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2′,5′-Oligoadenylate-peptide Nucleic Acids (2–5A-PNAs) Activate RNase L

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Abstract—To potentiate the 2–5A (2′,5′-oligoadenylate)-antisense and peptide nucleic acid (PNA) approaches to regulation of gene expression, composite molecules were generated containing both 2–5A and PNA moieties. 2–5A-PNA adducts were synthesized using solid-phase techniques. Highly cross-linked polystyrene beads were functionalized with glycine tethered through a *p*-hydroxymethylbenzoic acid linker and the PNA domain of the chimeric oligonucleotide analogue was added by sequential elongation of the amino terminus with the monomethoxytrityl protected *N*-(2-aminoethyl)-*N*-(adenin-1-ylacetyl)glycinate. Transition to the 2–5A domain was accomplished by coupling of the PNA chain to dimethoxytrityl protected *N*-(2-hydroxyethyl)-*N*-(adenin-1-ylacetyl)glycinate. Finally, (2-cyanoethyl)-*N,N*-diisopropyl-4-*O*-(4,4-dimethoxytrityl)butylphosphoramidite and the corresponding (2-cyanoethyl)-*N,N*-diisopropylphosphoramidite of 5-*O*-(4,4′-dimethoxytrityl)-3-*O*-(*tert*-butyldimethylsilyl)-N^6-benzoyladenosine were the synthons employed to add the 2 butanediol phosphate linkers and the four 2′,5′-linked riboadenylates. The 5′-phosphate moiety was introduced with 2-[[2-(4,4′-dimethoxytrityloxy)ethyl]sulfonyl]ethyl-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite. Deprotection with methanolic NH_3 and tetraethylammonium fluoride afforded the desired products, 2–5A-pnaA$_4$, 2–5A-pnaA$_8$ and 2–5A-pnaA$_{12}$. When evaluated for their ability to cause the degradation of two different RNA substrates by the 2–5A-dependent RNase L, these new 2–5A-PNA conjugates were found to be potent RNase L activators. The union of 2–5A and PNA presents fresh opportunities to explore the biological and therapeutic implications of these unique approaches to antisense. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

Antisense oligonucleotides have great potential as therapeutic agents since they are sequence-specific in their ability to inhibit gene expression.[1–4] Considerable effort has been directed toward stabilizing the phosphodiester backbone of oligonucleotides against attack by ubiquitous endo- and exonucleases that limit their in vivo potential.[4,5] Peptide nucleic acids (PNAs) are a particularly interesting example of a stabilized DNA analogue in which the sugar–phosphate backbone of the natural nucleic acid structure is replaced with a pseudopeptide linkage.[6] This class of antisense agents is resistant to both nuclease and protease attack, and hybridizes to the complementary nucleic acid strand with high affinity and improved selectivity.[7,8]

Unfortunately there is a major drawback to the PNA antisense approach. RNase H,[†] an enzyme that plays an important role in the conventional antisense mechanism of action, will not cleave the PNA/RNA hybrid. Thus, inhibition of mRNA translation by PNA antisense is limited since it is based solely on a secondary mechanism, the steric inhibition of translation.[8,9] However, it may be possible to overcome this PNA-antisense limitation through recruitment of 2–5A-dependent RNase L,[‡] which can degrade a targeted mRNA in the single-stranded region.[10]

Covalent conjugation of 5′-phosphorylated-2′,5′-linked oligoadenylate (2–5A,[§] **1**, Fig. 1) to a standard antisense oligomer (3′,5′-linked DNA) provides a novel reagent which effects the selective and specific cleavage of RNA both in cell-free systems and in intact cells.[10–17] The antisense portion of the chimera recruits a chosen RNA as substrate for cleavage, and the 2–5A portion of the chimera activates RNase L, thus providing a new approach for the targeted ablation of an mRNA and the protein which it specifies. This 2–5A-antisense strategy harnesses the natural 2–5A system which is

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[†] RNase H: (E.C. 3.1.26.4).
[‡] RNase L: (E.C. 3.1.26)-formerly known as 2–5A-dependent RNase.
[§] 2–5A, general formula $p_n5'A2'(p5'A2')_mp5'A$, herein $n=1$ and $m=2$, see **1** in Figure 1.

