Communications to the Editor

Exceptional and Selective Stabilization of A-T Rich DNA·DNA Duplexes by *N*-Methylpyrrole Carboxamide Peptides Conjugated to Oligodeoxynucleotides

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Received November 17, 1994

Netropsin and distamycin A are amidine-containing di- and tripeptides of *N*-methylpyrrolecarboxamide (MPC) which bind isohelically to the minor groove of DNA.[1] Association constants of 10^7-10^9 M^{-1} have been measured for complexes formed with A-T rich DNA.[2] These affinities are much greater than those exhibited by simple intercalating agents,[3] which bind to DNA with association constants of 10^4-10^6 M^{-1}. However, MPC peptides bind poorly to G-C rich DNA[2b,4] and to A-form[2b,5] nucleic acid.

The stabilization of short duplexes by intercalating agents has been recognized for many years. Hybrids formed by oligodeoxyribonucleotides (ODNs) with complementary DNA or RNA sequences are significantly stabilized by covalent attachment of these agents to one end of the oligonucleotide.[6] This modification has been shown to enhance the efficacy of oligonucleotides as antisense agents.

We now report that covalent attachment of a class of minor groove binding agents, the *N*-methylpyrrolecarboxamide (MPC) peptides, to short thymidylate or adenylate ODNs dramatically increases the stability of their duplexes. The degree of stabilization is a function of peptide length. The effect is highly selective, since DNA–DNA hybrids that contain G-C base pairs in the peptide binding region or DNA·RNA hybrids are not stabilized by a covalently linked MPC oligopeptide.

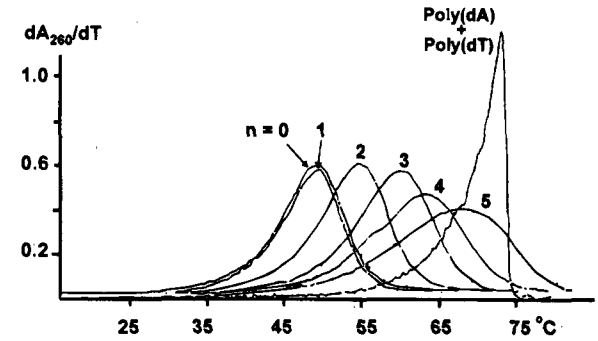

Figure 1. First derivative plots of helix–coil transitions at 260 nm for the $(dTp)_{16}$–MPC·poly(dA) duplex in 0.2 M NaCl, 0.1 mM EDTA, 0.01 M Na_2HPO_4, pH 7.0. [dAMP residues] = [dTMP residues] = 2×10^{-4} M. The number above each curve indicates the number of MPC residues in the peptide conjugated to the 3′ end of $(dTp)_{16}$.

The aminohexanoyl MPC peptides composed of one to five MPC subunits were synthesized by a published method.[7] To facilitate monitoring of the conjugation reaction with the ODN, every peptide contained a C-terminal 2-[[4-(phenylazo)benzyl]-thio]ethyl dye. All intermediates and final products were characterized by ^1H NMR spectrometry. The ODN–MPC conjugates were prepared by reacting a 3′-activated ODN[8] with the aliphatic NH_2 group of an MPC peptide. Conjugates were isolated from reaction mixtures by reverse phase HPLC. Conjugation yields were typically 30–50%. Purity of the conjugates was confirmed by analytical HPLC and gel electrophoresis, and the presence of the dye was verified by UV spectroscopy.

Figure 1 shows the first-derivative melting curves for a series of $(dTp)_{16}$–MPC conjugates hybridized to poly(dA). While the T_m of the duplex[9] was unaffected by one covalently appended MPC residue, longer peptides progressively raised the T_m. Conjugation of an MPC pentapeptide to the oligothymidylate strand stabilized the duplex by 20 °C. The loss of cooperativity observed with conjugates of the two longest peptides ($n = 4$ and 5) might be due to discontinuous hybrid formation on the poly(dA). If the conjugated minor groove binding peptide locks movement of the initial hybrid, single-stranded gaps cannot be removed by two-dimensional ODN sliding.

