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# A Specific Substrate-Inhibitor, a 2′-Deoxy-2′-fluorouridine-Containing Oligoribonucleotide, against Human RNase L

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**Abstract**—We examined the properties of RNA analogues containing 2′-deoxy-2′-α-fluorouridine (**1**) or 2′-*O*-methyluridine (**2**) as inhibitors against human RNase L, that cleaves a single-stranded RNA in the presence of 2′,5′-linked oligoadenylate (2-5A). The RNA analogue, FF, containing two molecules of **1** in place of uridine efficiently inhibited the RNase L-catalyzed RNA cleavage reaction, whereas the analogue, MM, containing two molecules of **2** was found not to have affinity for the enzyme. The $k_{cat}$ value for FF was 1/100 of that for an unmodified RNA, UU, whereas the $K_m$ value of FF was only twice as great as that of UU. Thus, it was found that the analogue, FF, containing **1** is an efficient inhibitor against human RNase L.


## Introduction

The 2-5A system is an RNA degradation pathway that is an important mechanistic component of interferon's action against viral infections.[1] The mechanism is as follows: (i) Interferon induces the expression of 2-5A synthetases, which, in the presence of double-stranded RNA, catalyze the formation of 2′,5′-linked oligoadenylates (2-5As); (ii) These 2-5As bind to and allosterically activate the latent RNase L; (iii) The activated RNase L cleaves viral and cellular RNAs on the 3′-sides of UpNp sequences;[2] (iv) The RNA degradation results in inhibition of protein synthesis and thereby inhibition of viral replication.

RNase L activity has been shown to be important in defense against viral infections and induction of apoptosis. Overexpression of wild-type RNase L suppressed the replication of several viruses, such as the encephalomyocarditis virus.[3] Conversely, the antiviral effect of interferon was suppressed by overexpression of a catalytically inactive mutant of RNase L in murine cells.[3] When RNase L was overexpressed, cell death, with morphological and biochemical characteristics of apoptosis, was also caused.[4] Furthermore, the expression of the inactive mutant of RNase L blocked staurosporine-induced apoptosis.[5]

Although RNase L plays an important role in the 2-5A system, no high-resolution structure of RNase L bound to its target RNA has been solved. In part, this situation arises from the necessarily fleeting nature of an enzyme–substrate complex. After binding to RNase L, the RNA substrate is immediately cleaved by the enzyme. Thus, it is difficult to analyze the structure of the complex between the enzyme and the RNA substrate. To overcome this problem, we have planned a synthesis of an RNA analogue forming a stable, long-lived complex suitable for structural analysis.

In this paper, we report the design, synthesis, and properties of inhibitors, that are comprised of oligoribonucleotides incorporating 2′-modified nucleoside analogues, against human RNase L.

## Results and Discussion

### Design and synthesis of RNA analogues

RNase L cleaves a single-stranded RNA preferentially on the 3′-side of a UpNp sequence. Carroll and coworkers reported that initial cleavage of a synthetic RNA, 5′-r($C_{11}U_2C_7$)-3′, occurs on the 3′-side of r($C_{11}U_2$) to yield an r($C_{11}$UpUp) fragment with a 3′-phosphate, and a second cleavage occurs on the 3′-side of r($C_{11}$U) to give an r($C_{11}$Up) fragment with a higher enzyme concentration or longer incubation time.[2] The result suggests that the RNase L-catalyzed RNA cleavage proceeds by the mechanism via a 2′,3′-cyclic phosphate as

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an intermediate similarly to other nucleases such as RNase A, RNase T1, and RNase T2.[6] Based on this background information, we designed an RNA analogue containing 2′-deoxy-2′-α-fluorouridine (**1**) or 2′-*O*-methyluridine (**2**) (Fig. 1) instead of uridine as an inhibitor against RNase L. These nucleoside analogues **1** and **2** adopt predominantly *N-type* sugar conformations the same as uridine.[7] However, the analogue **1** lacks a 2′-hydroxyl group, whereas the 2′-hydroxyl group of the analogue **2** is protected with the methyl group. Thus, we envisioned that the RNA containing **1** or **2** instead of uridine would be an inhibitor against RNase L which would be recognized but not cleaved by the enzyme.

