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5′-*O*-Dephosphorylated 2′,5′-oligoadenylate (2-5A) with 8-methyladenosine at the 2′-terminus activates human RNase L

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ABSTRACT

Human ribonuclease L (RNase L), an interferon-induced endoribonuclease, becomes enzymatically active after binding to 2-5A. The 5′-phosphoryl group of 2-5A is reportedly necessary for the conformational change leading to RNase L activation. However, we found that 5′-*O*-dephosphorylated 2-5A tetramer analogs with 8-methyladenosine at the 2′-terminus were more effective as an activator of RNase L than the parent 2-5A tetramer. Introduction of 8-methyladenosine is thought to induce a dramatic shift of 2-5A in the binding site of RNase L.

Interferons play a critical role in host immune responses against viral infections. One well-characterized mechanism of interferon action is the 2-5A system.[1,2] The biological action of the 2-5A system is mediated by RNase L, which is found in many eukaryotic cells. The 2-5A molecule functions by binding to RNase L, converting it from an inactive monomer to its catalytically active dimer. The activated RNase L cleaves single-stranded RNA preferentially on the 3′-side of UpNp.[3] This RNase L-mediated RNA degradation inhibits protein synthesis resulting in the suppression of viral replication.

To explore the potential for the development of an antiviral agent based on the 2-5A system, various 2-5A analogs were synthesized and their biological function and structure-activity relationship were investigated. A 5′-phosphoryl group, a minimum of three adenylyl residues in the 2′,5′-phosphodiester linkage[4] and the 3′-hydroxyl group of the second adenosine from the 5′-terminus[5] are reportedly necessary for the conformational change leading to RNase L activation. The adenine ring at the 5′-terminus of 2-5A is critical for the binding to RNase L, while the third adenine ring of 2-5A is required for the activation of RNase L (Fig. 1).[6]

We have previously reported the crystal structure of the *N*-terminal ankyrin repeat domain (ANK) of human RNase L complexed with activator 2-5A.[7] The crystal structure of the ANK/2-5A complex (Protein Data Bank accession code 1WDY) clearly shows that the bound 2-5A molecule directly interacts with ANK and that the third adenine ring of 2-5A is in a *syn* conformation (Fig. 6A).[7,8] The 2-5A analogs modified at the 8-position (e.g., 8-methyl) of the third adenine ring from the 5′-terminus, which is thereby forced into a *syn* conformation, are significantly more effective as an activator of RNase L than the unsubstituted 2-5A trimer.[9]

Here, we report the synthesis of 5′-*O*-dephosphorylated 2-5A analogs with an 8-methyladenosine residue and their ability to activate recombinant human RNase L. Among these analogs, analog **5** (A2′p5′A2′p5′A2′p5′(me^{8}A)) was more effective as an activator of RNase L than the parent 2-5A (**1b**). This increased RNase L activa-

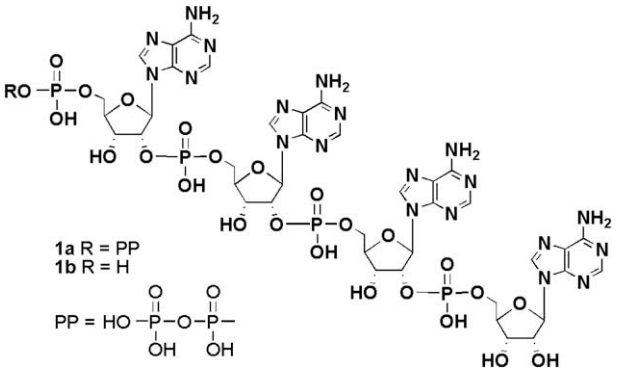

Figure 1. Structure of 2-5A.

