Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2511–2516

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

5'-PEPTIDYL SUBSTITUENTS ALLOW A TUNING OF THE AFFINITY OF OLIGODEOXYRIBONUCLEOTIDES FOR RNA

David A. Sarracino, Joshua A. Steinberg, Maxwell T. Vergo, Graeme F. Woodworth, Charles N. Tetzlaff and Clemens Richert*

Department of Chemistry, Tufts University, 62 Talbot Avenue, Medford, MA 02155, U.S.A.

Received 5 June 1998; accepted 28 July 1998

Abstract: The affinity of amide-linked 5'-aminoacyl and 5'-dipeptidyl DNA octamers for two RNA undecamers with 3'-overhangs was measured via UV melting analysis. A sequence-dependent increase in melting points was observed. At low ionic strength, two appended lysine residues elevate melting points more than two additional A:U base pairs.

The hybridization of synthetic oligodeoxynucleotides to complementary RNA and DNA strands is a key recognition event in molecular biology. It underlies the common protocols for finding genes, measuring their expression levels, and interfering with their expression via antisense inhibition.[1] The ability to tune this recognition event, i.e. to increase or decrease the affinity of a given hybridization probe for a target without altering its sequence specificity, is desirable, as it allows for the optimization of such procedures. Chemical modifications of oligonucleotides devised with this goal have mainly focused on the replacement of chemical groups in the nucleobase- and backbone-portion of oligonucleotides.[2] Recently, appendages to the termini of otherwise unmodified oligonucleotides have been prepared.[3] Among the possible appendages are peptides, whose conjugation to DNA produces peptide-DNA hybrids.[4] We and others[5] have focused synthetic efforts on preparing directly linked peptide-DNA hybrids, devoid of bifunctional linker moieties. We have recently reported how 5'-amide-linked peptide-DNA hybrids can be constructed through coupling of N-α-Fmoc amino acid building blocks to amino-terminal DNA synthesized on solid support.[6] Here we report how *L*-aminoacyl substituents of such amide-linked hybrids (either singly aminoacylated or dipeptidyl-hybrids) increase the affinity of the oligonucleotides for an RNA strand.

We chose the DNA octamer 5'-TGGTTGAC-3' as a typical mixed sequence hybridization probe and two complementary RNA undecamers, differing only in their trinucleotide overhang at the 3'-terminus, as our target sequences. The latter mimic longer RNA hybridization targets, such as mRNAs in antisense inhibition of gene expression. Screening of a number of singly 5'-aminoacylated oligodeoxyribonucleotides[7] attracted our attention to 5'-Trp-TGGTTGAC-3' (**1**, Figure 1), as one- and two-dimensional NMR experiments with Trp-TGCGCAC showed up-field shifts for the aromatic hydrogens of the tryptophan residue, pointing towards a stacking of the indole ring on the nucleobase(s).[8]

