the literature [2,4,5]. Both dangling ends stabilize the RNA duplexes, however, the 3′-dangling end effect is much stronger than the 5′-dangling end. The free energy increments (ΔG°_{37}) for the 3′-dangling ends oscillate between -0.8 and -1.7 kcal/mol, whereas in case of 5′-dangling ends they oscillate between -0.1 and -0.5 kcal/mol. The effect of the purine nucleotides as a 3′-dangling end is bigger (by approx. 0.6 kcal/mol) than the 3′-

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dangling end effect of the pyrimidine nucleotides. Moreover, for the helixes closed with a G-C base pair the 3′-dangling end effect is larger ($\Delta\Delta G^{\circ}_{37}$ = 0.4 kcal/mol) than for A-U closing base pair. Additionally, the 3′-dangling end increment of the helix closed by G-C is larger when terminal unpaired nucleotide is covalently bonded to cytidine ($\Delta\Delta G^{\circ}_{37}$ = 0.5 kcal/mol) than to guanosine. The 5′-dangling end does not indicate such large sequence dependence. Since the terminal unpaired nucleotides are not involved in hydrogen bonding, the stacking interaction and perhaps, to some degree, the hydrophobic and electrostatic interactions are responsible for the thermodynamic effects of the 5′- and 3′-dangling ends.

Recently, it was found that the 3′-dangling end contributes to stability of the tertiary RNA structure. The analysis of secondary structure of the yeast $tRNA^{Phe}$ indicated 12 unpaired nucleotides adjacent to base-paired regions. In the crystal structure of the $tRNA^{Phe}$, seven of those nucleotides stack on the top of adjacent base pair. In model studies, these stacked bases enhance stability of the adjacent helix by approximately 1 kcal/mol whereas the remaining five unstacked nucleotides enhance the duplex stability by less than 0.3 kcal/mol [6]. Moreover, recently Turner et al. analyzed many available X-ray and NMR structures of RNA and found that when the terminal unpaired nucleotide increased stability of the helix by 1 kcal/mol or more, the stacking interaction to the adjacent helix was observed [7].

The purpose of the experiments presented in this paper was to analyze the factors important for the 3′-dangling ends effect. These factors include the different 5-substituents of the uridine (various electronegativity and size of the substituents), the different positions of the methyl group in uridine (at position 3N, 5 and 6) and the size (aromaticity, the number of the π-electrons) of the heterocycle bases in unpaired terminal nucleotides position.

2. Experimental

2.1. The chemical synthesis of the oligoribonucleotides

The synthesis of the 5-substitued uridines as well as 6-methyl and 3N-methyluridine was described earlier [8–11]. 4-Desmethylwyosine and 5-amino-4-methylcarbamidoimidazole riboside derivatives were obtained as described in the literature [12,13]. The synthesis of the 3′-phosphoramidites and oligoribonucleotides as well as deprotection and purification were performed as published earlier [14–16]. The purity of oligoribonucleotides was analyzed by high performance liquid chromatography (HPLC) and was confirmed to be greater then 95%. To check 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 [17].

2.2. The melting experiments 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 [18,19]. Absorbance vs. 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 [20].

3. Results and discussion

In the previous paper, we described the effect of 5-substituted uridines as well as 6-methyl and 3N-methyluridine on the thermodynamic stability of the oligoribonucleotides, for $A\text{-}U^{Mod}$ base pair placed as internal or terminal base pairs of the duplexes [21]. Those results showed that substituents differently change the thermal stability of the RNA duplexes. Several factors (electronegativity and size of the substituent as well as position of substituted uridine within the oligomer) were responsible for the results. This multiplicity of the factors make explicit interpretation of the results difficult since used substituents could simultane-

Table 1
Thermodynamic parameters of helix formation with U^R 3′-dangling ends[a]

