Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 1357–1360

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Convergent Synthesis of Ribonuclease L-Active 2′,5′-Oligoadenylate-Peptide Nucleic Acids

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Received 18 February 2000; accepted 17 April 2000

Abstract—2-5A was conjugated to *N*-(2-aminoethyl)-glycyl PNA by periodate oxidization, followed by coupling with amino-derivatized PNA and final cyanoborohydride reduction. An adduct of 2-5A pentamer with tetrameric thymine PNA activated RNase L with the same potency as earlier versions of 2-5A-PNA or 2-5A-DNA.

Increasingly, RNA is being pursued as a target for drug discovery, especially since the realization that the decay of mRNA can be key in the post-transcriptional regulation of gene expression.[1] Drug design strategies employing RNA targets have included both small[2] and large[3] molecules as candidate therapeutic agents. Recently the oligonucleotide antisense drug Vitravene™ (formivirisen) for treatment of CMV-induced retinitis in AIDS patients, completed Phase III clinical trials, was approved by the FDA and is on the market.[4] To potentiate the biological activities of antisense molecules, we introduced the strategy of 2-5A-antisense.[5,6] This technology relies on an antisense sequence to target a specific RNA, followed by 2-5A [(p5′A2′)$_n$p5′A] activation of RNase L to effect the cleavage of targeted RNA.

Peptide nucleic acids (PNAs) are DNA mimetics in which the sugar-phosphate backbone is replaced by pseudopeptide linkages.[7–9] PNAs are very stable to enzymes and obey the Watson–Crick rules binding complementary DNA or RNA with higher affinity than their natural counterparts. Verheijen et al.[10] reported 2-5A-PNAs conjugates in which a 2-5A tetramer was connected to a PNA molecule with the aid of two butanediol phosphate linkers. Their potency was comparable to previous 2-5A-DNA conjugates, but the PNA backbone endowed such chimeric molecules with nuclease resistance. Most significantly, whereas PNAs are normally unable to act by a catalytic mechanism to degrade RNA, conjugation to 2-5A recruits the 2-5A-dependent RNase L for the degradation of targeted RNA. These 2-5A-PNA composite molecules were prepared by a stepwise approach that involved construction of a protected PNA followed by the addition of two butanediol phosphate linker moieties and then condensation of appropriately protected mononucleotidic synthons to generate 2′,5′-oligoadenylate tetramer. As a final step, 5′-monophosphorylation was effected. Deprotection gave the final 2-5A-PNA conjugate.[10]

As an alternative to the foregoing synthetic approach, we have explored the possibility of a convergent synthesis of 2-5A-PNA chimeras involving a direct condensation of a preformed and unprotected PNA with a unprotected 2-5A. This route would obviate the use of PNA protecting groups compatible with RNA protection strategy and might also be applicable to the generation of other interesting 2-5A-nucleic acid analogue chimeras. We employed an approach involving periodate oxidation of a 2′,5′-oligoadenylate to terminal dialdehyde, Schiff base formation with an aminolinker-bearing PNA, followed by cyanoborohydride reduction. This reaction sequence had proved successful in the generation of nuclease-stabilized 2-5A derivatives.[11]

Formation of a morpholine ring at the 3′ end of a nucleic acid has been used to form cross-links between RNAs and many kinds of molecules like peptides,[12] fluorescent groups[13,14] or a half-ribozyme.[15] Morpholino-

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0960-894X/00/$ - see front matter
PII: S0960-894X(00)00240-7

based oligonucleotides also have been employed as antisense agents.[16] The crux of these conjugations is based on the fact that the 2′,3′−C−C bond on the ribonucleoside at the 3′ end of nucleic acids can be cleaved by sodium periodate to give a dialdehyde. This subsequently reacts with an amino group to form a Schiff's base that can be reduced to a substituted morpholine. This method also was used to prepare exonuclease-resistant *N*-hexyl morpholine analogues of 2-5A.[11] It has been abundantly demonstrated that pH control in the steps of Schiff base formation and reduction is vital. The pH should be kept at about 8.6 in the Schiff base formation step and subsequently at 6.5 in the reduction. Sodium cyanoborohydride was used for the reduction because it reduces only the Schiff base while the aldehyde is not affected under these conditions.[17]

PNA tetramer **1** was assembled on a 2 μmol Fmoc-XAL-PEG-PS column with Fmoc-*N*-(2-aminoethyl) glycyl PNA monomers on an Expedite™ 8909 PNA synthesizer following the chemistry and protocols developed by the manufacturer (Perseptive Biosystems, Inc., Framingham, MA). After synthesis, the PNA were deprotected and removed from the polystyrene-resin by treatment with a mixture of 80% trifluoroacetic acid (TFA) containing 20% *m*-cresol for 5 min. The PNA pellet was precipitated by addition of ether and used for the reactions without further purification.