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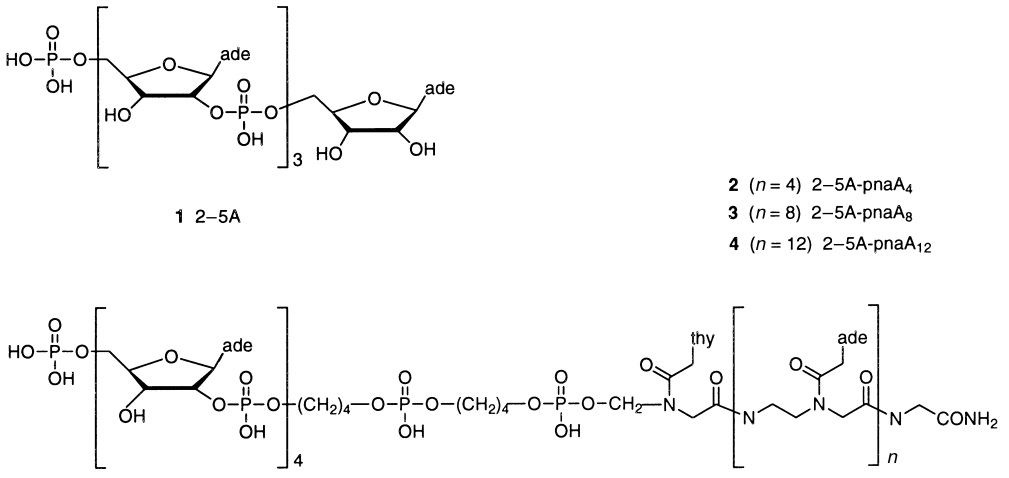


Figure 1. Structure of 2–5A (1), the 2′,5′-linked adenosine tetramer 5′-O-phosphoryladenylyl(2′→5′)adenylyl(2′→5′)adenosine; and of the 2–5A-PNA chimeras (2–4), having 4, 8, or 12 adenine bases in the antisense component (2–5A-pnaA$_n$).

one mechanism through which the cytokine interferon functions.[18]

The conjugation of PNA antisense oligomers to the 2–5A molecule could marry the advantages of both approaches to the control of gene expression. The PNA antisense domain would be stabilized to nuclease attack, the hybridization event would benefit from uncompromised stability and possibly improved selectivity, and the antisense effects would not be limited to steric blocking alone but would include the catalytic cleavage activity of 2–5A-dependent RNase L.

Herein we report the first synthesis of such novel reagents; namely, 2′,5′-oligoadenylate-peptide nucleic acids (2–5A-PNAs*). Furthermore, we demonstrate that these 2–5A-PNA chimeras bind to and activate RNase L to cleave RNA.

Results and Discussion

Synthesis

Retrosynthetic analysis (Scheme 1) revealed that the solid-phase assembly of target 2–5A-PNA adducts **2–4** could be attained starting from known building blocks **5–9** following a similar protocol as described for the synthesis of DNA–PNA chimeras.[20,21] Thus, highly cross-linked polystyrene beads were functionalized with a glycine unit via a *p*-hydroxymethylbenzoic acid linker, resulting in immobilized **10** (Scheme 2). The PNA part of the chimera was constructed by sequential elongation of the amino-terminus using monomethoxytrityl protected PNA adeninyl monomer **5**[22] and HATU† as the coupling reagent. The synthesis of the PNA sequence was completed by introduction of the 3′-linker by reaction of **6**[19] with the amino group of immobilized PNA **11** to give **12**. At this stage, elongation of the solid-phase bound oligomer was feasible using standard phosphoramidite chemistry. The butyl spacers were introduced by the *o*-nitrophenyltetrazole mediated reaction[23] of (2-cyanoethyl)-*N,N*-diisopropyl-4-*O*-(4,4′-dimethoxytrityl)-butylphosphoramidite **7**[24] with the free hydroxyl group in **12**. The 2′,5′-oligoadenylate part of the chimera was appended to the growing oligomer by elongation of **13** with the protected adenosine 2′-phosphoramidite **8** in four consecutive coupling cycles. The 5′-terminal phosphate triester was introduced by reaction of the free hydroxyl in resulting **14** with 2-[[2-(4,4′-dimethoxytrityloxy)ethyl]sulfonyl]ethyl-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite **9**, to afford fully protected 2–5A-PNA adduct **15**. In the final stage of the synthesis, the chimeric oligonucleotide **15** was released from the resin with concomitant deprotection using methanolic ammonia and desilylation with tetraethylammonium fluoride. The crude mixture was desalted by gel filtration and purified by reverse phase HPLC. This procedure furnished 2–5A-PNAs **2–4**, of the general formula 2–5A-pnaA$_n$, where $n = 4$, 8, or 12. The homogeneity and identity of the products were confirmed by HPLC analysis and mass spectrometry.