0960-894X/98/$ - see front matter
PII: S0960-894X(98)00449-1

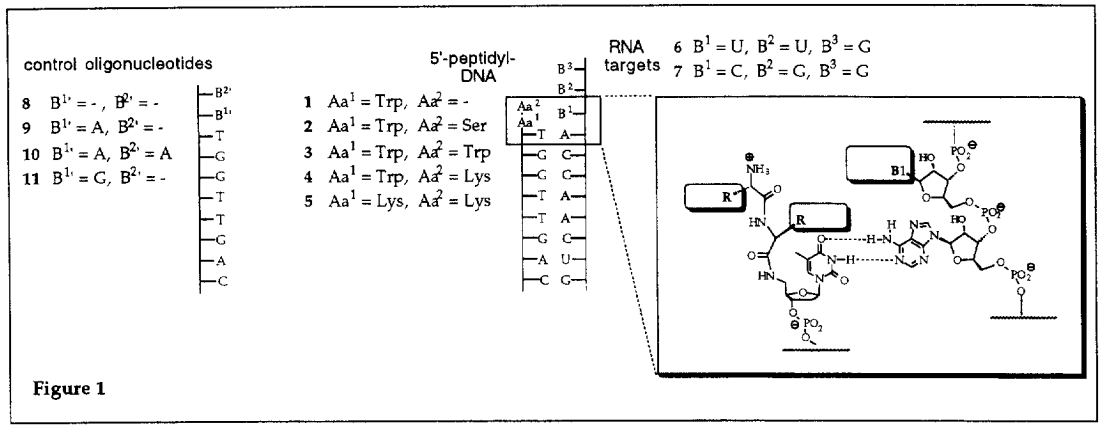

Figure 1

The duplexes between **1** and RNA target r(GUCAACCAUUG) (**6**) gave a CD-spectrum whose spectral features and melting transition (Figure 2) are typical for the A-type conformation expected for RNA:RNA and RNA:DNA duplexes,[9] indicating that the tryptophan residue is stacking on a largely undisturbed double helix.

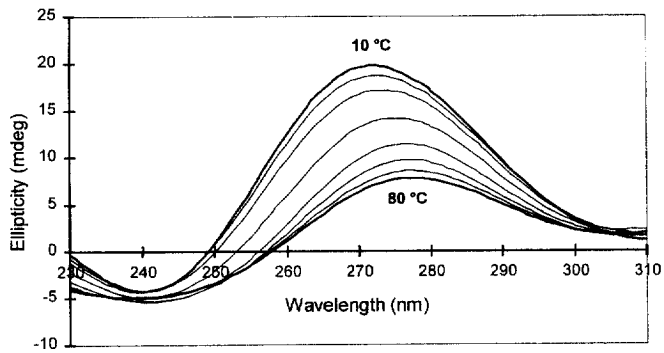

Figure 2. CD-spectra of *r*(GUCAACCAUUG) (**6**) and Trp-TGGTTGAC (**1**), each at 150 μM strand concentration, in 85 mM NH_4OAc buffer, pH 6.0 from 10 °C (top line) to 80 °C (bottom line) in 10 °C intervals.

Assuming that the tryptophan residue in **1** would be a suitable platform for interactions between additional amino residues and the nucleic acid portion of DNA/RNA duplexes, we prepared three dipeptidyl-hybrids, as well as unmodified DNA octamer **8** as a control. Hybrids **2**, **3**, and **4** bear a polar non-ionic, hydrophobic, and cationic side chain, respectively (Figure 1). Duplex dissociation[10] was first measured at 6 μM strand concentration and 10 mM salt (Figure 3). All aminoacylated nucleic acids exhibited a higher UV melting point (Tm) than unacylated DNA control compound **8**. The increase in Tm was +7 °C (**1**), + 8 °C (**3**), +9 °C (**2**), and +17 °C (**4**) under these conditions. The greater melting points increase for the lysinyl sequence suggested that electrostatic interactions between the amino acid side chains and the nucleic acids were energetically more significant than a hydrophobic effect associated with the stacking of the tryptophan residue and possible hydrogen bonding interactions. This assumption was confirmed in melting curves with dilysinyl-oligonucleotide **5**, which bears two cationic residues but no tryptophan residue, allowing for stronger

electrostatic interactions but less hydrophobic stabilization. Its melting point was found to be 21 °C higher than that of control DNA:RNA duplex **8+6** . This is more than the increase in melting point brought about by extending DNA octamer **8** to nonamer **9** with one, or decamer **10** with two additional adenosyl residues (Figures 1), which gave a ΔTm of +7 °C and +9 °C at this strand concentration and ionic strength.