To further quantitate the effects of the tethered MPC peptides, we synthesized a series of $(dTp)_8$ and $(dAp)_8$ conjugates in which the size of the peptide was varied from two to five MPC residues. Molecular modeling indicated that the pentapeptide should interact with no more than eight base pairs of duplex. First-derivative melting curves for the $(dAp)_8$·$(dTp)_8$–MPC_n ($n = 0-5$) duplexes are shown in Figure 2A. The melting temperatures for the respective hybrids are presented in Table

(1) For review, see: Zimmer, C.; Wähnert, U. *Prog. Biophys. Mol. Biol.* **1986**, *47*, 31 and references cited therein.

(2) (a) Luck, G.; Triebel, H.; Waring, M.; Zimmer, C. *Nucleic Acids Res.* **1974**, *1*, 503. (b) Wartell, R. M.; Larson, J. E.; Wells, R. D. *J. Biol. Chem.* **1974**, *249*, 6719. (c) Marky, L. A.; Breslauer, K. J. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 4359.

(3) (a) Isaacs, S. T.; Shen, C.-K.; Hearst, J. E.; Rapoport, H. *Biochemistry* **1977**, *16*, 1058. (b) Reinhardt, C. G.; Krugh, T. R. *Biochemistry* **1978**, *17*, 4845. (c) Hansen, J. B.; Koch, T.; Buchardt, O.; Nielsen, P. E.; Wirth, M.; Nordën, B. *Biochemistry* **1983**, *22*, 4878.

(4) Gursky, G. V.; Zasedatelev, A. S.; Zhuze, A. L.; Knorlin, A. A.; Grokhovsky, S. L.; Streltsov, S. A.; Surovaya, A. N.; Nikitin, S. M.; Krylov, A. S.; Retchinsky, V. O.; Mikhailov, M. V.; Beabealashvili, R. S.; Gottikh, B. P. *Cold Spring Harbor Symp. Quant. Biol.* **1983**, *47*, 367.

(5) (a) Zimmer, C.; Luck, G.; Thrum, H. *Stud. Biophys.* **1970**, *24/25*, 311. (b) Pullman, B. *Specificity and Biological Interaction. International Symposium at the Pontifical Academy of Sciences*; Chagas, C., Pullman, B., Eds.; Vatican Press: Vatican City, 1984; pp 1–20.

(6) Hélène, C.; Toulmé, J.-J. In *Topics in Molecular and Structural Biology. Oligodeoxynucleotides: antisense inhibitors of gene expression*; Cohen, J. S., Eds.; CRC Press: Boca Raton, 1989; pp 137–172.

(7) Grehn, L.; Ragnarsson, U. *J. Org. Chem.* **1981**, *46*, 3492.

(8) The 3′-terminal phosphate of the cetyltrimethylammonium salt of an ODN in organic solution was activated using triphenylphosphine/dipyridyl disulfide using a previously described method (Godovikova, T. S.; Zarytova, V. F.; Maltzeva, T. V.; Khalimskaya, L. M. *Bioorg. Khim.* **1989**, *15*, 1246).

(9) In all mixtures T and A residues were equimolar and $MgCl_2$ was absent. Under these conditions no triple-strand formation was expected for any of the Watson/Crick combinations studied here for the following reasons: (a) Others have shown that $(dA)_{19} + (dT)_{19}$ do not form triplexes in 0.2 M NaCl even when an A:T ratio of 1:2 is employed (Kibler-Herzog, L.; Kell, B.; Zon, G.; Shinozuka, K.; Mizan, S.; Wilson, W. D. *Nucleic Acids Res.* **1990**, *18*, 3545). (b) Free netropsin significantly destabilizes the poly(dT)·poly(dA)·poly(dT) triplex (Park, Y.-W.; Breslauer, K. J. *Proc. Natl. Acad. Sci U.S.A.* **1992**, *89*, 6653). (c) Our own unpublished results show that, in 10 mM $MgCl_2$, 140 mM KCl, and 20 mM HEPES-HCl (pH7.0), conjugates of $(dTp)_8$ and minor groove binders do not form T-A-T triplexes with poly(dA) when the T:A ratio is 1:1 or 2:1.

