Doxifluridine-conjugated 2-5A analog shows strong RNase L activation ability and tumor suppressive effect

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Abstract
RNase L is activated by 2',5'-oligoadenylates (2-5A) at subnanomolar levels to cleave single-stranded RNA. We previously reported the hypothesis that the introduction of an 8-methyladenosine residue at the 2'-terminus of the 2-5A tetramer shifts the 2-5A binding site of RNase L. In this study, we synthesized various 5'-modified 2-5A analogs with 8-methyladenosine at the 2'-terminus. The doxifluridine-conjugated 8-methyladenosine-substituted 2-5A analog was significantly more effective as an activator of RNase L than the parent 5'-monophosphorylated 2-5A tetramer and showed a tumor suppressive effect against human cervical cancer cells.

1. Introduction
Interferons are proteins with antiviral, antitumor, and immunomodulatory activities. The interferon-inducible 2',5'-oligoadenylates (2-5A) synthetase (OAS)/RNase L pathway, known as the 2-5A system, is a well-characterized activation mechanism of interferons. Treatment of cells with an interferon activates genes encoding one or several OAS isoforms, and RNase L. OAS isoforms are activated by binding to double-stranded RNA, a frequent by-product of viral infections. The activated OAS isoforms generate a series of 2-5A molecules from ATP. The 2-5A molecules [p5' (AP)n; x = 1–3, n ≥ 2] bind strongly to RNase L and convert it from an inactive monomer to a catalytically active dimer. Activated RNase L cleaves single-stranded RNA preferentially on the 3'-side of UpNp. RNase L-mediated RNA degradation inhibits protein synthesis resulting in cell apoptosis and thereby the suppression of viral replication. Furthermore, a positional cloning technique has identified RNase L as a candidate for a hereditary prostate cancer gene.

We previously reported the crystal structure of the 2-5A binding N-terminal ankyrin repeat domain (ANK) of human RNase L complexed with 2-5A. The crystal structure of ANK/2-5A complex (Protein Data Bank accession code 1WDY) clearly shows that the bound 2-5A molecule directly interacts with ANK, and the third adenine ring of 2-5A is in a syn conformation. Preferring a syn conformation, 2-5A trimer analogs with 8-methyladenosine residues in the third position (2'-terminus) [p5'A2p5'A2p5'(me8A)], ppp5'A2p5'A2p5'(me8A)] are significantly more effective activators of RNase L than the corresponding unsubstituted 2-5A trimers.

We recently found that 5'-monophosphorylated 2-5A tetramer analogs with 8-methyladenosine at the 2'-terminus [A2p5'A2p5'A2p5'(me8A)] were more effective activators of RNase L than the parent 5'-monophosphorylated 2-5A tetramer [p5'A2p5'A2p5'A2p5'], although a 5'-phosphoryl group is reported to be necessary for the conformational change leading to RNase L activation. Based on these results, the introduction of 8-methyladenosine is thought to induce a shift of 2-5A in the binding site of RNase L (Fig. 1). We synthesized various 8-methyladenosine-substituted 2-5A analogs (Fig. 2) to test this hypothesis. The ability of these analogs to activate recombinant human RNase L and their resistance to phosphatase were also investigated.

2. Results and discussion
The parent 5'-monophosphorylated 2-5A tetramer (1) and 2-5A analogs (2-9) were synthesized by the standard phosphoramidite
method with a DNA/RNA synthesizer. Controlled pore glass solid supports linked to 8-methyladenosine or adenosine, and phosphoramidite units of adenosine and uridine were prepared by previously reported methods. A phosphoramidite derivative of doxifluridine (5'-deoxy-5-fluorouridine, 5'dF5U), doxifluridine-2'-phosphoramidite was prepared in two steps from doxifluridine. The fully protected oligonucleotides linked to solid supports were treated according to a reported procedure. The obtained and 2-5A analogs (2–9) were purified by reversed phase high performance liquid chromatography (HPLC) and analyzed by matrix-assisted laser desorption/ionization coupled with time-of-flight mass spectrometry (MALDI-TOF/MS), and the observed molecular weights were in good agreement with their structures. The abilities of the parent 2-5A tetramer (1) and the 2-5A analogs (3–5) to activate RNase L were determined by monitoring the cleavage of a synthetic 5'-fluorescein (F)-modified RNA [F-5'-r(C11U2C7)-3']. Recombinant human RNase L was expressed in Escherichia coli and purified as previously reported. Reactions were analyzed by polyacrylamide gel electrophoresis. The half-maximal effective concentration (EC50) values and relative activities of 2-5A analogs in relation to (1) (EC50 of 1)/(EC50 of 2-5A analog) are summarized in Table 1.