| abbreviation | sequence |
|---|---|
| UU | 5′-CCC CCC CCC CCU UCC CCC CC-3′ |
| FU | 5′-CCC CCC CCC CC**1** UCC CCC CC-3′ |
| UF | 5′-CCC CCC CCC CCU **1**CC CCC CC-3′ |
| FF | 5′-CCC CCC CCC CC**1** **1**CC CCC CC-3′ |
| MU | 5′-CCC CCC CCC CC**2** UCC CCC CC-3′ |
| UM | 5′-CCC CCC CCC CCU **2**CC CCC CC-3′ |
| MM | 5′-CCC CCC CCC CC**2** **2**CC CCC CC-3′ |
| *UU | Flu-5′-CCC CCC CCC CCU UCC CCC CC-3′-Rho |
| *FF | Flu-5′-CCC CCC CCC CC**1** **1**CC CCC CC-3′-Rho |

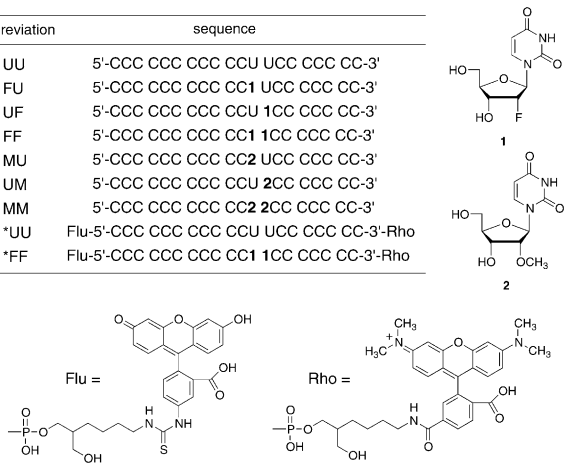


**Figure 1.** Sequences of oligonucleotides used in this study.

All RNA analogues containing **1** or **2** were synthesized by the standard phosphoramidite method with a DNA/RNA synthesizer.[8] RNA analogues, *UU and *FF (Fig. 1), modified with fluorescein and rhodamine derivatives were synthesized according to the reported method with a slight modification.[9]

## RNA cleavage by recombinant human RNase L

Recombinant human RNase L was expressed in *Escherichia coli* and purified according to the reported procedure.[10] The RNAs labeled at their 5′-ends with $^{32}$P were incubated with the enzyme that had been pre-incubated with 2′,5′-p$A_4$. The reactions were analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions[11] (Fig. 2). The densities of radioactive bands on the gel were determined with a bioimaging analyzer. Percentages of the RNAs cleaved in the presence of 60 nM human RNase L are represented by a histogram in Fig. 3. When the unmodified RNA, UU, was used as a substrate, RNA cleavage was observed on the 3′-sides of both uridines. On the other hand, when the RNA analogue, FU or UF, containing a molecule of **1** was used as a substrate, they were mainly cleaved on the 3′-side of uridine and not cleaved on the 3′-side of the analogue **1**. When two molecules of **1** were incorporated into RNA, the percentage of total cleavage of the RNA, FF, decreased to 25%. Furthermore, it is noteworthy that the RNA, FF, was not cleaved at all under the conditions (2 nM 2-5A and 120 nM RNase L), under which the unmodified RNA, UU, was cleaved completely (data not shown). Similarly,