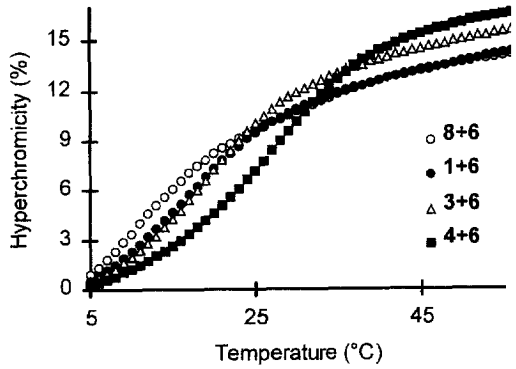

Figure 3. UV melting profiles of dipeptidyl-DNA/RNA duplexes (**3+6** and **4+6**) and the corresponding aminoacyl-DNA/RNA (**1+6**) and DNA/RNA duplex (**8+6**) at 6 μM strand concentrations in 10 mM ammonium acetate buffer at pH 6.0. The duplex of dipeptidyl-DNA hybrid **2** with **6** gave a curve very similar to that of **3+6** and is not shown for clarity.

The effect of the aminoacyl substituents on duplex stability was then tested at higher ionic strength. To ensure statistical significance, the experimental error was reduced by determining a total of at least four melting points, using an equal number of melting and cooling curves for each duplex. This in-depth study was performed at 1.3 μM strand concentration. The order among the modified oligonucleotides with a tryptophanyl residue linked to the 5'-terminus found at higher concentration (Lys-Trp > Ser-Trp ≈ Trp-Trp > Trp) was confirmed in these experiments. Plotting the melting points against the salt content (Figure 4a) also revealed the expected decrease of duplex stabilization with the loss of the electrostatic component in the interactions. When melting points with dilysinyl derivative **5** were compared to those with all-DNA octamer **8**, nonamer **9**, and decamer **10** (Figure 4b), the same trend was observed. At physiological ionic strength, the dipeptide appendage still produced the same melting point increase as one additional A:U base pair. Further, duplex stabilization by peptidyl-nucleic acid interactions at low ionic strength and by phosphate shielding through counterions at high ionic strength, together, make **5** a terminally modified hybridization probe with little sensitivity to ionic strength changes.

To test whether the duplex stabilization provided by the peptidyl substituents depends on the sequence of the single-stranded overhang on the RNA target, hybridization to RNA strand **7** bearing a 5'-CGG-3' overhang was measured. This called for DNA **11** as a control nonamer, whose 5' deoxyguanylate allows for formation of an additional G:C base pair with RNA target **7**. Comparing the melting points for all hybridization partners tested, the 3'-overhang of **7** led, on average, to ca. 1 °C higher melting points than those with the 5'-UUG-3' overhang of **6**, but the relative stabilizing effects of the aminoacyl substituents was almost identical to those observed with **6** (Table 1). This indicates that the ability to tune the hybridization properties of oligodeoxyribonucleotides with the aminoacyl substituents is not strongly dependent on the sequence flanking the RNA target site.

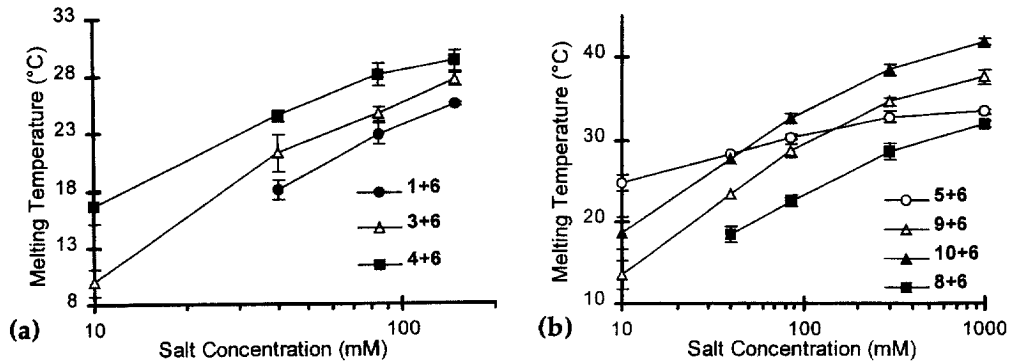

Figure 4. Effect of salt concentration on melting points of duplexes. (a) Duplexes between **6** and peptidyl-oligonucleotides **3** and **4**, and singly acylated **1** at up to 150 mM salt. (b) Duplexes of dilysinyl-oligonucleotide **5**, extended oligonucleotides **9** and **10**, and control DNA **8** at up to 1 M salt. Data points are average ± one S.D. of at least four melting curves at 1.3 μM strand concentration. Melting points of **1+6** and **8+6** were at 10 mM salt were too low to be determined at this strand concentration. Melting points of **2+6** are very similar to that of **3+6** and are not shown for clarity.

