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DISCRIMINATION BETWEEN RIBONUCLEASE H- AND RIBONUCLEASE L-MEDIATED RNA DEGRADATION BY 2′-*O*-METHYLATED 2-5A-ANTISENSE OLIGONUCLEOTIDES

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Abstract: 2′,5′-Oligoadenylate (2-5A) antisense chimeric oligonucleotides were synthesized containing varying 2′-*O*-methyl-ribonucleotide substitution patterns in the antisense domain. The ability of these composite oligonucleotides to mediate RNase H- and RNase L-catalyzed RNA degradation showed that these two enzymes have different activation requirements.

Antisense therapeutics[1] is based on the ability of a specific oligonucleotide or analogue thereof to base-pair with a targeted RNA and subsequently interfere with its biological function by a passive mechanism of "steric blocking" or by induced catalytic RNA degradation. RNA destruction has been realized through the intervention of RNase P, ribozymes and RNase H.[2] In fact, RNase H often has been held to be key to the effectiveness of oligonucleotides as antisense agents.[3] In a different approach, we reported that composite oligonucleotides containing 5′-monophosphorylated 2′,5′-oligoadenylate (2-5A) joined to an antisense oligonucleotide can address a selected RNA and effect its degradation through the activation of latent 2-5A-dependent RNase L.[4] This 2-5A-antisense strategy was conceived as a way to enhance the potency of antisense.[4] Through activation of RNase L, 2-5A-antisense would provide certain oligonucleotides, such as methylphosphonates, with a catalytic mode of action. For unmodified DNA oligonucleotides, capable of inducing target cleavage through hybrid formation and subsequent RNase H-induced scission of the RNA, 2-5A-antisense may enhance activity by addition of a second mechanism of RNA destruction.[5] Moreover, RNase L[5] possesses a different substrate specificity and subcellular distribution than RNase H[6] and therefore may be useful if the RNA target is not available to RNase H.

Because of such considerations, the following questions have been addressed herein. Since 2′-*O*-substitution is often used to enhance antisense oligonucleotides affinity for target RNA and to stabilize the antisense molecule against serum and cellular nuclease destruction,[7] what is the effect of antisense domain 2′-*O*-methylation on the ability of 2-5A-antisense to activate the 2-5A-dependent RNase L? What is the effect of the presence of the 2-5A tetramer and associated butanediol phosphate linkers[4] on the ability of the 2-5A-antisense to induce RNA degradation by the RNase H pathway? Is it possible to find a 2-5A-antisense chimera which would activate only RNase L, but not permit operation of the RNase H mechanism? An answer to the latter question may provide a tool that would permit discrimination of the relative contributions of the RNase H vs RNase L mechanisms to the biological activity of 2-5A-antisense. Thus, we report here the evaluation of a series of 2′-*O*-methylated 2-5A-antisense composite oligonucleotides and their spectrum of RNase L and RNase H activities.

Since the mRNA for the dsRNA-activated protein kinase PKR had been targeted successfully with 2-5A-antisense,[4] all composite oligonucleotides were synthesized containing the 19-mer antiPKR sequence and are named generically as 2-5A-antiPKRs. The structures, sequence, and 2′-*O*-methylation patterns of these 11 2-5A-