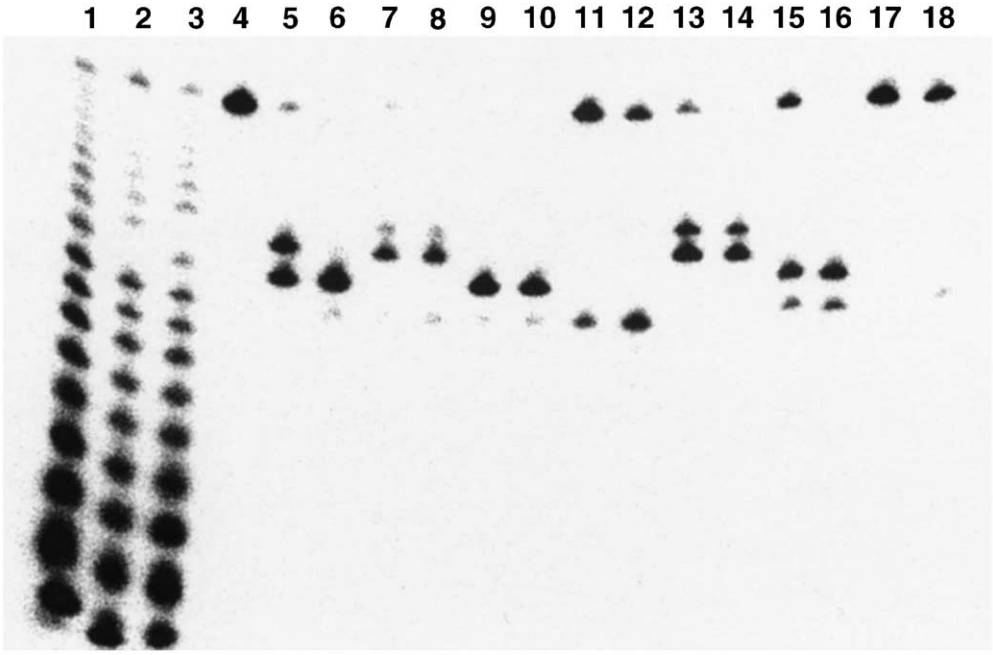


**Figure 2.** Polyacrylamide gel electrophoresis of 5′-$^{32}$P-labeled RNAs hydrolyzed by recombinant human RNase L activated with 2′,5′-p$A_4$. Lane 1: limited alkaline hydrolysis of 5′-$^{32}$P-labeled UU; lane 2: limited alkaline hydrolysis of 5′-$^{32}$P-labeled FU; lane 3: limited alkaline hydrolysis of 5′-$^{32}$P-labeled UM; lane 4: 5′-$^{32}$P-labeled UU; lanes 5 and 6: 5′-$^{32}$P-labeled UU + 2′,5′-p$A_4$ + RNase L; lanes 7 and 8: 5′-$^{32}$P-labeled FU + 2′,5′-p$A_4$ + RNase L; lanes 9 and 10: 5′-$^{32}$P-labeled UF + 2′,5′-p$A_4$ + RNase L; lanes 11 and 12: 5′-$^{32}$P-labeled FF + 2′,5′-p$A_4$ + RNase L; lanes 13 and 14: 5′-$^{32}$P-labeled MU + 2′,5′-p$A_4$ + RNase L; lanes 15 and 16: 5′-$^{32}$P-labeled UM + 2′,5′-p$A_4$ + RNase L; lanes 17 and 18: 5′-$^{32}$P-labeled MM + 2′,5′-p$A_4$ + RNase L. 60 nM (lanes 5, 7, 9, 11, 13, 15, and 17) or 120 nM (lanes 6, 8, 10, 12, 14, 16, and 18) of recombinant human RNase L was used in each experiment. The experimental conditions are given in the Experimental.

when a molecule of **2** was incorporated into RNA, the analogues, UM and MU, were mainly cleaved on the 3′-side of uridine (Fig. 3). When two molecules of **2** were introduced into RNA, the analogue, MM, was not cleaved by the enzyme at all.