RNA Duplex	Average of curve fits				T_M^{-1} vs. log C_T plots					
UCUAGAU^R ^{R}UAGAUCU	$-\Delta H^\circ$ (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	48.8±3.9	138.4±13.3	5.8±0.2	38.0	51.7±1.9	148.2±6.1	5.7±0.0	37.3	0.1	−0.8
R=Cl	50.8±4.1	144.6±13.8	6.0±0.1	39.1	54.1±1.4	155.2±4.5	6.0±0.0	38.9	−0.2	−1.1
R=Br	49.1±1.2	139.0±3.9	6.1±0.0	39.4	47.9±0.5	135.2±1.6	6.1±0.0	39.4	−0.3	−1.2
R=I	47.9±3.4	135.7±11.1	5.9±0.1	38.3	47.5±1.2	134.4±4.1	5.9±0.0	38.0	−0.1	−1.0
R=H	48.7±3.1	138.2±10.4	5.8±0.1	37.9	48.4±1.7	137.4±5.6	5.8±0.1	37.6	0	−0.9
R=Me	48.8±4.6	137.8±15.3	6.1±0.1	39.7	47.8±1.6	134.7±5.1	6.1±0.0	39.3	−0.3	−1.2
R=Et	50.2±1.8	143.3±6.2	5.8±0.1	37.6	49.7±1.4	141.8±4.7	5.7±0.0	37.4	0.1	−0.8
R=n-Pr	50.8±2.5	145.0±8.2	5.8±0.1	38.0	49.8±1.4	141.9±4.6	5.9±0.0	37.8	−0.1	−1.0
$U^{3NMec,b}$	52.2±2.4	147.6±8.3	6.5±0.1	41.8	59.9±1.7	172.3±5.7	6.5±0.0	41.1	−0.7	−1.6
R=5Me	48.8±4.6	137.8±15.3	6.1±0.1	39.7	47.8±1.6	134.7±5.1	6.1±0.0	39.3	−0.3	−1.2
$U^{6Me(N1)d}$	43.2±3.8	123.7±13.1	4.9±0.2	31.4	47.5±0.8	138.1±2.7	4.7±0.0	30.6	1.1	0.2
$U^{6Me(N3)e}$	44.0±4.6	126.6±15.5	4.7±0.2	30.4	43.4±1.5	124.8±5.1	4.7±0.1	30.0	1.1	0.2
UCUAGA AGAUCU	42.1±4.7	120.1±15.6	4.8±0.1	30.9	36.5±0.8	101.8±2.9	4.9±0.0	31.0	0.9	0

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.

ously change the hydrogen bonding or/and the stacking as well as hydrophobic interactions. The measured thermodynamic effect of the 3′-dangling ends, presented in this paper, reflect mainly the stacking interactions of the terminal unpaired nucleotides. In our experiments, we took under consideration three factors: (i) the influence of the seven different 5-substituents of uridine; (ii) the influence of the methyl substituent placed in various positions of the pyrimidine ring; (iii) size effect of the heterocycle bases (overlapping, aromaticity, number of the π-electrons) on 3′-dangling ends effects. All terminal unpaired nucleotides were positioned in the same oligoribonucleotide-$(UCUAGAX)_2$, where X represent modified nucleotides. The free energy ($\Delta\Delta G^\circ_{37}$) values collected in Table 1 concern the thermodynamic effect resulting from the presence of two 3′-dangling ends. However, the thermodynamic parameters discussed below are calculated for single 3′-dangling end effect.

3.1. The influence of the 5-substituted uridine on 3′-dangling end effect

The 5-modifications of the uridine include: fluoro, chloro, bromo, iodo, methyl, ethyl and *n*-propyl substituents. The comparison of the thermodynamic results (see Table 1) demonstrates that uridine derivative placed as 3′-dangling end enhance stability of $(UCUAGAU^{5R})_2$ by 0.10–0.15 kcal/mol (per modification), relatively to the core oligomer $(UCUAGA)_2$.

Similarly, as for terminal and internal A-U^{5Hal} base pairs, the effect of the 5-fluorouridine base pair is opposite to remaining halogens and reduces 3′-dangling end stabilization by 0.05 kcal/mol [21]. The 5-chloro, 5-bromo and 5-iodouridine increase 3′-dangling end effect up to 0.15 kcal/mol.