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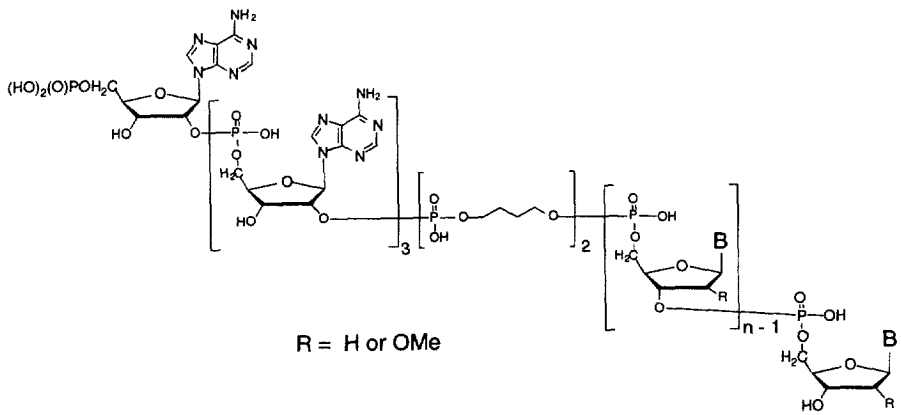
antiPKR chimeras are shown in Figure 1. Synthesis was accomplished using phosphoramidite chemistry with reagents, procedures and purification methodologies described previously[4,8] but with the inclusion of 2′-*O*-methyl-nucleoside phosphoramidites (Glen Research, Sterling, VA) as required. Capillary gel electrophoresis was used to establish purity which was >90%. The nucleotide composition of each 2-5A-antisense chimera was determined as described previously[7] by digestion with snake venom phosphodiesterase followed by product analysis using ion-exchange HPLC. Results of such analyses are shown in Table 1.



1 (p5′A2′)$_3$p5′A2′p[O(CH$_2$)$_4$Op]$_2$dGdTdAdCdT dAdCdTdCdC dCdTdGdCdT dTdCdTdG

2 (p5′A2′)$_3$p5′A2′p[O(CH$_2$)$_4$Op]$_2$dGdTdAdCdT dAdCdTdCdC dCdTdGdCdT $U_mC_mU_mG_m$

3 (p5′A2′)$_3$p5′A2′p[O(CH$_2$)$_4$Op]$_2$dGU_mdAC_mdT A_mdCU_mdCC_m dCU_mdGC_mdT U_mC_mU G_m

4 (p5′A2′)$_3$p5′A2′p[O(CH$_2$)$_4$Op]$_2$dGdT$A_m$$C_m$dT dA$C_m$$U_m$dCdC $C_m$$U_m$dGdC$U_m$ $U_mC_mU_mG_m$

5 (p5′A2′)$_3$p5′A2′p[O(CH$_2$)$_4$Op]$_2$dGdTdAdCdT dA$C_m$$U_m$$C_m$$C_m$ $C_m$$U_m$$G_m$$C_m$$U_m$ $U_mC_mU_mG_m$

6 (p5′A2′)$_3$p5′A2′p[O(CH$_2$)$_4$Op]$_2$$G_m$$U_m$$A_m$$C_m$$U_m$ $A_m$$C_m$$U_m$$C_m$$C_m$ dCdT dGdCdT dT$C_m$$U_m$$G_m$

7 (p5′A2′)$_3$p5′A2′p[O(CH$_2$)$_4$Op]$_2$$G_m$$U_m$$A_m$$C_m$$U_m$ dAdCdTdCdC dC$U_m$$G_m$$C_m$$U_m$ $U_mC_mU_mG_m$

8 (p5′A2′)$_3$p5′A2′p[O(CH$_2$)$_4$Op]$_2$$G_m$$U_m$$A_m$$C_m$dT dAdCdT$C_m$$C_m$ $C_m$$U_m$$G_mCU_m$ $U_mC_mU_mG_m$

9 (p5′A2′)$_3$p5′A2′p[O(CH$_2$)$_4$Op]$_2$$G_m$$U_m$$A_m$$C_m$$U_m$ $A_m$$C_m$$U_m$$C_m$$C_m$ $C_m$$U_m$dGdCdT dT$C_m$$U_m$$G_m$

10 (p5′A2′)$_3$p5′A2′p[O(CH$_2$)$_4$Op]$_2$$G_m$$U_m$$A_m$ $C_m$$U_m$ A_m $C_m$$U_m$C C CdT $G_m$$C_m$$U_m$ $U_mC_mU_m$ G_m

11 (p5′A2′)$_3$p5′A2′p[O(CH$_2$)$_4$Op]$_2$ $G_mU_mA_mC_mU_m$ $A_mC_mU_mC_mC_m$ $C_mU_mG_mC_mU_m$ $U_mC_mU_mG_m$

Figure 1. Structure of 2′,5′-A-antisense composite nucleic acids. Antisense domain sequences are written in the 5′ -> 3′ direction and a space is inserted after each fifth nucleotide. 2′-*O*-methylation is denoted by the subscript m.