Table 1. Melting points [°C] of duplexes with RNA undecamer **7**.

Sequence Salt concentration:	10 mM	40 mM	85 mM	150 mM
8 (control octamer)	< 10	18.6±1.3	23.3±0.7	25.6±1.0
1	< 10	19.2±1.5	22.4±1.6	26.7±0.5
2	15.5±3.3	22.4±1.1	26.2±0.8	28.3±0.6
3	14.2±0.3	21.2±1.4	25.0±1.6	27.8±1.2
4	19.1±1.4	24.6±1.2	27.6±1.1	29.6±0.8
5	22.6±1.2	29.2±0.3	30.8±1.0	32.0±0.8
11 (control nonamer)	24.3±2.0	34.2±0.6	38.9±0.2	41.4±0.8

Melting points are averages (± one S.D.) from at least four melting curves.

The results reported here are noteworthy in several respects. They demonstrate that aminoacyl-appendages increase the stability of duplexes of DNA with RNA in a sequence-dependent manner, allowing for an exquisite tuning of the affinity for an antisense target by choosing the sequence of the appendage. On a "per residue" basis, the directly linked lysinyl residues appear to have a stronger electrostatic effect than ornithine- and arginine chains appended[11] or incorporated[12] into DNA via xenobiotic linkers or an SPKK motif appended to DNA.[13] In fact, two cationic residues seem to have a similar net stabilizing effect as the combined counterions shielding seven internucleotide phosphodiester groups at high salt concentration, even though the peptidyl ligand is localized at the terminus of the DNA. Due to this localization at the terminus, the appendages should not interfere with interactions of the central and distal portions of the oligonucleotides. For antisense applications,

this is expected to result in accessibility of the DNA:RNA duplexes to RNase H attack. Besides their increased affinity, which may be based on a faster on-rate,[14] the 5'-peptidyl-oligonucleotide are also resistant to exonuclease attack from the 5'-terminus, adding to their attractiveness as antisense agents. Additional protection against 3'-exonuclease attack, the major form of nuclease activity in human blood, is found for the 3',5'-dipeptidyl-hybrids recently prepared in these laboratories.[15]

Further, these results are interesting in the context of naturally occurring aminoacyl-nucleic acids. Assuming that the effects of 3'- and 5'-aminoacylation on the binding properties of nucleic acids are similar, the single-stranded acceptor region of tRNAs should experience a sequence-dependent increase in affinity for complementary strands when charged with amino acid residues. While duplex formation with the mRNA is restricted to the anticodon region of present-day tRNAs, probably because an aminoacyl sequence-dependent binding would be detrimental to the expedient and unambiguous translation of the genetic information, such interactions could have been important for primitive precursors of tRNA in the early evolution of the translational machinery.[16]

In conclusion, dipeptidyl-oligodeoxyribonucleotides, DNA analogs readily synthesized without detectable racemization by automated DNA assembly followed by peptide couplings,[6] can be tuned to an increased affinity for RNA target strands with a reduced ionic-strength sensitivity that cannot be achieved with natural nucleotide building blocks alone. The less costly amino acid residues are therefore interesting alternatives to appendages constructed with backbone-modified nucleic acids. The initial characterization of just a very few of the many possible peptidyl-appendages indicates that these analogs have properties that should make them attractive for a number of biomedical applications for which tailor-made hybridization probes are needed.

