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This study assessed the effects of aritificial cationic oligopeptides on a DNA/RNA hybrid duplex. An oligopeptide containing the octamer of L-2,4-diaminobutyric acid (Dab8) was found to enhance both RNase A resistance and RNase H activity of the DNA/RNA hybrid, which are important for developing nucleic acid drugs.

Oligopeptides, which consist of a few to a few dozen amino acids, are easy to synthesize with diverse sequences. A variety of functional oligopeptides have been identified including antimicrobial peptides, [1] ligand peptides (which can bind to a specific protein),[2] and cell-penetrating peptides (CPP). [3] Among them, some oligopeptides contain many cationic functional groups, which exhibit their properties by interacting with negatively charged functional groups. For example, some lysine- and arginine-rich peptides exhibit antimicrobial properties by interacting with phospholipids in cell membrane.[1] Arginine-rich peptides such as R8 are well known CPP and their properties have been extensively studied for drug delivery systems (DDS) using a wide variety of therapeutic candidates.[3,4] Whereas Trans-Activator of Transcription (TAT) peptides are also CPP, they are originally mimic molecules of RNA binding region of HIV tat protein.[5] Nucleic acids as well as phospholipids are either oligo- or poly-anionic molecules. Therefore, interactions between cationic oligopeptides and DNA or RNA are important for the application of cationic oligopeptides; DDSs using nucleic acid-based drugs with cationic oligopeptides have been enthusiastically studied.[6] Reportedly, both the membrane permeability and nuclease resistance of nucleic acids improve by the conjugation of cationic oligopeptides and nucleic acids.[7] Strömberg's group and we previously reported cationic oligopeptides with varying side-chain lengths that bind to RNA duplexes forming A-type duplex structures.[8] Additionally, oligopeptides containing an octamer of L-2,4-diaminobutyric acid (Dab8) and L-ornithine (Orn8) induced a greater increase in the melting temperature (T_m) of an RNA duplex than that induced by the oligopeptides containing the octamer of L-2,3-diaminopropionic acid (Dap8) and L-Lysine (Lys8). Oligopeptides containing the octamer of L-2-amino-3-guanidinopropionic acid (Agp8) induced a greater increase in the T_m of an RNA duplex than that induced by the oligopeptides containing the octamer of L-2-amino-4-guanidinobutyric acid (Agb8) and L-arginine (Arg8) (Figure 1).

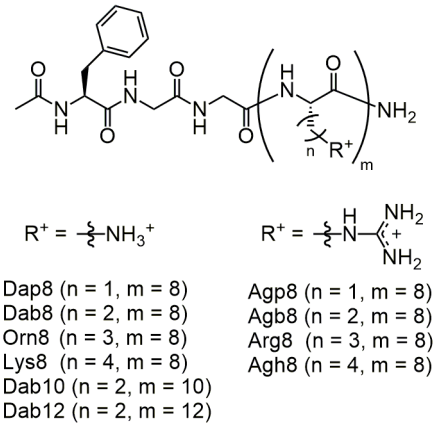

Figure 1 Structures of oligopeptides used in this study

In this study, oligopeptides were studied to determine their effects on the properties of a DNA/RNA hybrid duplex. DNA/RNA hybrids are important for developing single-stranded antisense nucleic acid therapeutics with ribonuclease H (RNase H) dependent antisense mechanisms,[9] because once a single-stranded antisense nucleic acid binds to its targeted RNA, the RNase H specifically recognizes the DNA/RNA hybrid regions, ultimately cleaving the RNA strand. Therefore, RNase H activity

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of DNA/RNA hybrids is quite important for the efficacy of these nucleic acid drugs. Recently, in addition to single-stranded antisense drugs, heteroduplex oligonucleotides (HDOs) containing DNA/RNA hybrid duplexes have been reported as another type of antisense nucleic acid-drug candidates.[10] Therefore, DNA/RNA hybrid duplexes are important not only for the mechanisms of antisense drugs but also themselves as HDO-type antisense drugs.