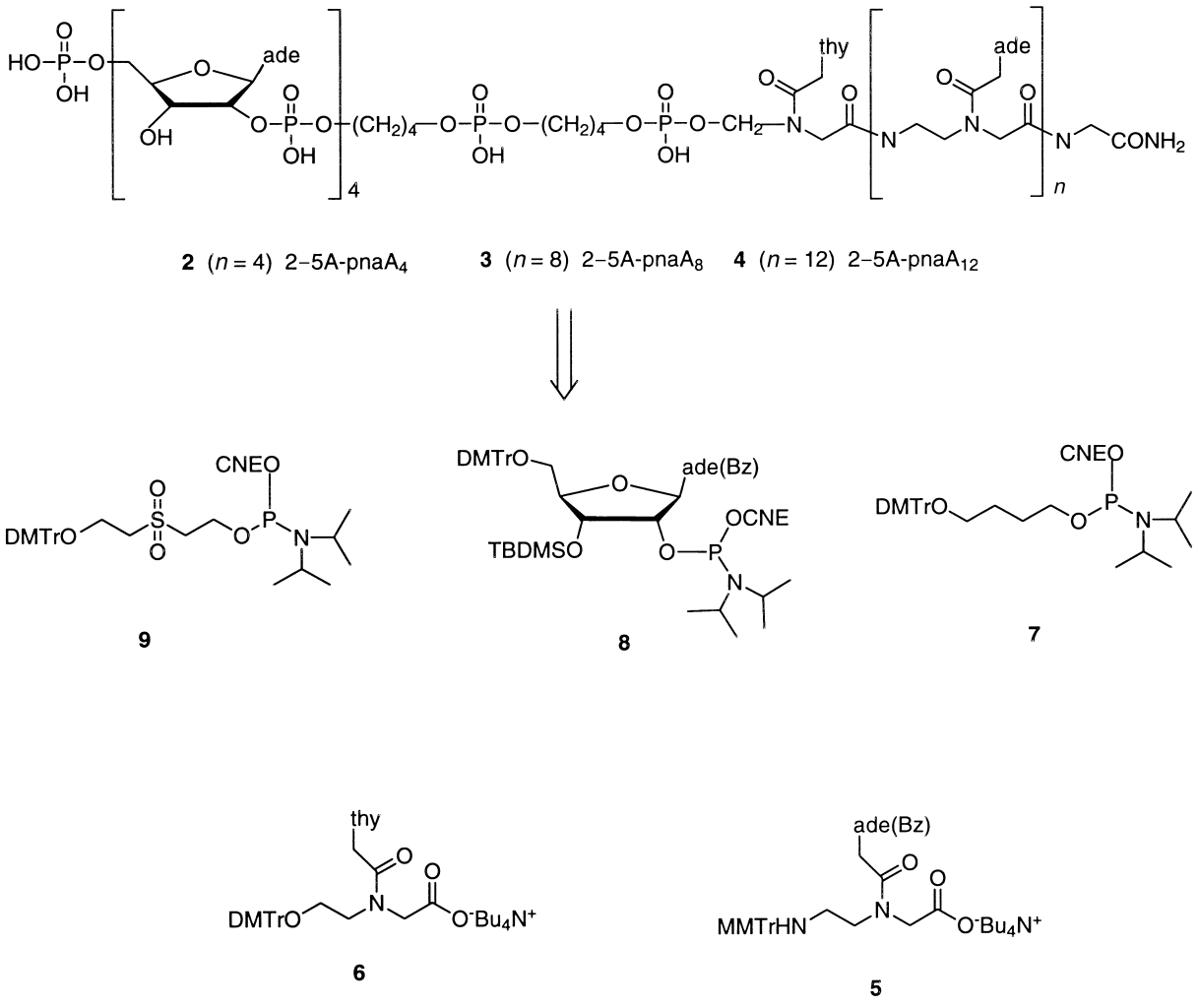
Activation of RNase L cleavage

To ascertain the ability of the 2–5A-PNA chimeras to activate RNase L, two independent assays were used. One assay employed poly(U) as the substrate, and in the other, a smaller substrate with fewer cleavage sites was used, r(pC$_{11}$U$_2$C$_7$). In these in vitro experiments, the sequence specific targeting of cleavage was not addressed, as the prototype synthetic RNA in the reaction was the only substrate available to the 2–5A-activated enzyme.

In the first assay, the 2–5A tetramer (**1**) activated pure recombinant human RNase L to degrade poly(U)-[^{32}P]pCp, with 50% cleavage occurring at a concentration (EC_{50}) of 0.4 ± 0.04 nM (Fig. 2). Each of the three 2–5A-PNA analogues showed a concentration-dependent

* 2–5A-pnaA$_n$, chimera of 2–5A attached at the 2′ end, via a dibutyl linker, to an oligoadenylate moiety of *n* bases with a PNA backbone, see **2–4** in Figure 1.