Acknowledgments. The authors wish to thank Ina Schwope and Colleen Bleczinski for preparing peptidyl-oligonucleotides, and M. Uesugi and G. Verdine (Harvard University) for access to a CD spectropolarimeter. Support by the N.I.H., National Institutes of General Medical Sciences, grant GM54783 to C.R., is gratefully acknowledged.

Note Added in Proof. After submission of our manuscript, a paper by Harrison and Balasubramanian has appeared (*Nucleic Acids Res.* **1998**, *26*, 3136-3145), in which the binding properties of 49 different 5'-peptide-DNA conjugates with an SMCC linker between the 5'-hexylamino oligonucleotide and the side chain of a cysteinyl residue are being reported. At 110 mM salt, heptapeptide conjugates with 2-5 lysinyl residues gave a ΔTm of 3.1-9.7°C (1.6-1.9°C per residue) for duplexes with a hexadecamer DNA target and 0.7°C per residue for duplexes with an octamer DNA target.

References and Notes

1. Selected reviews on antisense therapy: (a) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 544-584. (b) Milligan, J. F.; Matteucci, M. D.; Martin, J. C. *J. Med. Chem.* **1993**, *36*, 1923-1937. (c) Stein, C. A.; Cheng, Y.-C. *Science* **1993**, *261*, 1004-1012. (d) DeMesmaeker, A.; Haener, R.; Martin, P; Moser, H. E. *Acc. Chem. Res.* **1995**, *28*, 366-374. (e) Agrawal, S.; Iyer, R. P. *Pharmacol. Ther.* **1997**, *76*, 151-160.
2. (a) Sanghvi, Y. S.; Cook, P. D., Eds.; *Carbohydrate Modifications in Antisense Research*; ACS Symposium Series: Washington, DC: 1994, pp 1-22. (b) Hunziker, J.; Leumann, C. In *Modern Synthetic Methods*, Ernst, B.; Leumann, C., Eds.; Verlag Helvetica Chimica Acta: Basel, 1995; pp 333-417.
3. Selected references: (a) Monia, B. P.; Lesnik, E. A.; Gonzalez, C.; Lima, W. F., McGee, D.; Guinosso, C. J.; Kawasaki, A. M.; Cook, P. D.; Freier, S. M. *J. Biol. Chem.* **1993**, *168*, 12514-14522. (b) Manoharan, M.; Tivel, K. L.; Andrade, L. K.; Mohan, V.; Condon, T. P.; Bennett, C. F.; Dan Cook, P. *Nucleosides Nucleotides* **1995**, *14*, 969-973. (c) Yu, D.; Iyer, R. P.; Shaw, D. R.; Lisziewicz, J.; Li, Y.;

Jiang, Z. W.; Roskey, A.; Agrawal, S. *Bioorg. Med. Chem.* **1996**, *4*, 1685-1692. (d) Szewczyk, J. W.; Baird, E. E.; Dervan, P. B. *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1487-1489. (e) Robles, J.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1997**, *119*, 6014-6021.