When cationic oligopeptides bind to DNA/RNA hybrids, they alter the thermodynamic stability of the hybrid duplex and might prevent the binding of nucleases to the hybrids. These alterations possibly affect the cleavage reactions of the DNA/RNA hybrids by a variety of endogenous nucleases. For example, paromomycin, which is one of aminoglycosides, were previously reported to increase RNase A resistance but to decrease RNase H activity of a DNA/RNA hybrid duplex.[11] Recently, we reported some artificial cationic oligosaccharides which could increase RNase A resistance without decreasing RNase H activity of an HDO.[12] Although the cationic oligopeptides in our previous report seem to be slightly inferior to the oligosaccharide system in interacting selectivity to A-type nucleic acid duplexes, the oligopeptides could be advantageous over oligosaccharides from the viewpoint that the oligopeptides tend to more thermodynamically stabilize A-type nucleic acid duplexes and they can be easier to synthesis. An ideal cationic oligopeptide should increase the nuclease resistance and not decrease the RNase H activity of the hybrids. In this study, we assessed the effect of cationic oligopeptides using a 12mer DNA/RNA hybrid, $d(CAGT)_3/r(ACUG)_3$, using Dab8, Dab10, Dab12, Orn8, Dap8, Lys8, Agp8, Agb8, Arg8, and Agh8 (Figure 1).

Table 1 ΔT_m values of the DNA/RNA hybrid duplex in the presence of cationic oligopeptides relative to the T_m value in the absence of the peptides in 10 mM phosphate buffer containing 100 mM of NaCl, pH = 7.0. [DNA] = [RNA] = [cationic oligopeptide] = 4 μM; DNA: CAGTCAGTCAGT; RNA: ACUGACUGACUG.

Entry	Peptide	ΔT_m
1	Dap8	+ 1.7
2	Dab8	+ 10.5
3	Orn8	+ 6.9
4	Lys8	+ 6.3
5	Agp8	+ 13.0
6	Agb8	+ 9.3
7	Arg8	+ 5.2
8	Agh8	+ 2.8
9	Dab10	+14.1
10	Dab12	+18.7

First, we performed UV melting analysis to evaluate the effect of the cationic oligopeptides on the thermodynamic stability of

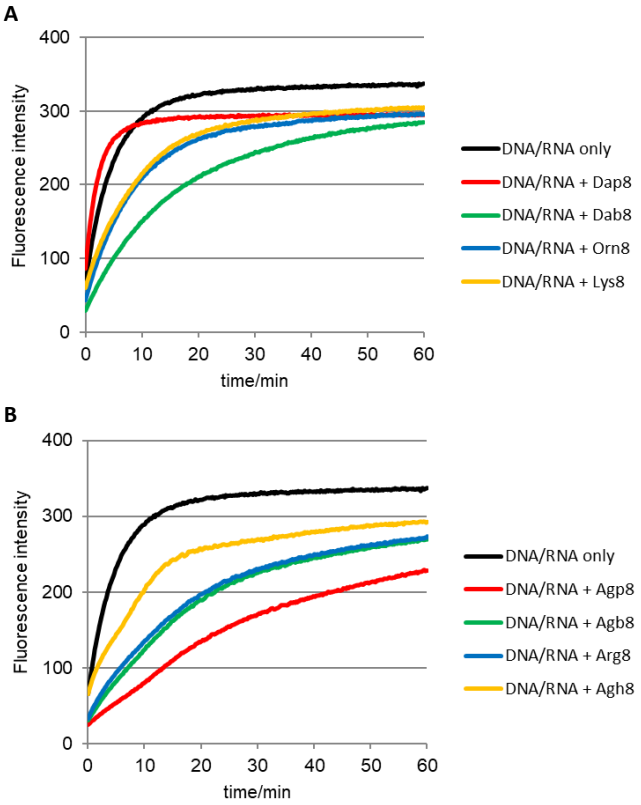

Figure 2 Fluorescence signals as a function of time. The DNA/RNA hybrid was treated with 0.5 μg/mL of RNase A at 30 °C and pH = 7.3. The initial concentration of the DNA/RNA hybrid was 100 nM in Tris-HCl buffer containing 100 mM of NaCl; DNA: CAGTCAGTCAGT; RNA: FAM-ACUGACUGACUG-Dabcyl; A) The results in the absence or presence of 200 nM oligoaminopeptides. B) The results in the absence or presence of 200 nM oligoguanidinopeptides.