The relative activities of 5 [p5'U2'p5'A2'p5'A2'p5'(me8A)] and 6 [U2'p5'A2'p5'A2'p5'(me8A)] were 4.8 and 5.4, respectively. These analogs had almost the same ability as 3 [A2'p5'A2'p5'A2'p5'(me8A)] to induce RNase L activity. On the other hand, 5'-UMP (uridine monophosphate)-modified 2-5A analogue bearing adenosine at the 2'-terminus 4 [p5'U2'p5'A2'p5'A2'p5'A] showed only a slight ability to activate RNase L under the same conditions. These results strongly supported the hypothesis that the introduction of an 8-methyladenosine residue at the 2'-terminus induces a shift of the 2-5A binding site of RNase L. Interestingly, substitution of the 2',5'-phosphodiester bond at the 5'-side by a 3',5'-phosphodiester

Figure 1. Structures of 2-5A analogs and a plausible model of the binding patterns for the 2-5A analogs (A: adenine ring; R: ribose; P: phosphate).

Figure 2. Structures of 2-5A analogs synthesized in this study.
Table 1
Activation ability of 2-5A analogs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>EC50b (nM)</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>p5’A2’p5’A2’p5’A2’p5’A</td>
<td>12.9</td>
<td>1</td>
</tr>
<tr>
<td>2c</td>
<td>A2’p5’A2’p5’A2’p5’A</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>A2’p5’A2’p5’A2’p5’(me8A)</td>
<td>2.3</td>
<td>5.6</td>
</tr>
<tr>
<td>4</td>
<td>p5’U2’p5’A2’p5’A2’p5’A</td>
<td>84.0</td>
<td>0.15</td>
</tr>
<tr>
<td>5</td>
<td>p5’U2’p5’A2’p5’A2’p5’(me8A)</td>
<td>2.7</td>
<td>4.8</td>
</tr>
<tr>
<td>6</td>
<td>U2’p5’A2’p5’A2’p5’(me8A)</td>
<td>2.4</td>
<td>5.4</td>
</tr>
<tr>
<td>7</td>
<td>A3’p5’A2’p5’A2’p5’(me8A)</td>
<td>20.3</td>
<td>0.64</td>
</tr>
<tr>
<td>8</td>
<td>U3’p5’A2’p5’A2’p5’(me8A)</td>
<td>134</td>
<td>0.1</td>
</tr>
<tr>
<td>9</td>
<td>(5’dfU)2’p5’A2’p5’A2’p5’(me8A)</td>
<td>0.45</td>
<td>28.9</td>
</tr>
</tbody>
</table>

a A, me8A, U and 5’dfU denote adenosine, 8-methyladenosine, uridine and doxifluridine, respectively.
b Concentration of 2-5A analogs required for cleavage of half of a synthetic RNA.
c Our previous data.
d Cleavage of the synthetic RNA was not observed.

The strong effect of the 8-methyladenosine substituent could induce a shift of 2-5A in the binding site of ANK. In fact, the ability of both 5 and 6 to activate RNase L equaled that of 3, even though these analogs contain UMP and uridine, respectively. On the other hand, substitution of the 2’,5’-phosphodiester bond at the 5’-side by a 3’,5’-phosphodiester bond could decrease interactions with the C site of ANK. From comparing the RNase L activation ability of 7 and 8, it is suspected that there is another interaction site (D site).

On the basis of these findings, we designed 5’-doxifuridine-modified 2-5A analogue 9 [(5’dfU)2’p5’A2’p5’A2’p5’(me8A)], a 2-5A–doxifuridine hybrid. Doxifuridine is a synthesized fluoropyrimidine anticancer drug, which is an oral prodrug of 5-fluorouracil. It is activated to 5-fluorouracil by pyrimidine nucleoside phosphorylase in tumor cells. The 2-5A–doxifuridine conjugate should maintain high RNase L activity and show anti-tumor effects. Therefore, the hybrid 2-5A analogue could be a therapeutic agent against cancer derived from oncogenic viruses such as human papillomavirus, which is associated with cervical cancer, and hepatitis C virus, a cause of liver cancer. As described above, the 2-5A analogue 9 was synthesized using standard phosphoramidite method and RNase L activity was examined. Surprisingly, the ability of 9 (EC50 = 0.45 nM) to elicit RNase L activity was actually 30 times greater than that of the parent 2-5A tetramer 1. This result appears to support the presence of a D site. In the case of 9, a fluorine atom on the uracil ring interacts strongly with amino acid residues of the D site to activate RNase L (Fig. 4). This interaction is not thought to occur in the case of 2-5A analogs 3, 5, and 6.