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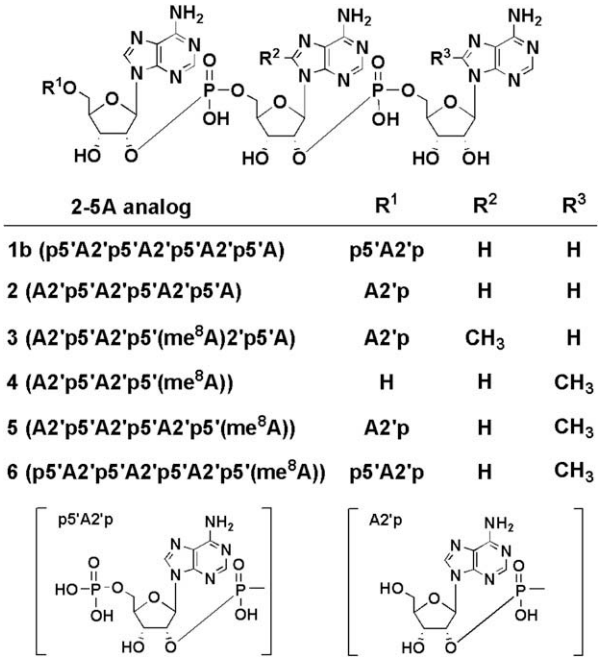

Figure 2. Structures of 2-5A analogs.

tion ability is thought to arise from a shift in the 2-5A binding site of RNase L compared with the binding site for the parent 2-5A (**1b**).

Analog **1b** (p5′A2′p5′A2′p5′A2′p5′A) and 2-5A analogs were synthesized by the standard phosphoramidite method with a DNA/RNA synthesizer. The phosphoramidite unit of 8-methyladenosine was prepared by a previously reported method.[10,11] The fully protected oligonucleotides linked to the solid support were treated according to a reported procedure.[11] The obtained **1b** and 2-5A analogs (**2–6**) were purified by reversed phase high performance liquid chromatography (HPLC) and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). The observed molecular weights were in agreement with the expected structures (Fig. 2).[12]

Table 1
Activation ability of 2-5A analogs

2-5A analog	EC_{50}[a] (nM)	Relative activity
1b	9.7	1
2	—[b]	—
3	>50	<0.19
4	>50	<0.19
5	2.5	3.9
6	2.5	3.9

[a] Concentration of 2-5A analogs required for cleavage of half of a synthetic RNA.
[b] Cleavage of the synthetic RNA was not observed.

The ability of the parent 2-5A (**1b**) and 2-5A analogs (**2-6**) to activate RNase L was determined by monitoring the cleavage of a synthetic RNA (Fig. 3). In this study, 5′-fluorescein-r($C_{11}U_2C_7$)-3′ was used as the substrate RNA. Recombinant human RNase L was expressed in *Escherichia coli* and purified as previously reported.[13,14] The reaction was analyzed by polyacrylamide gel electrophoresis. The EC_{50} values and relative activities of 2-5A analogs (**5** and **6**) in relation to **1b** ([EC_{50} of **1b**]/[EC_{50} of 2-5A analog]) are summarized in Table 1. The EC_{50} of **1b** was 9.7 nM. The relative activities of analogs **5** and **6** with 8-methyladenosine residing in the 2′-terminus position were each 3.9. The ability of analogs **5** and **6** to elicit RNase L activity was greater than that of **1b**. 5′-*O*-Dephosphprylated 2-5A analog **2** exhibited no RNase L activation ability and the 2-5A analogs **3** and **4** had slight RNase L activation ability under the same conditions.

The stability of the 8-methylated 2-5A analog **5** against nucleolytic hydrolysis by snake venom phosphodiesterase (SVPD) was also investigated.[15] As shown in Figure 4, the half-lives of the parent 2-5As (p5′A2′p5′A2′p5′A and **1b**; p5′A2′p5′A2′p5′Ap5′A) were 18 and 14 min, respectively. However, the half-life of the 2-5A analog **5** was greater than 90 min. Thus, analog **5** was more stable against SVPD digestion than the parent 2-5As (Fig. 4).