the DNA/RNA hybrid. The results are shown in Table 1. Dap8, Dab8, Orn8, and Lys8 increased the T_m value of the hybrid by 1.7 °C, 10.5 °C, 6.9 °C, 6.3 °C, and Agp8, Agb8, Arg8, and Agh8 increased the T_m by 13.0 °C, 9.3 °C, 5.2 °C, and 2.8 °C, respectively. Among the octaamino and octaguanidinopeptides, Dab8 and Agp8 provided the most thermodynamic stability to the hybrid; similar results were previously obtained for the stabilization of an RNA/RNA duplex.[8b] We also conducted circular dichroism (CD) experiments for studying the cause of the differences of the stabilization ability between individual oligopeptides (Figure S4-S5). In all cases, CD spectra of the DNA/RNA hybrid were nearly unchanged by addition of any cationic oligopeptides. In addition, because CD spectra of each oligopeptide alone did not show those of specific secondary-order structures, they were suggested to form random-coiled structures (data not shown). Therefore, Dab8 and Agp8 could be to most entropically favoured over other oligopeptides with longer or shorter side chain length because the distance between amino groups of Dab8 and Agp8 (9Å and 11Å, respectively, distance between two nitrogen atoms) are close to the major groove width (8-15 Å, distance between oxygen atoms of two phosphate groups calculated from NMR solution structures[13]). Slight Increase or decrease in peak intensity around 210 nm in the presence of cationic oligopeptides might

reflect a specific secondary-order structure of the peptides in the complexes.

Dab10 and Dab12, which have 10 and 12 amino groups, respectively, increased the T_m value of the hybrid more than Dab8 (ΔT_m values were 14.1, 18.7 °C, respectively). Then, we also conducted fluorescence anisotropy measurements to evaluate the affinity of Dab8 and Agp8 to the DNA/RNA hybrid. The dissociation constants of Dab8 and Agp8 were calculated to be 1.7×10^{-8} and 3.1×10^{-8} M, respectively, using the hybrid duplex of 12mer DNA and FAM-modified RNA (Figure S6-S7). In addition, from the Job plots, binding ratios were suggested to be approximately 1 in both cases (Figure S8-S9).

The RNase A resistance of the DNA/RNA hybrid was monitored using a fluorescence resonance energy transfer (FRET) system.[14] A 12mer RNA bearing a FAM- and a Dabcyl group at each terminus was used to induce FRET. If the RNA strand is cleaved, FRET does not occur, and the fluorescence intensity increases. Although RNase A is an endonuclease specific to single-stranded RNA, it is also known to cleave the RNA strand in a duplex.[15] As shown in Figure 2A, increase in the fluorescence intensity was observed following RNase treatment, and the intensity reached saturation in approximately 20 min (black line). This increase was not inhibited in the presence of Dap8. On the other hand, other oligoaminopeptides, Dab8, Orn8, and Lys8 reduced the rate of fluorescence increase. Among them, Dab8 was the most effective in lowering the rate of fluorescence increase. Figure 2B shows the variations in fluorescence intensity in the presence or absence of oligoguanidinopeptides. In these cases, Agh8 was the least and Agp8 was the most effective in lowering the rate of fluorescence increase. Because kinetic parameters of these reactions were difficult to calculate due to that the cleavage reaction occurs at multiple sites to generate multiple fragments, apparent $t_{1/2}$ was calculated by the fluorescence intensity (Table S2). These results were almost similar to those obtained by the UV melting analysis; which indicated that a peptide that could thermodynamically stabilize the DNA/RNA hybrid duplex was more effective in inhibiting the rate of the fluorescence increase by RNase A cleavage. For example, Dab8 and Agp8, which increased the T_m values most among the octaaminopeptides and octaguanidinopeptides respectively, also mostly increased the apparent $t_{1/2}$. As RNase A cleaves the single-stranded RNA in a hybrid duplex in equilibrium between a duplex and a partial single-stranded structure, it is reasonable to assume that the peptides that thermodynamically stabilize the duplex structures tend to increase the RNase A resistance of the DNA/RNA hybrid by preventing the formation of single stranded structures. On the other hand, we now do not know why only Dap8 accelerate this RNase A cleavage reaction.