When the 2-5A-antisense chimeras (**1-11**) were hybridized to complementary PKR sense RNA, the duplexes' melting temperature (T_m) increased in accordance with rising percentage of 2′-*O*-methylation (Table 2) with only minor sequence-specific effects (e.g., **3** vs **4**, **5** vs **6** and **7**, **8** vs **9** and **10**). The increased T_m reflects the greater thermodynamic stability resulting from the 2′-*O*-methylations favoring a 3′-*endo* conformation for the ribose while preorganizing the oligonucleotide for formation of the A-form of the heteroduplex.[9]

Table 1
Characterization of 2-5A-AntiPKR Oligonucleotides

	Snake Venom Phosphodiesterase Digestion Products[a]							
Oligo	dCMP	C_mMP	TMP	U_mMP	dGMP	G_mMP	dAMP	A_mMP
1 calc	7	0	7	0	3	0	2	0
obs	7.0	0	7.5	0	3.4	0	2.1	0
2 calc	6	1	5	2	2	1	2	0
obs	5.7	1.0	5.1	2.2	2.2	1.1	2.1	0
3 calc	3	4	2	5	2	1	1	1
obs	2.6	3.7	2.0	5.5	2.2	1.3	1.0	1.0
4 calc	3	4	2	5	2	1	1	1
obs	2.9	3.8	1.9	5.6	2.2	1.1	1.0	1.0
5 calc	1	6	2	5	1	2	2	0
obs	1.3	6.9	2.7	7.5	1.5	3.1	2.4	0
6 calc	2	5	3	4	1	2	0	2
obs	2.1	5.2	3.0	4.5	1.3	2.6	0	2.1
7 calc	4	3	1	6	0	3	1	1
obs	4.1	3.1	1.0	6.6	0	3.7	1.2	1.1
8 calc	1	6	2	5	0	3	1	1
obs	0.9	5.5	1.9	6.0	0	3.6	1.1	1.1
9 calc	1	6	2	5	1	2	0	2
obs	1.0	6.0	2.3	6.3	1.3	2.5	0	2.0
10 calc	3	4	1	6	0	3	0	2
obs	2.7	3.7	0.9	6.9	0	3.5	0	2.0
11 calc	0	7	0	7	0	3	0	2
obs	0	6.4	0	7.9	0	3.5	0	1.9

[a] 2-5A-AntiPKR oligonucleotides (**1-11**) were digested with snake venom phosphodiesterase using published[8] conditions. Separation and quantitation was by HPLC as described earlier[8]. For each compound (**1-11**), the theoretical calculated (**calc**) component nucleotide composition is given followed by the experimentally determined (**obs**) value. In accord with a reviewer's suggestion, we have not listed specific results for two digestion products which were common to all chimeras. The observed values for the AMP product ranged from 2.6 to 3.0. Also the relative ratio of AMP bearing intact butane diol linker (pABuBu) ranged from 0.8 to 1.0.

For determination of the ability of RNase H to cleave PKR RNA when hybridized with each of the 2-5A-antisense chimeras (**1-11**), the following conditions were employed. The appropriate 2-5A-antisense chimera (0.6 A_{260} unit) and complementary 19-mer PKR sense RNA (0.5 A_{260} unit) were dissolved in 175 μL RNase-free H_2O plus 20 μL of a 10x RNase H cleavage buffer (100 mM HEPES, pH 7.5, 1000 mM KCl, 50 mM $Mg(OAc)_2$, 10 mM ATP, and 143 mM β-mercaptoethanol. RNase H [5 μL (10 units), *E. coli* enzyme, 2100 units/mL, Sigma, St. Louis, MO] was added and the reaction mixture was incubated at 37 °C. Aliquots were removed at 0 time, 15 min, 1 h, 4 h and 24 h and added to 1.5 volumes of 20 mM EDTA (pH 7.5) and stored at -70 °C for later analysis.