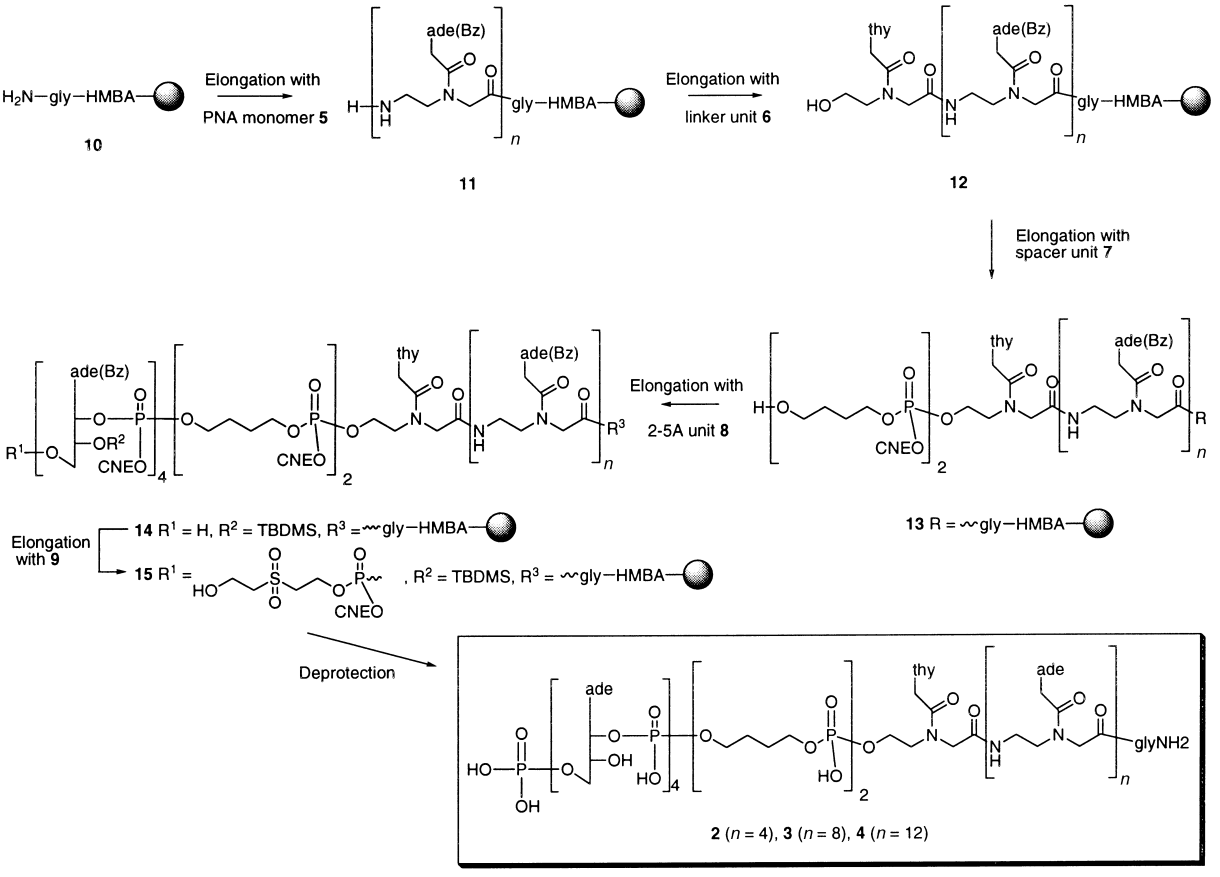
† HATU, *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate.



Scheme 1. Retrosynthetic analysis of target 2–5A-PNAs 2–4. (DMTr, dimethoxytrityl; MMTr, monomethoxytrityl; TBDMS, *t*-butyldimethylsilyl; CNE, cyanoethyl; HMBA, *p*-hydromethylbenzoic acid.)

ability to cause cleavage of the poly(U). The most effective was the tetraadenylate derivative (**2**), which possessed an EC_{50} of 4 ± 0.5 nM. Only slightly less effective was the octaadenylate (**3**), with an EC_{50} of 7 ± 3 nM. Significantly less effective was the chimera that contained the adenylate 12-mer (**4**), with an EC_{50} of 25 ± 4 nM. For comparison the term C_{rel} was used which defines the relative activity of the 2–5A tetramer as 1, and compares (ratio of EC_{50}'s) the relative concentration of other activators needed to effect 50% cleavage. The greater the C_{rel}, the less effective the activator. These data thus indicate the following (decreasing) order of effectiveness: 2–5A (**1**) ($C_{rel} = 1$); 2–5A-pnaA$_4$ (**2**) ($C_{rel} = 10$); 2–5A-pnaA$_8$ (**3**) ($C_{rel} = 17$); 2–5A-pnaA$_{12}$ (**4**) ($C_{rel} = 62$) (Table 1). Thus, while none of the chimeras was as potent as the 2–5A standard, each brought about 50% RNA substrate cleavage at concentrations within one to two orders of magnitude of 2–5A. In addition, the PNA congeners exhibited decreasing activation potency as the length of the PNA antisense oligomer increased (Fig. 2).