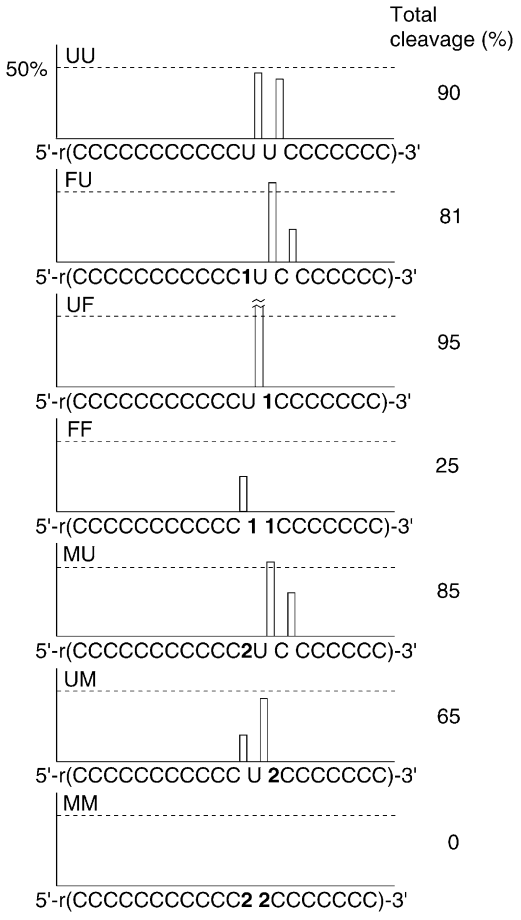


**Figure 3.** The cleavage sites on the RNA analogues by human RNase L.

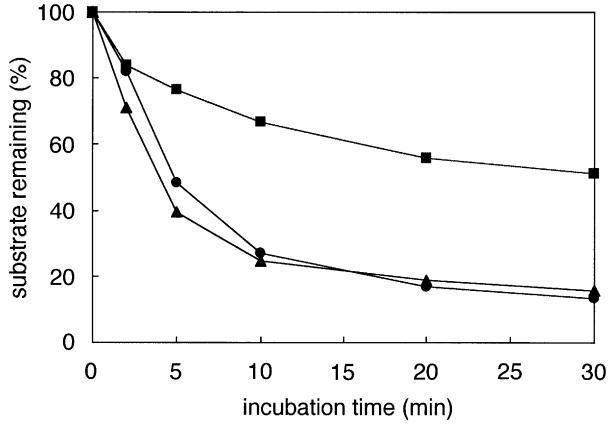


**Figure 4.** Inhibition of the RNase L-catalyzed RNA cleavage by the RNA analogues. ●: 5′-$^{32}$P-labeled UU (100 nM) + 2′,5′-p$A_4$ (2 nM) + RNase L (120 nM); ■: 5′-$^{32}$P-labeled UU (100 nM) + FF (100 nM) + 2′,5′-p$A_4$ (2 nM) + RNase L (120 nM); ▲: 5′-$^{32}$P-labeled UU (100 nM) + MM (100 nM) + 2′,5′-p$A_4$ (2 nM) + RNase L (120 nM). The experimental conditions are given in the Experimental.

## Inhibitory effect of the RNA analogues against RNase L-catalyzed RNA cleavage reaction

Next, we tested the inhibitory effect of the RNA analogues, FF and MM, against RNA cleavage by human RNase L. A series of experiments was carried out in a buffer containing the unmodified RNA (100 nM) labeled at its 5′-end with $^{32}$P, the RNA analogue (100 nM), 2-5A (2 nM), and human RNase L (120 nM). After being incubated at 30 °C, the products were analyzed by PAGE under denaturing conditions. The densities of radioactive bands on the gel were determined with a bio-imaging analyzer. As shown in Figure 4, the analogue, FF, effectively inhibited the RNase L-catalyzed RNA cleavage reaction. On the other hand, the analogue MM did not inhibit the reaction at all. This result indicates that the analogue FF containing **1** is an efficient inhibitor against RNase L, whereas the analogue MM containing **2** does not have affinity for the enzyme.