The RNase H activity of the DNA/RNA hybrid duplex in the absence and presence of the cationic oligopeptides was evaluated using the same DNA/RNA hybrid used in RNase A experiments. The experimental results are shown in Figure 3. In the absence of cationic peptides, the fluorescence intensity increased with time following the addition of RNase H and was saturated in approximately 25 min (black line). The fluorescence intensity profiles in the presence of Dap8, Orn8, or Lys8 were quite similar to those observed in the absence of the cationic peptides (Figure 3A, Table S2). However, the increase in fluorescence intensity clearly accelerated in the presence of Dab8. This accelerating effect was not observed in the presence of other oligopeptides. As shown in Figure 3B, Agp8, Agb8, Arg8, and Agh8 slightly reduced the rate of fluorescence increase. Figure 3C compares the fluorescence intensity exhibited in the presence of Dab peptides of different chain lengths; the fluorescence increase rates were found to decrease with an increase in the oligopeptide length. In contrast to the results obtained for RNase A cleavage, these results in Figure 3 did not correlate with those obtained from the UV melting analysis. One reason why some cationic oligopeptides did not inhibit

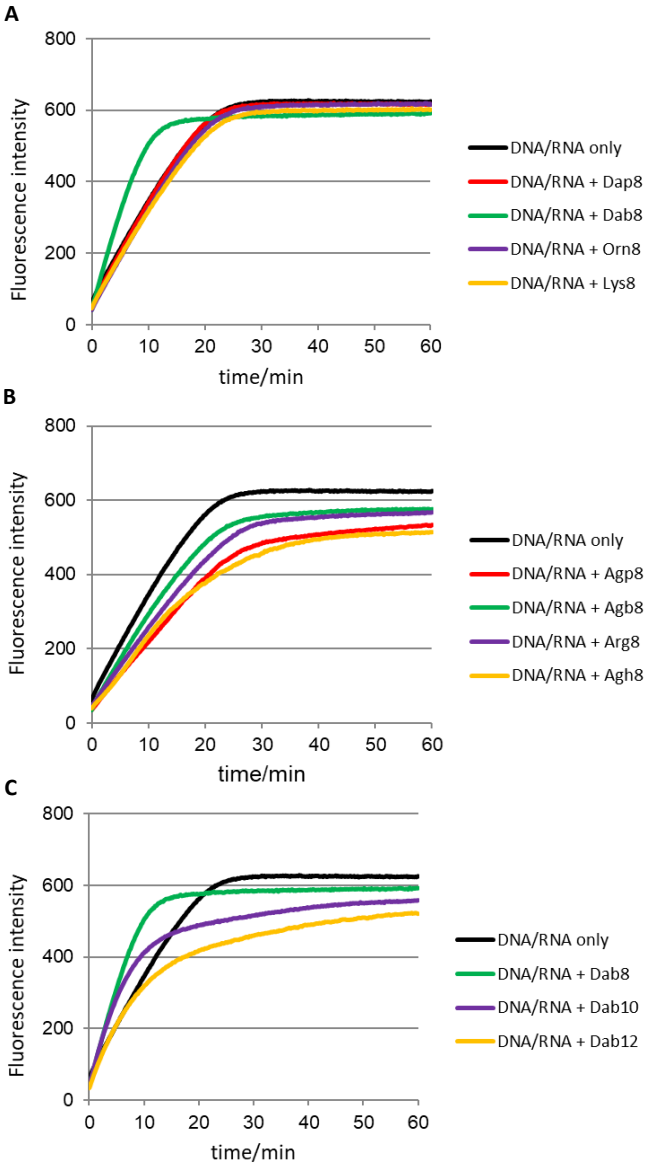

Figure 3 Fluorescence signals as a function of time. The DNA/RNA hybrid was treated with 6.7U/mL of RNase H at 20 °C, pH = 7.5. The initial concentration of the DNA/RNA hybrid was 100 nM in a Tris-HCl buffer containing 100 mM of NaCl; DNA: CAGTCAGTCAGT; RNA: FAM-ACUGACUGACUG-Dabcyl; A) The results in the absence or presence of 200 nM oligoaminopeptides. B) The results in the absence or presence of 200 nM oligoguanidinopeptides. C) The results in the absence or presence of 200 nM Dab8, Dab10, and Dab12.