The optimal stacking is observed when interacting heterocycle bases are parallel. If only electronegativity of the halogen is taken under

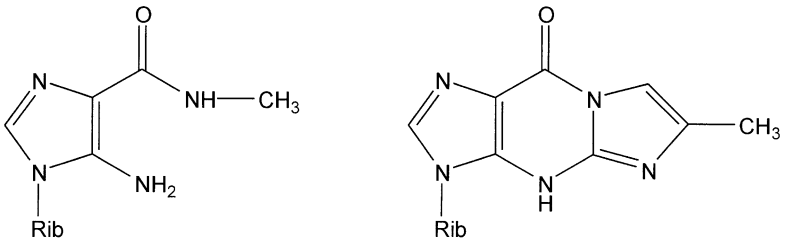

Fig. 1. The structures of 5-amino-4-methylcarbamidoimidazole (1) and 4-desmethylwyosine (2).

consideration, we should expect the largest stability of duplex containing 5-iodouridine as a 3′-dangling end. The comparison of the size of the halogen suggests that the 5-fluoro substituent should be less disturbing to the parallel orientation of the adjacent nucleobases. The analysis of the results in Table 1 shows that the strongest stabilizing effect is observed for 5-bromouridine ($\Delta\Delta G^{\circ}_{37} = -0.15$ kcal/mol). It could mean that the final 3′-dangling end effect depends simultaneously on electronegativity and size of the 5-substituent.

The same method of analysis can be applied for 5-alkyluridine derivatives. The 5-methyluridine increases stability by 0.15 kcal/mol, relative to $(UCUAGAU)_2$. For 5-ethyl and 5-*n*-propyluridine the change of stability by the 3′-dangling end is negligible.

3.2. The influence of methyl substituent position in the uracil residue on 3′-dangling end effect

The next question concerns the problem how the same substituent (in our case the methyl) at different positions of the uracil base, affects the thermal stability of the RNA duplex. We compared the influence of the 3N-methyl, 5-methyl- and 6-methyluridine (both N1 and N3 isomers). In the previous paper, we described the effect of those uridine derivatives on RNA stability when A-U^{Mod} base pairs were placed at internal or terminal position within duplexes [21]. Both, 3N-methyl and 6-methyluridines placed in internal position prevented the duplex formation and the single stranded character of the melting curves was observed. However, when the same modifications were placed in terminal position, both derivatives stabilize the duplex. The 5-methyluridine stabilized the RNA duplex when placed in both terminal and internal position within the oligomer.

The thermodynamic studies of $(UCUAGAU^{R})_2$, where R = H, 3NMe, 5Me, 6Me(N1) and 6Me(N3) demonstrated that thermal stability of the duplexes depends on position of the methyl in the uridine. The results demonstrated that 3N-methyl and 5-methyl substituent stabilize the duplex by 0.35 and 0.15 kcal/mol, respectively (see Table 1). Increased stability of the duplex with 3N-methyluridine as a 3′-dangling end presumably reflects stronger stacking interaction. The electrodonating character of the methyl substituent presumably is only in part responsible for the observed effect since the 5-methyluridine stabilized the duplex by 0.2 kcal/mol less then 3N-methyluridine. The increased stability caused by the 3N-methyluridine could result from hydrophobic interaction of the 3N-methyluridine with base pair closing the duplex and/or some changes of electrons distribution in uracil residue due to ‘protection’ of the lactam system.

Both (N1 and N3) isomers of the 6-methyluridine destabilize duplex similarly. The free energies (ΔG°_{37}) are −5.8, −4.7 and −4.7 kcal/mol for $(UCUAGAU)_2$, $[UCUAGAU^{6Me(N1)}]_2$ and $[UCUAGAU^{6Me(N3)}]_2$, respectively. The 6-methyl substituent in uridine (N1 isomer) changes the glycosidic bond orientation from anti to syn which has been proved by NMR and X-ray studies [22]. That could be the reason for the destabilization effect for 6-methyluridines placed as 3′-dangling

end. The alternative interpretation of this effect could result from the electrostatic interaction of 6-methyluridine and closing base pair [7].

3.3. The influence of heterocycle base size on 3′-dangling end effect

The model studies of the 3′-dangling end demonstrate that the purines stabilize the RNA duplex by approximately 0.6 kcal/mol more than pyrimidines [5]. This can be explained by a larger overlapping of 3′-purines than 3′-pyrimidines due to the size of the base.

To test this assumption, we synthesized and measured the thermodynamic stability of the RNA duplexes containing, as a 3′-dangling end, nucleotides of a different size of the heterocycles. Those heterocycles included derivatives of imidazole, pyrimidine, purine and a derivative of the purine with an additional five-member ring (4-desmethylwyosine). The thermodynamic parameters collected in Table 2, show correlation between the size of the heterocycle base and the 3′-dangling end effect. The bigger size of the heterocycle base results in a larger 3′-dangling end effect. Stabilization effect ($\Delta\Delta G^{\circ}_{37}$) of the 3′-dangling end is -0.35, -0.45, -0.9 and -1.5 kcal/mol for imidazole (5-amino-4-methylcarbamidoimidozole), pyrimidine (uridine), purine (guanosine) and 4-desmethylwyosine, respectively (see Fig. 1). The comparison of 3′-dangling end effect of cytidine and uridine as well as adenosine and guanosine demonstrate a similar effect of both pyrimidines and purines [4,5]. It means that the 3′-dangling end effect of tested heterocycles is mostly due to a different size, not different functional groups present within the heterocycles.