In a second independent approach, activation of the 2–5A-dependent RNase L by the various 2–5A-PNA chimeras was ascertained by monitoring the ability of pure recombinant human RNase L to cleave the radiolabelled synthetic ribonucleotide, r([^{32}P]pC$_{11}$U$_2$C$_7$). Use of this oligonucleotide was introduced by Carroll and co-workers[25] who found that RNase L caused scission of the short RNA 3′ to both uridine nucleotides, with initial cleavage occurring to yield r(pC$_{11}$UpUp), and a second cleavage to give r(C$_{11}$Up) at higher enzyme concentration or longer incubation times. In the present assays, activity has been represented in terms of the concentration of 2–5A or analogue required to effect 50% cleavage to r([^{32}P]pC$_{11}$UpUp) (EC_{50}). Under these assay conditions, the 2–5A tetramer (**1**) possessed a mean EC_{50} of 0.3 ± 0.01 nM (Fig. 3). The 2–5A-PNA chimera tetramer (**2**) showed a mean EC_{50} of 3 ± 1 nM, a 10-fold reduction in RNase L activation ability. The octaadenylate congener (**3**) gave an average EC_{50} of 5 ± 1 nM, and compound **4** (the dodecaadenylate chimera) had a mean EC_{50} of 32 ± 1 nM, a 100-fold reduction in activation compared to the tetrameric 2–5A standard. Based on the above data, the 2–5A-peptide nucleic acids could be ranked in the following order of decreasing ability to activate RNase L, using a C_{rel} value as defined for the poly(U) cleavage assay: 2–5A



Scheme 2. Synthesis of 2–5A-PNA adducts 2–4. (Abbreviations are defined in Scheme 1.)

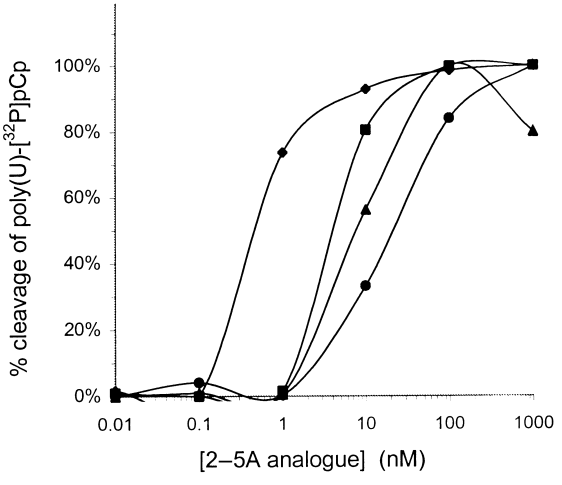


Figure 2. A representative experiment showing the effect of 2–5A or 2–5A-PNA chimera on the ability of RNase L to cleave a radiolabeled poly(U) substrate: (◆), 2–5A; (■), 2–5A-pnaA$_4$; (▲), 2–5A-pnaA$_8$; (●), 2–5A-pnaA$_{12}$.

(**1**) ($C_{rel} = 1$) > 2–5A-pnaA$_4$ (**2**) ($C_{rel} = 11$)~2–5A-pnaA$_8$ (**3**) ($C_{rel} = 14$) > 2–5A-pnaA$_{12}$ (**4**) ($C_{rel} = 99$) (Table 1).

Binding to RNase L

The ability of the 2–5A-PNA chimeras to bind to RNase L was evaluated in a nitrocellulose filter assay that depended on the competition of the radioligand, 2–5A-[^{32}P]pCp, with the unlabeled 2–5A analogues, for binding to the RNase L in CEM* lysate. The results are presented as IC_{50} values (the molar concentration of 2–5A or analogue required to displace 50% of the radioactive probe). Also, the data were normalized to the 2–5A standard (defined as 1), and the relative IC_{50} concentrations (C_{rel}) are given in Table 1.

Table 1. Relative activities (C_{rel})[a] of 2–5A-PNA chimeras and the parent 2–5A compound in cleavage activity and radiobinding assays

Compound	Poly(U) Cleavage	$pC_{11}U_2C_7$ Cleavage	Radiobinding
2–5A	1	1	1
2–5A-pnaA$_4$	10 ± 1 (5)	11 ± 2 (3)	3 ± 1 (3)
2–5A-pnaA$_8$	17 ± 8 (4)	14 ± 3 (3)	8 ± 7 (4)
2–5A-pnaA$_{12}$	62 ± 10 (5)	99 ± 2 (3)	43 ± 26 (6)

[a]C_{rel} is the EC_{50} (or IC_{50}) of the 2–5A-PNA chimera divided by the EC_{50} (or IC_{50}) of the 2–5A standard, with 2–5A arbitrarily assigned a C_{rel} value of 1. These values are given as the mean ± standard deviation, with the number of experiments (n) given in brackets. The experimental EC_{50} values for 2–5A were 0.4 ± 0.04 nM (n = 5; poly(U) assay) and 0.3 ± 0.01 nM (n = 3; $pC_{11}U_2C_7$ assay). Similarly, the experimental IC_{50} for probe displacement by 2–5A in the radiobinding assay was 3.3 ± 1.5 nM (n = 6).

The binding ability of the 2–5A-PNA adducts showed length-dependence (Fig. 4, Table 1). In conditions

* CEM, a human T-lymphoblastoid cell line originating from a patient with acute lymphoblastic leukemia.