Conjugation of PNA tetramer 1 with adenosine or AMP

Compound **2** was synthesized from excess adenosine and **1**. Adenosine[18] (32 O.D or 2.1 μmol in 300 μL water) was oxidized by 0.1 M $NaIO_4$ (25 μL) with ice cooling. After 40 min, 45 O.D of **1** in 400 μL water was added and the pH was adjusted to about 8.5 with 0.1 N NaOH. Stirring was continued on ice for 1 h. Then 83 μL of 0.5 M $NaBH_3CN$ was added to the mixture. The pH was controlled at about 6.5 with 1% HOAc and the solution was stirred for 2 h. HPLC analysis showed the reaction was complete. The compound was purified by reverse-phase HPLC on an ODS column.[18]

Chimera **3** was prepared in a similar manner. The structures of both compounds were corroborated with electrospray mass spectroscopy (ESIMS). Calc for **2**: $C_{66}H_{92}N_{24}O_{24}$: 1605.6; found 1605.5; Calc for **3**: $C_{66}H_{93}N_{24}O_{27}P$: 1685.6; found 1685.5.

Enzyme digestion of 3

0.5 O.D of **3** was incubated with bacterial alkaline phosphatase (1 unit) in 100 μL of 50 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$ at 37 °C for 6 h. The solution was transferred to a Microcon-10 tube, and was centrifuged to remove bacterial alkaline phosphatase. The digestive product was analyzed by reverse-phase HPLC and demonstrated to be identical with **2**, which was prepared from adenosine and **1**.

Coupling between 2-5A dimer and PNA compound 1

To the cooled solution of p5′A2′p5′A(5.9 O.D, 0.23 μmol) dissolved in 42 μL water was added the 0.1 M solution of $NaIO_4$ (4.0 μL). The oxidation was carried out in the dark for 1 h. Then a 2-fold excess of Na_2SO_3 over $NaIO_4$ was

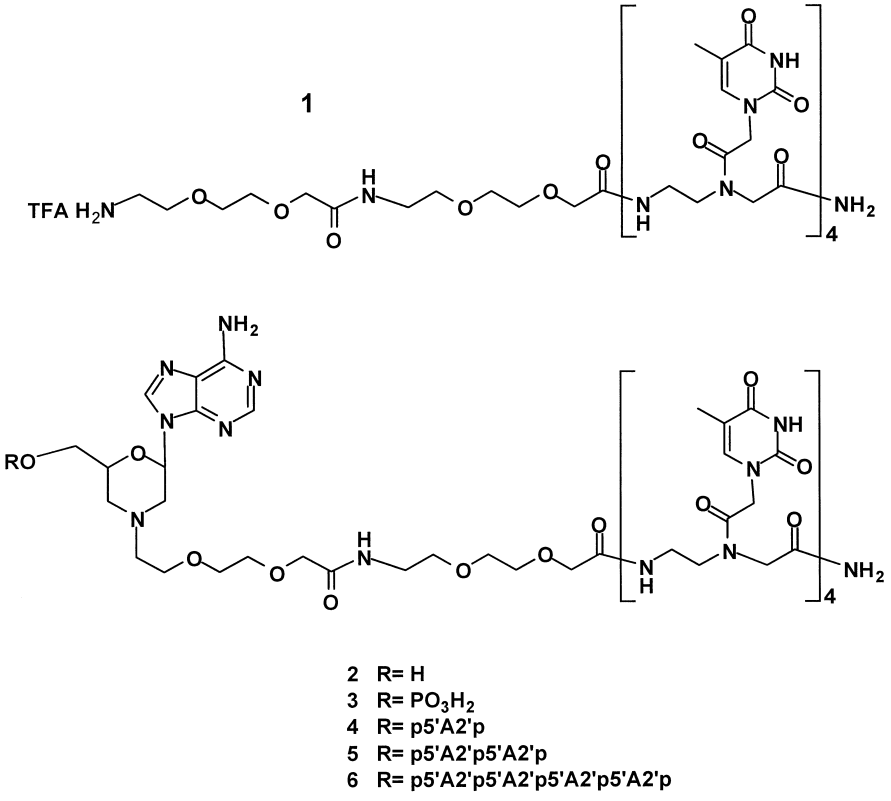

Figure 1. Structures of compounds synthesized.

added to the solution to remove the excess oxidant. The reaction mixture was stirred for 15 min. After that, 16 O.D of **1** (dissolved in 33 μL water) was added, and the pH was adjusted to about 8.6 with 0.1 N NaOH. After 1 h 9.0 μL of 0.5 M $NaBH_3CN$ were added, and the pH was adjusted to about 6.6. After 4 h, the product was purified by HPLC to afford chimera **4**. The molecular mass was determined by electrospray ionization mass spectroscopy (ESIMS). Calc for $C_{76}H_{105}N_{29}O_{33}P_2$: 2014.8; found 2014.6.

Preparation of 5 and 6

The reaction between p5′A2′p5′A2′p5′A or p5′A2′p5′A2′p5′A2′p5′A2′p5′A and **1** was carried out under similar reaction conditions. ESIMS: calc for **5**, $C_{86}H_{117}N_{34}O_{39}P_3$: 2344.0; found 2343.7. Calc for **6**, $C_{106}H_{141}N_{44}O_{51}P_5$: 3002.4; found 3002.1.