4. (a) Haralambidis, J.; Duncan, L.; Angus, K.; Tregear, G. W. *Nucleic Acids Res.* **1990**, *18*, 493-499. (b) Arar, K.; Monsigny, M. Mayer, R. *Tetrahedron Lett.* **1993**, *34*, 8087-8090. (c) Bongartz, J.-P.; Aubertin, A.-M.; Milhaud, P. G.; Lebleu, B. *Nucleic Acids Res.* **1994**, *22*, 4681-4688. (d) Truffert, J.-C.; Lorthioir, O.; Asseline, U.; Thuong, N. T.; Brack, A. *Tetrahedron Lett.* **1994**, *35*, 2353-2356. (e) Robles, J.; Pedroso, E.; Grandas, A. *Nucleic Acids Res.* **1995**, *23*, 4151-4161.
5. (a) Bergmann, F.; Bannwarth, W. *Tetrahedron Lett.* **1995**, *36*, 1839-1842. (b) Guibourdenche, C.; Seebach, D.; Natt, F. *Helv. Chim. Acata* **1997**, *80*, 1-13. (c) Bruick, R. K.; Dawson, P. E.; Kent, S. B. H.; Usman, N.; Joyce, G. F. *Chem Biol.* **1996**, *3*, 49-56.
6. Tetzlaff, C. N.; Schwope, I.; Bleczinski, C. F.; Steinberg, J. A.; Richert, C. *Tetrahedron Lett.* **1998**, *39*, 4215-4218.
7. Aminoacyl- and dipeptidyl-oligodeoxyribonucleotides were synthesized as previously reported (reference 6). Briefly, DNA synthesis using commercially available phosphoramidites T, dC^{Bz}, dA^{Bz} (Perseptive Biosystems), dG^{dmf} (Perkin Elmer), and known 5'-deoxy-5'-aminothymidine phosphoramidite (Bannwarth, W. *Helv. Chim. Acta* **1988**, *71*, 1517) was followed by peptide assembly using Fmoc-Trp-OH, Fmoc-Ser-OH (Advanced ChemTech), Fmoc-Lys(Alloc)-OH (Perseptive Biosystems), and Fmoc-Lys(Fmoc)-OH (Novabiochem). Peptidyl-DNA hybrids were purified to > 95% purity by HPLC (RP_{18}, CH_3CN gradient/0.1 M TEAA). Tricationic peptidyl-DNA **5** required degradation of DNA failure products with calf spleen phosphodiesterase prior to HPLC purification to obtain the desired purity. Oligoribonucleotides **6** and **7** and RNA-DNA hybrids **12-14** were prepared using 2'-*O*-Fpmp phosphoramidite building blocks (Cruachem USA), and for the latter DNA phosphoramidite building blocks as given above. Chain assembly, deprotection, and purification were performed according to the supplier's recommendations (Cruachem Technical Bulletin No. 037R01). All oligonucleotides were characterized by MALDI-TOF mass spectrometry.
8. Ho, W.; Steinbeck, C.; Richert, C., unpublished results.
9. Saenger, W. *Principles of Nucleic Acid Structure*. Springer-Verlag: New York, 1983.
10. Melting and cooling curves were measured at 260 nm in nuclease-free water with ammonium acetate buffer (Ambion, pH 6.0) at 0.1 mM EDTA over a temperature range of 5 to 65° C at a rate of 1 ° C per minute. Background absorbance changes, determined from the concurrent acquisition of a control sample containing buffer alone, were subtracted, and melting points were determined using Perkin Elmer UV TempLab® 1.2. Curves were smoothed with a 25 point moving average and melting points taken as the extrema of the 91 point first derivative.
11. Zhu, T.; Wei, Z.; Tung, C.-H.; Dickerhof, W. A.; Breslauer, K.; Georgopoulos, D. E.; Leibowitz, M. J.; Stein, S. *Antisense Res. Dev.* **1993**, *3*, 265-275.
12. Wei, Z.; Tung, C.-H.; Zhu, T.; Dickerhof, W. A.; Breslauer, K. J.; Georgopoulos, D. E.; Leibowitz, M. J.; Stein, S. *Nucleic Acids Res.* **1996**, *24*, 655-661.
13. Truffert, J. C.; Asseline, U.; Thuong, N. T.; Brack, A. *Prot. Pept. Lett.* **1995**, *2*, 419-424.
14. Corey, D. *J. Am. Chem. Soc.* **1995**, *117*, 9373-9374.
15. Schwope, I.; Bleczinski, C. F., Richert, C., unpublished results.
16. (a) Orgel, L. E. *J. Mol. Evol.* **1989**, *29*, 465-474. (b) Schimmel, P.; Henderson, B. S. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11283-11286. (c) Di Giulio, M. *J. Mol. Evol.* **1997**, *45*, 571-578. (d) Henderson, B. S.; Schimmel, P. *Bioorg. Med. Chem.* **1997**, *5*, 1071-1079.