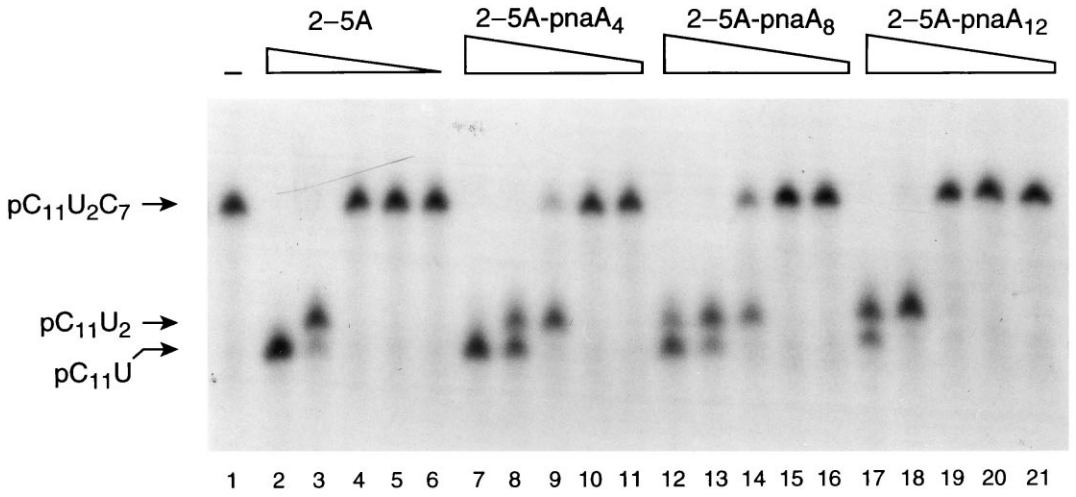


Figure 3. A representative autoradiograph showing the effect of 2–5A or 2–5A-PNA chimera on the ability of RNase L to cleave the synthetic oligonucleotide r([^{32}P]pC$_{11}$U$_2$C$_7$): lane 1, no 2–5A; lanes 2–6, 10^{-7}–10^{-11} M 2–5A; lanes 7–11, 10^{-5}–10^{-9} M 2–5A-pnaA$_4$; lanes 12–16, 10^{-5}–10^{-9} M 2–5A-pnaA$_8$; lanes 17–21, 10^{-5}–10^{-9} M 2–5A-pnaA$_{12}$.

where the 2–5A tetramer (**1**) gave an IC_{50} of 3 ± 1 nM, the tetraadenylate and octaadenylate congeners (**2** and **3**) were approximately equivalent in binding affinity as judged by their respective IC_{50}'s of 8 ± 3 nM and 21 ± 13 nM. However, the extension of the adenylate sequence to a dodecamer, giving an overall 13-mer as the antisense domain in the 2–5A-PNA chimera (**4**), with an IC_{50} of 123 ± 61 nM, caused a significant decrease in binding. Therefore, the binding data were in accord with the data from the two activation assays (Figs 2 and 3) since there was a correspondence between a diminished ability to activate RNase L and the ability to bind to RNase L.

Conclusions

The experiments presented above demonstrate that 2′,5′-oligoadenylate-peptide nucleic acid conjugates are capable of binding to and activating the 2-5A-dependent RNase L. Although the adducts evaluated (**2–4**) were 1–2 orders of magnitude less effective than parent 2–5A (**1**) in activation of RNase L, their potency is comparable to previous 2′,5′-oligoadenylate-3′,5′-oligodeoxynucleotide conjugates. For instance, Maitra et al.[14] found that 2–5A-antiPKR* was twofold less effective than 2–5A alone at inducing the degradation of PKR RNA by pure human RNase L, but that the corresponding 2–5A-sensePKR construct or a chimera against an unrelated RNA sequence (2–5A-antiHIV†) were 10–100 fold less potent than the parent 2–5A tetramer. Similarly, 2–5A-antiBCR‡ and 2–5A-*iso*-antiBCR (a ''tail-to-tail'' chimera) were 10-fold less potent at inducing degradation of the non-targeted oligonucleotide rC$_{11}$U$_2$C$_7$,[26] and the 2-5A-antisense anti-RSV§ chimera that inhibits RSV replication,[16] as well as the 2-5A-anti-*gag*(HIV) chimera that causes targeted degradation of a *gag* RNA,[17] were an order of magnitude less effective than 2–5A itself. The mechanism underlying this difference has not yet been investigated, but may involve steric interference by the antisense moiety in the interaction of 2–5A and the enzyme, or in the process of enzyme dimerization.[27]

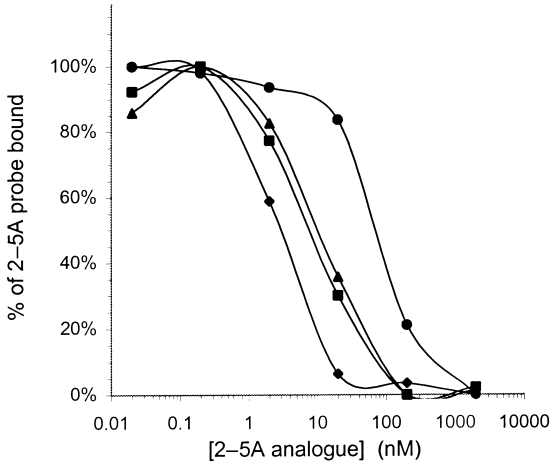


Figure 4. A representative experiment showing the displacement of probe (2-5A-[^{32}P]pCp) from binding sites in CEM lysate by 2–5A analogues: (◆), 2–5A; (■), 2–5A-pnaA$_4$; (▲), 2–5A-pnaA$_8$; (●), 2–5A-pnaA$_{12}$.