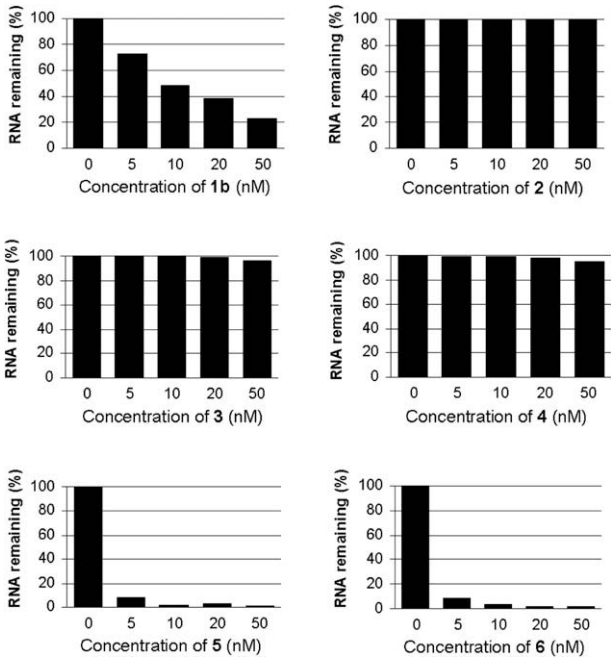

Figure 3. RNA cleavage by recombinant human RNase L activated with 2-5A analogs. Concentration of RNA substrate: 200 nM. Concentration of RNase L: 50 nM. Incubation time: 30 min. Graph shows percentage of RNA remaining.

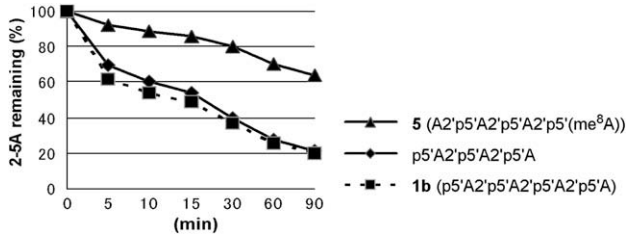

Figure 4. Stability of methylated 2',5'-oligoadenylate against snake venom phosphodiesterase. Concentration of 2-5A analogs: 9 μM. Concentration of SVPD: 0.004 unit/mL. Reaction buffer: 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$ at 37 °C. The reaction mixture was analyzed by HPLC. The residual fragment ration of 2-5A analogs to the initial amount was determined from their peak area ratios.

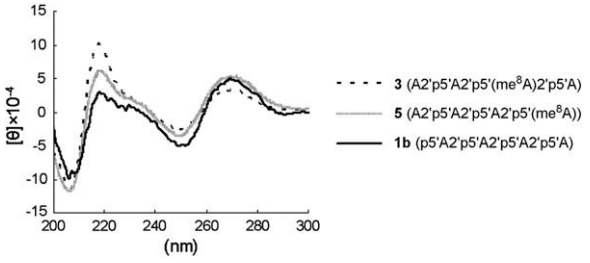

Figure 5. CD spectra of 2-5A analogs in 100 mM sodium chloride, 10 mM sodium phosphate (pH 7.0) at 22 °C.

The circular dichroism (CD) spectra of the parent 2-5A (**1b**) and 2-5A analogs (**3** and **5**) at 22 °C are shown in Figure 5. The spectra show a negative band around 250 nm and two positive bands around 220 nm and 270 nm. Analogs **3** and **5** did not possess a conformation notably different from **1b**.

We speculate about the binding pattern of the parent 2-5As (**1a** and **1b**) and 2-5A analogs (**2** and **5**), respectively, as shown in Figure 6. The parent 2-5As (**1a** and **1b**) are assumed to bind initially to the A site of ANK. The binding of three adenosine nucleotides from the 5′-terminus to RNase L contributes significantly to the ability of 2-5A to warp the ANK structure, thus inducing a conformational change in the enzyme and/or unmasking an interaction domain, permitting dimerization and activation of RNase L (Fig. 6B and C). **2** had no RNase L activation ability under the same conditions. However, the ability of **5** and **6** to activate RNase L were approximately fourfold higher than that of **1b**. It has been suggested that introduction of 8-methyladenosine into the 2′-terminus can, due to the 8-methyl substituent, force the nucleoside to adopt a *syn* conformation around the base-sugar glycosidic bond and consequently enhance the interactions between the B site of ANK and 8-methyladenosine of the 2′-terminus; the three adenosine nucleotides from the 2′-terminus play an important role in the activation of RNase L (Fig. 6E). Furthermore, the phosphodiester group, without the 5′-monophosphoryl group, is thought to be involved in RNase L activation (Fig. 6D and E).