RNase H cleavage might be different binding modes of the oligopeptides and RNase H to the DNA/RNA hybrid. The cationic oligopeptides were designed as major groove binders to A-type nucleic acid duplexes and recently we demonstrated that the major groove binding of Dab8 to an RNA/RNA duplex by NMR study.[16] On the other hand, RNase H are known to access from minor groove of DNA/RNA hybrids.[17] As shown in Figure 4A, Dab8 are expected to bind to phosphate groups near the 5′ termini of both strands, which compose a major groove, and RNase H are expected to recognize phosphate groups near the 3′ termini of both strands from the X-ray crystal structure.[17a] Therefore, these bindings could be difficult to compete against each other. Additionally, Dab8 could increase the thermodynamic stability of the DNA/RNA hybrid. In other words, Dab8 increased the rate of the hybrid duplex structure in equilibrium between the duplex and single-stranded structures. Therefore, this stabilization might be preferable for RNase H to bind to the DNA/RNA hybrid because the substrate of this enzyme is the hybrid duplex structure and this might cause the enhancement of RNase H activity. On the other hand, Dab12 could be easier to compete against RNase H than Dab8 in the binding to the DNA/RNA hybrid because of its longer peptide length even if Dab12 bound to the major groove of the hybrid duplex (Figure 4B). This hypothesis is consistent with the fact that the fluorescence increase rates decreased with an increase in the oligopeptide length of Dab peptides.

Our results suggest that oligopeptides with adequate side-chain lengths and number of amino groups can enhance the RNase H cleavage as well as RNase A resistance of a DNA/RNA hybrid. In other words, RNase A resistance and RNase H activity do not trade-off by the use of some adequate oligocationic molecules. These results are vital for the development of nucleic acid-based drugs.

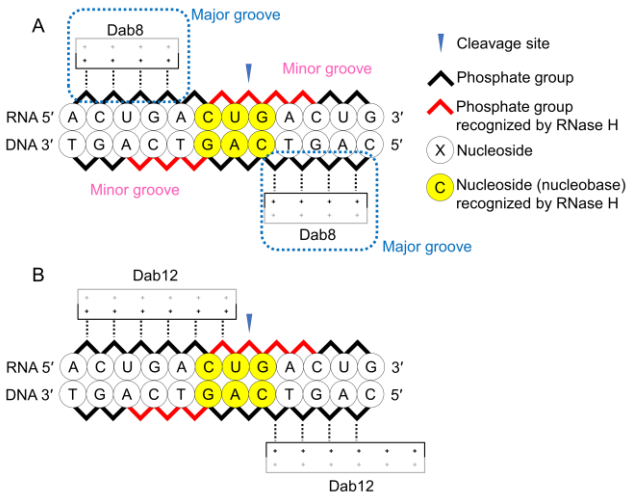

Figure 4 Plausible typical binding sites of RNase H and cationic oligopeptides in a DNA/RNA hybrid duplex; (A) in the case of Dab8. (B) in the case of Dab12. The RNase H recognition sites was based on the crystal structure of human RNase H1-DNA/RNA complex.[17a]

Conflicts of interest

There are no conflicts to declare.

Acknowledgement

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