## Fluorescence resonance energy transfer (FRET) analysis of RNase L-catalyzed RNA cleavage

Geselowitz et al. reported that a FRET analysis of RNase L-catalyzed RNA cleavage reaction is of great use for determination of the kinetic parameters of the reaction.[9] Thus, we determined the kinetic parameters of the cleavage reaction of FF using the FRET method, and compared them with the kinetic parameters of UU. In this study, RNAs, *UU and *FF (Fig. 1), modified with fluorescein and rhodamine derivatives were used as the substrates.

The intensity of the fluorescence of the substrate, *UU, rose by mixing it with a buffer containing RNase L and 2′,5′-p$A_4$. Eventually, the intensity of the fluorescence became constant at a value about 2.2 times the starting value (Fig. 5). On the other hand, when *FF was used as a substrate, the intensity of the fluorescence of the reaction mixture increased slowly compared to the case of *UU. The Michaelis–Menten kinetic parameters were calculated from individual reaction curves by preparing

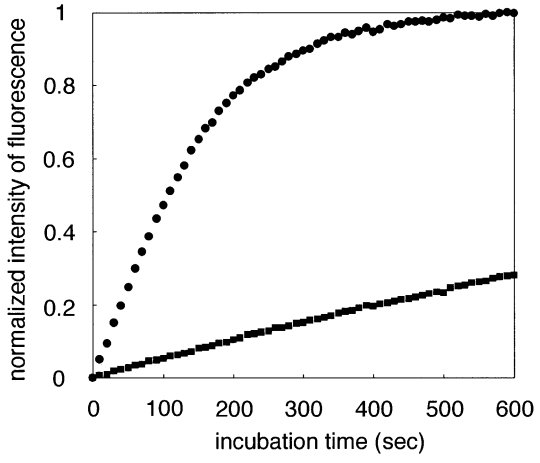


**Figure 5.** Profiles of FRET analysis of RNase L-catalyzed RNA cleavage reaction. ●: *UU; ■: *FF. The experimental conditions are given in the Experimental.

**Table 1.** Kinetic parameters of the RNase L-catalyzed RNA clevage reaction

| Substrate | $K_m$ (nM) | $V_{max}$ (nM/s) | $k_{cat}$ (1/s) | $k_{cat}/K_m$ (1/s/nM) |
|---|---|---|---|---|
| *UU | 264 | 1.75 | 0.364 | $1.38\times10^{-3}$ |
| *FF | 531 | 0.213 | $3.54\times10^{-3}$ | $6.67\times10^{-6}$ |

Walker–Schmidt plots.[9] The parameters are summarized in Table 1. The turnover number ($k_{cat}$) and the Michaelis constant ($K_m$) for *UU were 0.364 $s^{-1}$ and 264 nM, respectively. On the other hand, those for *FF were $3.54\times10^{-3}$ $s^{-1}$ and 531 nM, respectively. The $k_{cat}$ value for *FF decreased by 2-order compared to that for *UU, whereas the $K_m$ value of *FF was only twice greater than that of *UU.

In order to confirm whether the hydrolyzed product, 5′-**1**$_2$C$_7$-3′, inhibits the RNase L-catalyzed RNA cleavage reaction of *FF, we next synthesized 5′-**1**$_2$C$_7$-3′ and performed the cleavage reaction of *FF in the presence of 1 equivalent of 5′-1$_2$C$_7$-3′. The reaction conducted in the presence of 5′-**1**$_2$C$_7$-3′ showed a very similar reaction profile to that conducted in the absence of 5′-**1**$_2$C$_7$-3′. The result indicates that the hydrolyzed product, 5′-**1**$_2$C$_7$-3′, almost does not influence on the cleavage reaction of *FF. From these results, it was found that the analogue, FF, containing **1** is an efficient inhibitor against human RNase L.