The stability of the 2-5A–doxifuridine hybrid 9 against nucleolytic hydrolysis by snake venom phosphodiesterase was also investigated (Fig. 5). The half-lives of the parent 2-5A 1 and

![Figure 3](Image)

Figure 3. Presumed model of binding patterns for 2-5A analogs 4, 5, 7 and 8 (A: adenine ring; U: uracil ring; R: ribose; P: phosphate). Values in parentheses described following each sequence indicate relative activity to 1.

![Figure 4](Image)

Figure 4. Presumed model for the binding patterns of 6 and 9 (A: adenine ring; U: uracil ring; R: ribose; P: phosphate). Values in parentheses described following each sequence indicate relative activity to 1.
were 6.4 and 9.0 min, respectively; thus, 9 was more resistant to the enzyme than was the parent 2-5A.

We next examined the growth inhibitory effect of 2-5A–doxifluridine hybrid 9 in human cervical cancer HeLa cells (Fig. 6). HeLa cells were transfected with 1, 9, and doxifluridine at concentrations of 0.1 μM and 1 μM for 6, 24, and 48 h. The parent 2-5A tetramer 1 slightly suppressed cell proliferation. In contrast, 9 inhibited cell growth in a dose-dependent manner similar to doxifluridine alone. At a concentration of 1 μM, cell growth was approximately 25% that of the control. Although further investigation is required, 9 should be a potentially long-acting anticancer agent with low adverse effects.

3. Conclusions

In conclusion, we synthesized various 5′-modified 2-5A analogs with 8-methyladenosine at the 2′-terminus to prove the hypothesis that the introduction of an 8-methyladenosine residue at this position induces a shift of the 2-5A binding site of RNase L. It was also found that RNase L activation ability was reduced by substitution of the 2′,5′-phosphodiester bond at the 5′-side with a 3′,5′-phosphodiester bond. The doxifluridine-conjugated 8-methyladenosine-substituted 2-5A analogue had an approximately 30-fold greater ability to activate RNase L and greater resistance to nucleolytic hydrolysis in comparison with the parent 2-5A tetramer. Furthermore, the 2-5A–doxifluridine hybrid suppressed the growth of human cervical cancer cells in a dose-dependent manner. These observations will contribute significantly to the design of 2-5A-based antiviral and anticancer agents.

4. Experimental

4.1. General remarks

All reactions were carried out under an argon atmosphere, unless otherwise noted. All reagents and solvents were purchased from commercial vendors and used without further purification, unless indicated otherwise. 1H and 13C NMR spectra were recorded on a JEOL JNM AL-400 spectrometer or JNM ECS-400 spectrometer (400 MHz for 1H NMR, 100 MHz for 13C NMR, and 162 MHz for 31P NMR). Chemical shifts (δ) were expressed in parts per million and internally referenced (7.26 ppm for CDCl3 for 1H NMR, 77.0 ppm for CDCl3 for 13C NMR and 0.00 ppm for H3PO4/CDCl3 for 31P NMR). Direct analysis in real time (DART) coupled with TOF mass spectra were taken on a JMS T100TD instrument. MALDI-TOF mass spectra were taken on a Shimadzu AXIMA-CFR plus instrument. Flash column chromatography was performed using silica gel 60N [spherical neutral (63–210 μm)] from Kanto Chemical Co., Inc.