HPLC was used to assess RNase H enzyme digests. Analysis was on a Ultrasphere C18 reverse phase column using the following elution program: 30 min gradient of 5-45 % solvent B in solvent A where solvent A was triethylammonium acetate, 0.1 M, pH 7.0, and solvent B was triethylammonium acetate, 0.1 M, pH 7.0 and CH_3CN (1:1, v/v). The substrate PKR sense RNA, the 2-5A-antiPKR chimera (**1**), and the duplex PKR sense RNA: 2-5A-antiPKR chimera (**1**) were separated cleanly when the temperature of the column was 25 °C. However, when the temperature of the column was increased to 50 or 70 °C, only the non-hybidized oligonucleotides, PKR sense RNA and the chimera (**1**) were present since the duplex had dissociated. Thus, RNase H digests of PKR sense RNA mediated by 2-5A-antisense chimeras **1**-**11** were analyzed using the latter conditions in which the disappearance of the substrate PKR RNA as well as the appearance of resultant cleavage products could be monitored with the unaffected 2-5A-antisense chimera serving as an internal standard.

Table 2
Properties of 2′-*O*-Methylated 2-5A-antiPKR Composite Oligonucleotides

Oligo	% 2′-O-Me	T_m [a]	Degradation of PKR RNA by RNase H $t_{1/2}$	Activation[b] of RNase L IC_{50}
1	0	72.1	<< 15 min	$3.4 \times 10^{-8} \pm 1.4 \times 10^{-8}$
2	21	74.4	<< 15 min	$2.1 \times 10^{-8} \pm 0.3 \times 10^{-8}$
4	58	79.8	>> 24 h	$2.6 \times 10^{-8} \pm 0.6 \times 10^{-8}$
3	58	78.3	>> 24 h	$2.7 \times 10^{-8} \pm 1 \times 10^{-8}$
6	68	81.9	~ 15 min	ND
7	68	81.2	~ 15 min	ND
5	68	82.9	<< 15 min	ND
10	79	83.7	~ 1 h	ND
8	79	82.7	<< 15 min	ND
9	79	83.7	~ 15 min	ND
11	100	87.9	>> 24 h	$2.9 \times 10^{-8} \pm 0.3 \times 10^{-8}$

[a] T_m values were measured at 260 nm with an HP 8452 spectrophotometer in a buffer of 0.15 M NaCl, 0.01 M sodium cacodylate (pH 7.5), 1 mM $MgCl_2$. The concentrations of 2-5A-antisense chimera and PKR RNA complement were 0.25 A_{260}/mL in each.

[b] IC_{50} is defined as the concentration (M) required to effect 50% cleavage of RNA substrate.

When evaluated for their ability to mediate RNase H-catalyzed cleavage of PKR sense RNA, the all DNA antisense domain chimera **1** caused the rapid degradation of complementary PKR RNA whereas the 2-5A-antiPKR chimera **11**, with an all 2′-*O*-methylated antisense domain, was completely devoid of activity (Table 2), in line with earlier observations that such 2′-modified oligonucleotides are incapable of supporting RNase H action[11]. When the 2′-*O*-methylation was confined to just the four 3′-terminal nucleotides of the antisense domain, the resulting 2-5A-antiPKR chimera **2** was fully competent at supporting RNase H-catalyzed RNA degradation. Although they contained 42 % of normal DNA nucleotides, chimeras **3** and **4** were without ability to effect PKR sense RNA scission under the influence of RNase H. This finding parallels results with non-2-5A conjugated oligonucleotides which required a minimum of 4 contiguous deoxyribonucleotides to mediate cleavage of RNA by RNase H[11]. Chimeric oligonucleotides **8**, **9**, and **10** contained four contiguous deoxyribonucleotides and supported RNase H cleavage in accord with previous reports on non-2-5A oligonucleotides,[12] albeit with variation in their effectiveness (Table 2). Finally, oligonucleotides **5**, **6** and **7** all possessed a stretch of six contiguous deoxyribonucleotides and were highly active in mediating PKR sense RNA degradation.