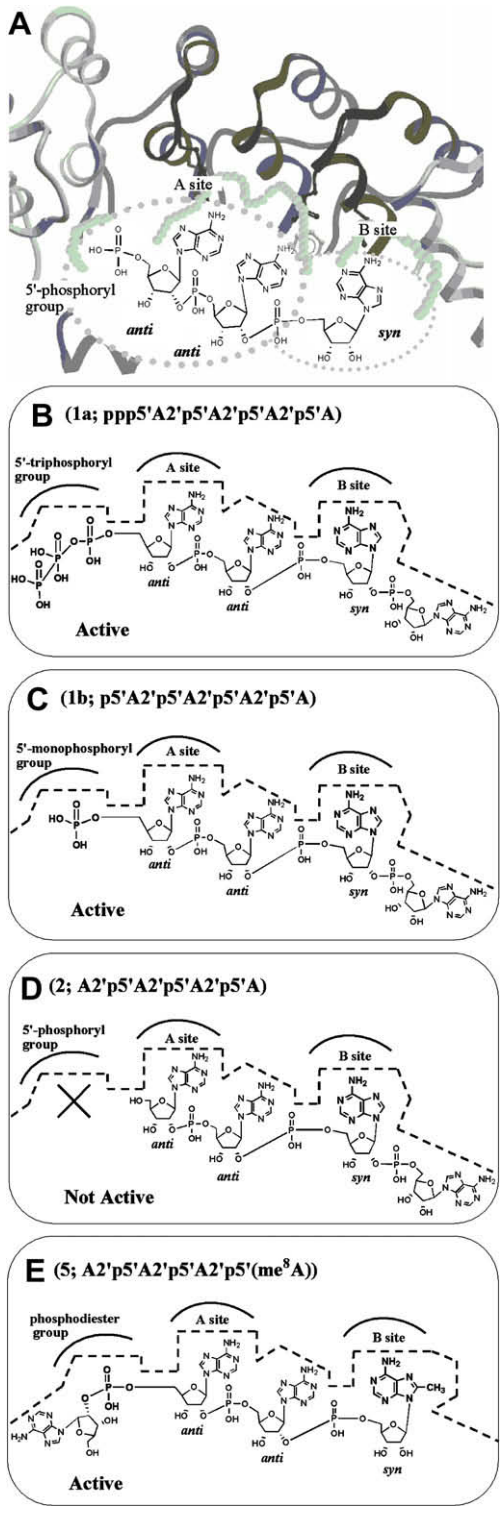

Figure 6. (A) Crystal structure of ANK complexed with 2-5A.[7] (B–E) Presumed model for binding pattern of the parent 2-5As (**1a** and **1b**) and 2-5A analogs (**2** and **5**), respectively.

In conclusion, we synthesized 2-5A analogs containing 8-methyladenosine. The ability of these analogs to activate recombinant human RNase L and their phosphatase resistance were investigated. The analog **5** (A2′p5′A2′p5′A2′p5′(me^{8}A)) was significantly more effective as an activator of RNase L than **1b**. This increased RNase L activation ability of **5** leads to the hypothesis that the introduction of an 8-methyladenosine residue at the 2′-terminus of the 2-5A tetramer induces a shift of the 2-5A binding site against RNase L. Furthermore, **5** was more resistant to nucleolytic hydrolysis by snake venom phosphodiesterase than **1b**. These observations will contribute significantly to the design of potent activators of RNase L as antiviral agents.

Acknowledgments

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