4.2. Preparation of doxifluridine-2′-phosphoramidite (10)

A solution of tert-butyldimethylsilyl chloride (301 mg, 2.0 mmol) in THF (2 mL) was added dropwise to a solution of doxifluridine (246 mg, 1.0 mmol) and imidazole (204 mg, 3.0 mmol) in DMF (2 mL), and then the mixture was stirred at room temperature for 3 h. The reaction was quenched by the addition of saturated aqueous NaHCO3 solution. The reaction mixture was partitioned between CH2Cl2 and H2O. The organic layer was washed with brine, dried over Na2SO4 and concentrated under reduced pressure. The residue was purifed by column chromatography on silica gel (n-hexane/EtOAc, 10:1–3:1) to give 2′-O-(tert-butyldimethylsilyl)doxifluridine (150 mg, 42%) and 3′-O-(tert-butyldimethylsilyl)doxifluridine (120 mg, 33%) as both colourless solids. N,N-Diisopropylethylamine (230 μL, 1.32 mmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (120 μL, 530 μmol) were added to a solution of 3′-O-(tert-butyldimethylsilyl)doxifluridine...
(95 mg, 260 μmol) in THF (1.3 mL), and then the mixture was stirred at room temperature for 1 h. The reaction mixture was partitioned between CHCl3 and saturated NaHCO3 aqueous solution. The organic layer was washed with brine, dried over Na2SO4, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (n-hexane/EtOAc, 3:2:1:1) to give 2′-O-(tert-butyldimethylsilyl)-3′-O-[2-cyanoethoxy](N,N-diisopropylamino)phosphoryl)doxifuridine (doxifuridine-2′-phosphoramidite; 10) as a colourless solid (120 mg, 79%); 31P NMR (162 MHz, CDCl3) δ = 150.0, 149.7.

4.2.1. 2′-O-(tert-butyldimethylsilyl)doxifuridine 1H NMR (400 MHz, CDCl3) δ = 8.27 (s, 1H, NH), 7.37 (d, J = 6.4 Hz, 1H, H-6), 5.71–5.70 (d, J = 2.8 Hz, H-1′), 4.17 (q, J = 2.8 Hz, 1H, H-2′), 4.04 (quin, J = 6.5 Hz, 1H, H-4′), 3.68–3.63 (m, 1H, H-3′), 2.45 (d, J = 8.0 Hz, 1H, OH), 1.45 (d, J = 6.5 Hz, 3H, H-5′), 0.93 (s, 9H, C(CH3)3), 0.17 (s, 3H, SiCH3), 0.13 (s, 3H, SiCH3); 13C NMR (100 MHz, CDCl3) δ = 237.0 Hz), 123.6 (d, J = 34.0 Hz), 90.6, 80.0, 75.7, 74.9, 25.6, 18.2, 18.0, -4.6, -5.3; MS (DART) m/z 361 [M+H]+, HRMS (DART) Calcd for C15H26FN2O5Si [M+H]+: 361.1595. Found: 361.1546.

4.2.2. 3′-O-(tert-butyldimethylsilyl)doxifuridine 1H NMR (400 MHz, CDCl3) δ = 8.52 (s, 1H, NH), 7.36 (d, J = 6.0 Hz, 1H, H-6), 5.66 (d, J = 4.0 Hz, 1H, H-1′), 4.09–4.05 (m, 1H, H-2′), 4.02 (quin, J = 6.0 Hz, 1H, H-4′), 2.96 (d, J = 4.8 Hz, 1H, OH), 1.38 (d, J = 6.5 Hz, 3H, H-5′), 0.94 (s, 9H, C(CH3)3), 0.16 (s, 3H, SiCH3), 0.15 (s, 3H, SiCH3); 13C NMR (100 MHz, CDCl3) δ = 156.8 (d, J = 27.0 Hz), 148.8, 140.5 (d, J = 237.0 Hz), 123.6 (d, J = 34.0 Hz), 90.6, 80.0, 75.7, 74.9, 25.6, 18.2, 18.0, -4.6, -4.8; MS (DART) m/z 361 [M+H]+, HRMS (DART) Calcd for C15H26FN2O5Si [M+H]+: 361.1595. Found: 361.1583.

4.3. Cell growth inhibition assay

HeLa cells were grown at 37 °C in a humidified atmosphere of 5% CO2 in air in Dulbecco’s modified Eagle medium (D-MEM) (Wako) supplemented with 10% bovine serum (Sigma). Each 2-SA analogue and doxifuridine (1 μM or 0.1 μM) was transfected to the cells using Transfast (Promega) (10 μM) and Opti-MEM (Life Technologies). After 1 h, D-MEM (250 μL) containing 10% bovine serum was added to each well, and cells were further incubated at 37 °C. After 6, 24, and 48 h, viable cells in each well were counted by a Counting Chamber (Erma). The results were confirmed by three independent transfection experiments with two cultures each, and are expressed as the averages from three experiments as means ± standard deviation.

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References and notes

1. For a review of the 2′-5A system, see: Silverman, R. H. J. Virol. 2007, 81, 12720.