From this set of RNase H-inactive and RNase H-active 2-5A-DNAs, five were selected for evaluation for their potency as 2-5A-dependent RNase L activators. Methodology for determination of the ability of 2-5A-antiPKR chimeras to activate RNase L was as published.[13] The 20-mer [^{32}P]5′-pC$_{11}$UUC$_7$ was generated by polynucleotide kinase-catalyzed [γ-^{32}P]-labelling of the RNA 20-mer and served as a homogenous recombinant human RNase L substrate to yield a 13-mer [P^{32}]5′-pC$_{11}$UUp. The reaction conditions were: 14-18 μL cleavage buffer (25 mM Tris - HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 100 μM ATP, 10 mM DTT) 2 μL of test compound at 10 x the desired final concentration, 2 μL of the pure human RNase L (14 μg/mL, 160 nM final concentration) and 2 μL of substrate 20-mer [^{32}P]5′-pC$_{11}$UUC$_7$ (10 nM final concentration). Enzyme reaction mixtures were incubated for 10 min on ice and then at 30 ^{0}C for 15 min. Quantitation involved separation of products and substrate by electrophoresis on a 20% polyacrylamide/8 M urea gel at 300 V for 6 h at 1-5 °C followed by autoradiography. To quantitate cleavage products, the autoradiograph was scanned to a TIFF file using Adobe Photoshop software at 600 dpi. ImageTool software (University of Texas Health Sciences Center, San Antonio, TX) was employed to determine the background-subtracted integrated density for an area including each unreacted substrate cleavage product. The % cleavage was defined as (background-subtracted integrated density of product divided by the background-subtracted integrated densities of uncleaved product plus cleavage product) x 100%. The % cleavage was graphed *vs* concentration of 2-5A tetramer [(p5′A2′)$_4$] or 2-5A-antisense chimera and the concentration (IC_{50}) necessary to effect 50% substrate cleavage was determined.

From Table 2, it was clear that all of the evaluated 2-5A-antiPKR chimeras were equally capable of supporting RNase L-catalyzed degradation of PKR sense RNA. It did not matter whether the antisense domain was fully 2′-*O*-methylated (chimera **11**), partially 2′-*O*-methylated (oligonucleotides **2, 3** and **4**) or completely unmethylated (chimera **1**). Thus, 2′-*O*-methylation in the antisense domain of 2-5A-antiPKR chimeras does not adversely affect the ability of these oligonucleotides to activate RNase L.

These results show that 2′-*O*-methylation in the antisense domain of 2-5A-antiPKR does not impede the ability of such conjugates to activate the 2-5A-dependent RNase L. Just as significantly, the 2-5A and linker domains do not interfere with the enhanced affinity of 2′-*O*-methylated oligoribonucleotides for their target RNA. Second, the presence of the 2-5A moiety and butanediol phosphate linkers elements at the 5′-terminus of oligonucleotides does not affect the latter's capacity to support RNase H-catalyzed degradation of complementary RNA. These latter findings mean that 2′-*O*-methylation can be employed as a means to enhance 2-5A-antisense affinity for its target RNA as well as an approach to stabilize the chimera to degradation by endonucleases.

The third and most important result is that several 2-5A-antisense chimeras have been identified that activate RNase L, but do not mediate the action of RNase H. Thus, it should be possible to dissect out the relative contribution of the two separate enzyme contributions to the intact cell biological activity of 2-5A-antisense. These findings may be applied profitably to various other 2′-modified antisense oligonucleotides such as 2′-fluoro, 2′-propoxy and 2′-methoxyethoxy. These substituents also increase nuclease resistance and/or target RNA affinity; however, just as with 2′-*O*-methylation, complete replacement of the 2′-hydroxy moiety with such substituents disables the RNase H pathway for complementary RNA destruction. 2-5A-antisense versions of such RNase H-inactive analogues may recruit RNase L activity to the targeted RNA, thereby potentiating their activity.

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