Insofar as 2–5A-PNA conjugates possess an RNase L activation potency similar to 2–5A-antisense chimeras with an all nucleic acid backbone, they represent an important advance in the application of both the 2–5A-antisense and PNA strategies to the control of gene expression. Most importantly, the 2–5A-dependent RNase L augments the PNA-antisense agents with a catalytic mode of targeted RNA destruction. The in vivo lifetime of these chimeras will be improved since

* PKR, a dsRNA-dependent protein kinase.
† HIV, human immunodeficiency virus.
‡ BCR–ABL, an oncogenic protein tyrosine kinase underlying chronic myelogenous lukemia. It is formed from a mutant gene in which a translocated *abl* gene is fused to the *bcr* gene.
§ RSV, respiratory syncytial virus.

the PNA backbone is highly resistant to attack by the nucleases present in cells and sera,[7] and this longevity is gained without compromise to the affinity or selectivity of target hybridization.[8] Another advantage of the PNA-antisense oligomer is the synthetic flexibility of the pseudopeptide linkage which will facilitate further modifications, for example directed toward improved cellular uptake of the chimera.[5,28,29] Finally, this general 2–5A-antisense approach may be applied profitably to a number of other antisense oligonucleotide analogues, such as α-oligonucleotides or methylphosphonates, that display desirable properties of cell permeation or resistance to degradation, but are not compatible with an RNase H-mediated catalytic ablation of RNA.

Experimental

Synthesis of the 2–5A-PNAs

All solvents (Biosolve, DNA synthesis grade) were used as received. Solid-phase syntheses were performed on a Pharmacia Gene Assembler using highly cross-linked polystyrene beads as the solid support (loading: 26–28 μmol/g) on a 1 μmol scale. The support was functionalized with a glycine moiety via a *p*-hydroxymethylbenzoic acid linker. Assembly of the PNA part was established using solutions of 0.3 M of monomers **5** and **6**, 0.3 M DiPEA* and 0.3 M HATU in acetonitrile/dimethylformamide (1/1, v/v). Prior to coupling, the monomers were pre-activated for 1 min by mixing equal amounts of the PNA monomer (15 equiv per μmol support), HATU and DiPEA solutions. The protocol for one PNA chain extension cycle consisted of (1) wash: acetonitrile/dimethylformamide (1/1, v/v), 2.5 mL; (2) coupling: PNA + HATU + DiPEA in acetonitrile/dimethylformamide (1/1, v/v), 15 min; (3) wash: acetonitrile/dimethylformamide (1/1, v/v), 2.5 mL, acetonitrile, 2.5 mL; (4) capping: Ac_2O/lutidine/*N*-methylimidazole/tetrahydrofuran (1/1/1/7, v/v/v/v), 2.0 mL; (5) wash: acetonitrile, 2.5 mL, dichloromethane, 3.5 mL; (6) detritylation: 2% trichloroacetic acid in dichloromethane, 3 min; (7) wash: dichloromethane, 2.5 mL, acetonitrile, 5 mL. The introduction of the phosphate bonds was carried out with 15 equiv of known cyanoethyl phosphoramidites **7–9**[24] using 5-(*o*-nitrophenyl)tetrazole (8 equiv) as the activator. Standard DNA capping, washing, oxidation and detritylation cycles were used. Coupling yields were gauged spectrophotometrically (254 nm) by the absorption of the released trityl cation after each deprotection step. After the last elongation step, the oligomers were cleaved from the support with concomitant deprotection of the phosphate groups and exocyclic amino groups by treatment with methanolic ammonia (1.5 mL) at 50 °C for 16 h. The samples were filtered and the silyl protective groups were removed by treatment with tetraethylammonium fluoride (0.5 M in dry acetonitrile) at ambient temperature for 16 h. Desalting was established using a G-25 column with a 0.15 M solution of ammonium bicarbonate as the eluting agent. RP-HPLC purification and analysis were carried out on a Jasco HPLC system equipped with a LiChrospher 100 RP-18 endcapped column (10.0×250 mm and 4.0×250 mm, respectively). Gradient elution was performed at 40 °C by building up a gradient starting with buffer A (50 mM triethylammonium acetate in water) and applying buffer B (50 mM triethylammonium acetate in acetonitrile/water, 1/1, v/v) with a flow rate of 1.0 mL/min or 5.0 mL/min for analysis and purification, respectively. The identity of the oligomers was confirmed using mass spectrometry (electrospray). For 2–5A-pnaA$_4$ (**2**), calculated for $C_{105}H_{138}N_{53}O_{49}P_7$: 3143; found 3142.7. For 2–5A-pnaA$_8$ (**3**), calculated for $C_{149}H_{190}N_{81}O_{57}P_7$: 4244; found 4244.5. For 2–5A-pnaA$_{12}$ (**4**), calculated for $C_{193}H_{242}N_{109}O_{65}P_7$: 5345.6; found 5347.0.