To determine the biological activity, we employed two different methodologies.[19] The first provided a measure of the ability of the modified A-, AMP-, or 2-5A-PNA to bind to human recombinant RNase L as determined by competition of the analogue A-, AMP-, or 2-5A-PNA with p5′A2′p5′A2′p5′A2′p5′A3′ [^{32}P]p5′C3′p.[19] In this assay, the four analogues (**3**–**6**) displayed quite different binding affinities for RNase L (Table 1). PNA derivatives bearing only adenosine or AMP completely failed to bind to RNase L. Compound **5**, with a trimeric 2-5A moiety conjugated to the PNA tetramer showed effective binding to RNase L, albeit somewhat reduced from that shown by compound **6**, which bound as well as parent unmodified 2-5A trimer. These results are congruent with earlier reports that a minimum of three intact adenine rings are needed for full RNase L binding ability.[11,20] The affinity of 2-5A-PNA chimera **6** for RNase L also was comparable to that reported earlier for a 2-5A-PNA conjugate with a dual butanediol phosphate linker.[10]

The second assay[19] addressed the relative ability of the analogues to activate RNase L to cleave the test substrate poly(U)p[^{32}P]Cp. As would be expected from the lack of binding interaction, neither chimera **3** or **4** could activate RNase L to degrade poly(U)pCp. However, chimera **5**, which did show good binding to RNase L, was at least 476-fold less active than parent 2-5A trimer in eliciting nuclease activity. This latter result may be considered somewhat surprising in view of the earlier observation that a trimeric 2-5A, with the 2′-terminal ribose modified to an *N*-hexylmorpholine ring, was virtually as effective an activator of RNase L as was the parent unmodified trimer p5′A2′p5′A2′p5′A.[11] The combination of these results suggests that the significantly longer pendant morpholine N-substituent peptide nucleic acid may interfere with activation in the case of the modified trimer (compound **5**). Congruent with this line of reasoning, compound **6** was only 13-fold reduced in ability to activate RNase L as compared to the standard trimer. In fact, this activity is quite similar to that reported for a 2-5A-PNA conjugate in which the linker moiety consisted of two butanediol phosphates.[10] It would appear that the additional spacing between the PNA molecule and the third (from the 5′-terminus) nucleotide of 2-5A optimizes the activity of this genre of chimeric constructs.

Table 1. Relative activities (C_{rel}[a]) of various PNA chimeras and trimeric 2-5A in cleavage and binding Assays

Compound	Binding, C_{rel}[a]	Poly(U) cleavage, C_{rel}[a]
p5′A2′p5′A2′p5′A	1	1
3	No binding detected	No cleavage detected
4	No binding detected	No cleavage detected
5	0.7 ± 0.2	476[b]
6	1.0 ± 0.1	14 ± 3.2

[a]C_{rel} is the IC_{50} (or EC_{50}) of the 2-5A-PNA chimera divided by the IC_{50} (or EC_{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. The experimental EC_{50} value for 2-5A was 0.21 ± 0.05 nM. Similarly, the experimental IC_{50} for probe displacement by 2-5A in the radiobinding assay was 13 ± 3 nM.
[b]Complete cleavage of poly(U)pCp substrate was not obtained.

The results presented herein show that a convergent approach to 2-5A-PNAs and perhaps other related molecules is possible. Moreover, the type of morpholino linkage generated by this periodate oxidation/Schiff base formation/cyanoborohydride reduction approach is compatible with reasonable activation of RNase L. Finally, these results suggest that the nature of the linker between the PNA and 2-5A moiety may be of minimal importance; however, the spacing between the antisense domain and the third nucleotide (from the 5′-terminus) of 2-5A may be much more critical. This latter finding is compatible with the demonstrated requirement for 2′,5′-oligoadenylate-RNase L interactions with the adenine ring of this third nucleotide residue.[5]

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18. Experimental details. All HPLC analyses and purifications were performed on an HP1050 instrument equipped with Chemstation software. Beckman Ultrasphere ODS analytical (4.6 × 250 mm) and semi-preparative (10 × 250 mm) columns were respectively used for the analysis and purification with the following buffers: A: 0.1M NH_4OAc; B: 22.5% CH_3CN/0.1 M NH_4OAc; Linear gradient elution was performed at 45 °C with a flow rate of 1 mL/min or 3 mL/min. In the purification of all compounds by HPLC, the fractions corresponding to the peak containing chimera were pooled and evaporated in a Speedvac, and then the residue was desalted with a Sep-Pak C18 cartridge (Waters) or by dialysis (Spectra, MWCO 500). For reactions, AMP, $NaBH_3CN$ were purchased from Aldrich. $NaIO_4$ was from Sigma. BAP was from Sigma (P-4069, 100 units in 0.4 mL).
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