## Conclusion

In this paper, we have reported the design and synthesis of inhibitors against human RNase L. The RNA analogue, FF, containing two molecules of 2′-deoxy-2′-α-fluorouridine (**1**) in place of uridine efficiently inhibited the RNase L-catalyzed RNA cleavage reaction, whereas the RNA analogue, MM, containing two molecules of 2′-*O*-methyluridine (**2**) was found not to have affinity for the enzyme. The $k_{cat}$ value for FF was 1/100 of that for UU, whereas the $K_m$ value of FF was only twice greater than that of UU. Thus, it was found that the analogue, FF, containing 1 was suitable for structural analysis of a complex between RNase L and an RNA substrate. Analysis of the function of human RNase L using the RNA analogue, FF, is currently under way in our laboratory.

## Experimental

### RNA synthesis

All RNAs were synthesized using phosphoramidite units purchased from GLEN Research. The synthesis was carried out with an Applied Biosystems DNA/RNA synthesizer (Model Expedite). The synthesized RNAs were purified as reported previously.[12] The fluorescent RNAs, *UU and *FF, were synthesized using Fluorescein Phosphoramidite, 3′-Amino-Modifier C7 CPG, and TAMRA NHS Ester, which were purchased from GLEN Research, according to the reported procedure.[9] Recombinant human RNase L was expressed and purified according to the reported procedure with a slight modification.[10] The 5′-ends of the RNAs were labeled with [γ-$^{32}$P]ATP and T4 polynucleotide kinase A-19 (Takara Shuzo Co. Ltd.).

RNA cleavage by recombinant human RNase L. 2′,5′-p$A_4$ (final concentration, 12 nM), was mixed with recombinant human RNase L (final concentration, 60 or 120 nM) in a buffer comprising 20 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate, 8 mM 2-mercaptoethanol, 90 mM KCl, and 0.1 mM ATP (total, 19 μL), and then the mixture was incubated on ice. After 30 min, 1 μL of a solution containing 2 μM RNA labeled with $^{32}$P at the 5′-end was added to the mixture (total, 20 μL), and then the solution was incubated at 30 °C. After 30 min, an aliquot of the reaction mixture (5 μL) was added to a loading solution (10 μL) containing 7 M urea. The solution was analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea. The densities of radioactive bands on the gel were determined with a Bio-imaging Analyzer (Bas 2000, Fuji Co., Ltd.).

### Inhibition of RNase L-catalyzed RNA cleavage by the RNA analogue

2′,5′-p$A_4$ (final concentration, 2 nM), was mixed with recombinant human RNase L (final concentration, 120 nM) in a buffer comprising 20 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate, 8 mM 2-mercaptoethanol, 90 mM KCl, and 0.1 mM ATP (total, 45 μL), and then the mixture was incubated on ice. After 30 min, 2.5 μL of a solution containing 2 μM RNA labeled with $^{32}$P at the 5′-end and 2.5 μL of a solution containing 2 μM RNA analogue was added to the mixture (total, 50 μL), and then the solution was incubated at 30 °C. At appropriate periods, an aliquot of the reaction mixture (5 μL) was added to a loading solution (10 μL) containing 7 M urea. The solution was analyzed as described above.

### FRET analysis of RNase L-catalyzed RNA cleavage

*UU and *FF, modified with fluorescein and rhodamine derivatives were synthesized according to the reported method with a slight modification.[9] Fluorescence measurements were performed using a fluorometer (Fluorescence Spectrophotometer F4500, Hitachi Inc.). 2′,5′-p$A_4$ (final concentration, 100 nM or 1 μM), was mixed with recombinant human RNase L (final concentration, 4.8 or 60 nM) in a buffer comprising 1.15 mM HEPES (pH 7.5), 10.4 mM magnesium chloride, 0.5 mM DTT, 10.4 mM KCl, 0.02% PEG-6000, and 0.12 mM ATP (total, 150 μL), and then the mixture was incubated at 25 °C. After 10 min, 50 μL of a solution containing 1 μM *UU or *FF was added to the mixture (total, 200 μL), and then the solution was incubated at 25 °C. Samples were analyzed using a 485-nm excitation filter and a 518-nm emission filter.

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