* DiPEA, diisopropylethylamine.

RNase L activation and cleavage of a poly-uridine (Poly(U)) substrate

Pure recombinant human RNase L was prepared by a modification of a previously described procedure.[30] Poly(U) was obtained commercially as a mixture of high molecular weight uridine polymers. Using T4 RNA ligase, the poly(U) was 3′-labeled with 5′-[^{32}P]pCp and then HPLC purified. This procedure and the assay have been described previously.[30,31] 2 μL of a 10× cleavage buffer (100 mM HEPES, pH 7.5, 1.0 M KCl, 50 mM $Mg(OAc)_2$, 10 mM ATP, and 143 mM 2-mercaptoethanol) and 12–16 μL of RNase-free water were used in each cleavage reaction. To this, 2 μL of a 10× solution of 2–5A analogue (final concentrations 10^{-5} to 10^{-10} M) and recombinant RNase L enzyme (final concentration of 130 nM) were added, and lastly 2 μL of poly(U)-[^{32}P]pCp substrate (final concentration 10 μM in UMP equivalents) to make a final volume of 20 μL. After a 15 min incubation at 30 °C, 4 volumes of 5 mg/mL carrier (yeast) RNA was added, and then 10 M ammonium acetate to a final concentration of 2–2.5 M. After mixing with 2 volumes of cold ethanol, the reaction mixtures were left on ice for 30 min, and the precipitated RNA pelleted with a brief spin at 4 °C (12,000 g for 2 min). The presence of cleaved fragments of poly(U)-[^{32}P]pCp was assessed by counting aliquots of the supernatant in scintillation fluid.

RNase L activation and cleavage of a synthetic oligonucleotide substrate

This assay relied upon the RNase L-induced cleavage of the synthetic oligonucleotide, r$C_{11}U_2C_7$ as described by Carroll et al.[25] Synthetic oligoribonucleotide, r$C_{11}U_2C_7$, was prepared by Midland Certified Reagent Co. (Midland, TX) and 5′-labeled with polynucleotide kinase and γ-^{32}P-ATP (DuPont-NEN, Wilmington, DE). For cleavage assays, the cleavage buffer was 25 mM Tris–HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 100 μM ATP, and 10 mM DTT. For each assay, components were added in the following order: 14–18 μL cleavage buffer, 2 μL 2–5A-antisense chimera at 10× the desired final concentration, and 2 μL of an RNase L solution to give a final concentration of 130 nM RNase L. Cleavage reaction mixtures were held for 10 min on ice after addition

of RNase L. The substrate [^{32}P]pC$_{11}$U$_2$C$_7$ (2 μL of a solution to give a final substrate concentration of 10 nM) was added last and the mixture incubated at 37 °C for 15 min. 20 μL of RNase-free loading buffer was then added, and the samples applied to a 1 mm 20% PAGE/8M urea gel for electrophoresis at 350 V for 6 h at 1 °C. The gels were exposed to film, and the films developed and scanned to quantify cleavage densitometrically.[30,31]

For all RNase L-catalyzed RNA degradation experiments, the EC_{50} was defined (after background subtraction) as the effective concentration that brought about 50% degradation of RNA substrate.

Radiobinding assays

Human CEM cell cytoplasmic extracts were prepared according to previously described methodology.[32] The radiobinding assay probe p5′A2′(p5′A2)2 p5′A3′[^{32}P]p 5′C3′p, was synthesized by the T4 RNA ligase-catalyzed addition of [^{32}P]5′pCp to the 3′ end of 2–5A (**1**), using a published procedure with subsequent HPLC purification.[30,31] To assay a given 2–5A analogue, serial dilutions were prepared in water. Each binding assay consisted of 5 mL of 2–5A or 2–5A analogue, 5 μL of CEM cell lysate, and 15 μL of a master mix that consisted of one part of buffer A [20 mM Tris–HCl (pH 7.6), 85 mM KCl, 5 μM Mg(OAc)$_2$ 1 mM ATP, 5% (v/v) glycerol] and two parts of buffer B (same as buffer A, but without ATP) and sufficient 2–5A-[^{32}P]pCp probe to give 10^4 to 2×10^4 cpm/assay. The order of addition for each assay was 2–5A or analogue, then master mix, then lysate. Assay mixtures were incubated at 4 °C for 2 h, after which they were applied to nitrocellulose filters that were subsequently washed (3×) with water. The filters were placed in scintillant and counted in a liquid scintillation counter.[30,31]

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