The stacking interactions between heterocycle bases of the nucleic acids are the effect of the induced dipole–dipole interactions [23,24]. The smaller value of the 3′-dangling end could be the consequence of the differences in the induced dipole–dipole interactions. Tested heterocycles are also different in the number of π-electrons of the heterocycles. It is difficult to explain unmistakable observed effects only by one type of the interactions. Presumably the whole thermodynamic effect is a sum of a few interactions, however, their contribution is not necessarily the same.

4. Conclusions

The 3′-dangling end is one of the structural motives of RNA and influences the total thermodynamic stability of molecules [5]. The model studies show that natural nucleotides as a 3′-dangling end always stabilize the RNA duplexes. The studies presented herein expand our understanding of this phenomenon and are particularly interesting since they concern 14 different nucleotides tested as 3′-dangling ends. Several factors in modified nucleosides such as the electronegativity, the size of the 5-substituents, position of the methyl group in the uracil residue and the size of

Table 2
Thermodynamic parameters of helix formation with 3′-dangling ends[a]

RNA duplex	Average of curve fits				T_M^{-1} vs. log C_T plots				
UCUAGA*X* *X*AGAUCU	$-\Delta H^{\circ}$ (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)
X=Imd[c]	50.3±3.6	143.9±11.6	5.7±0.1	36.9	54.4±2.0	157.4±6.7	5.6±0.0	36.5	−0.7
X=Uridine	48.7±3.2	138.3±10.4	5.8±0.1	37.9	48.4±1.7	137.4±5.6	5.8±0.1	37.6	−0.9
X=Guanosine	55.9±2.2	158.7±7.3	6.7±0.1	43.0	61.7±2.7	177.1±8.8	6.7±0.0	42.5	−1.8
X=DesMeV[d]	60.9±4.4	170.9±14.1	7.9±0.1	48.6	63.9±2.1	180.7±6.7	7.9±0.1	48.1	−3.0
UCUAGA AGAUCU	42.1±4.7	120.1±15.6	4.8±0.1	30.9	36.5±0.8	101.8±2.9	4.9±0.0	31.0	0

[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] Imd=5-amino-4-*N*-methylcarbamidoimidazole riboside.
[d] DesMeV=4-desmethylwyosine.

the nucleobase have been tested. That allows for analysis of the data from various points of view.

First, the effect of 5-substituent of uridine is the result of electronegativity and the size of the substituents. Each tested 5-substituent of uridine changes stability of the core duplex when placed as a 3′-dangling end, however, the observed effect is not very large. The largest stabilization effect occurs for 5-bromo, 5-chloro and 5-methyluridine. For the other tested 5-substituents (fluoro, iodo, ethyl and *n*-propyl) this effect is negligible.

Second, the position of the methyl substituent in uracil residue influences the 3′-dangling end effect. The largest stabilization effect occurs for 3N-methyluridine. The 5-methyluridine also stabilizes the duplex, however, $\Delta\Delta G^{\circ}_{37}$ is smaller by 0.2 kcal/mol than the one observed for 3N-methyluridine. Surprisingly, both isomers of the 6-methyluridine destabilize in the same way as the core duplex-$(UCUAGA)_2$. The reason for this phenomenon is not clear at the present moment but perhaps it can be linked to syn conformation of the glycosidic bond of the 6-methyluridine or electrostatic interactions.

Third, the size of the heterocycle base placed at terminal unpaired nucleotide position changes the 3′-dangling end effect. The bigger heterocycle nucleobase causes the stronger stabilization of RNA duplex when placed as 3′-dangling end.

Finally, obtained results demonstrate that the thermodynamic effect of the 3′-dangling end is the function of many factors. Choosing the type of the 3′-nucleotide substituent, we can change the stability of the RNA duplex even by 1.6 kcal/mol through the 3′-dangling end effect.

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