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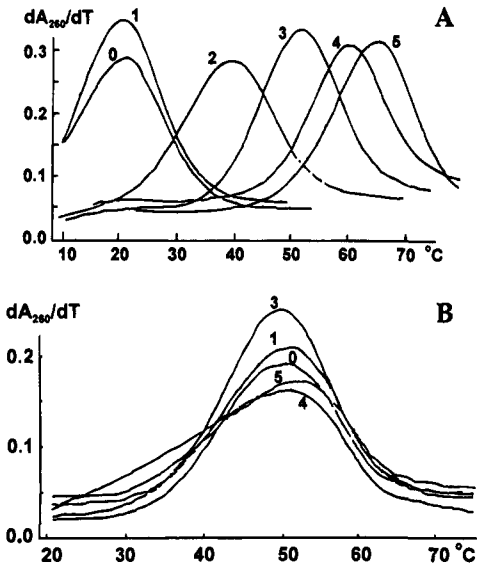


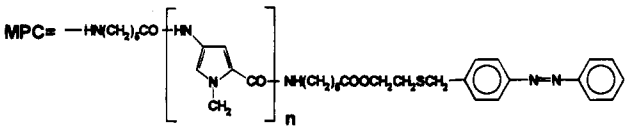
Figure 2. First derivative plots of helix−coil transitions at 260 nm for (A) the $(dTp)_8$−MPC_n·$(dAp)_8$ duplex (n = 0−5) and (B) the d(ApGpCpGpGpApTpGp)·d(CpApTpCpCpGpCpTp)−MPC_n duplex (n = 0, 1, 3, 4, 5). The concentration of each ODN was 2.5×10^{-5} M. See Figure 1 caption for the buffer.

1. Thermostability was again dependent upon the length of the MPC peptide, and the MPC peptide was a more effective stabilizing agent when tethered to $(dTp)_8$ than to $(dAp)_8$. The MPC pentapeptide conjugated to the $(dTp)_8$ increased the T_m of the duplex by 44 °C, while its conjugation to the $(dAp)_8$ strand increased the T_m by 32 °C.

The stabilization of $(dTp)_8$·$(dAp)_8$ by oligothymidylate-linked MPC peptides was greater than that achieved with free distamycin or covalently linked intercalating agents. Free distamycin, which contains a positively charged amidine group,[1] increased the T_m of the same duplex by 26 °C whereas the electrostatically neutral MPC tripeptide linked to $(dTp)_8$ provided 31 °C of stabilization[10] (Table 1). Conjugation of ethidium bromide[11] to the 3′ end of one or both strands of the $(dTp)_8$·$(dAp)_8$ duplex provided 9 or 22 °C of stabilization, respectively.

G-C rich DNA and DNA·RNA hybrids are not stabilized by conjugated MPC peptides. As shown in Figure 2B, the T_m of a hybrid formed by two G-C rich complementary 8-mer ODNs was not significantly shifted by the presence of a covalently appended MPC peptide. Similarly, hybrids formed between poly(rA) and $(dTp)_8$−MPC (n = 0−5) had identical T_m's of approximately 23 °C (data not shown). It appears that, like free netropsin and distamycin,[2,4,5] conjugated MPC peptides are unable to bind to the minor groove of these duplexes. We propose that A-T rich ODN−MPC conjugates may be useful as probes for the efficient and selective detection of complementary DNA sequences. Conjugation of other minor groove binding agents with different base pair binding specificities could modify the type of hybrid stabilized. We have found that a variety of nucleic acid hybrids can be stabilized by conjugation to other such agents and are investigating the scope of that interaction.

Table 1. Melting Temperatures of $(dTp)_8$·$(dAp)_8$ Duplexes Bearing MPC Oligopeptides[a]



Duplex	T_m°C	ΔT_m°C[b]
$(dAp)_8$ + $(dTp)_8$	21.1	-
$(dAp)_8$ + $(dTp)_8$ + Distamycin A[c]	47.1	26.0
$(dAp)_8$ + $(dTp)_8$-MPC_n n = 2	39.4	18.3
n = 3	51.7	30.6
n = 4	60.2	39.1
n = 5	65.4	44.3
$(dTp)_8$ + $(dAp)_8$-MPC_n n = 2	29.1	8.0
n = 3	39.0	17.9
n = 4	42.7	21.6
n = 5	52.6	31.5
$(dTp)_8$ + $(dAp)_8$-EtBr[d]	30.5	9.4
$(dTp)_1$-EtBr + $(dAp)_8$-EtBr	42.9	21.8

[a] Optical melts were conducted in 0.2 M NaCl, 0.1 mM EDTA, 0.01 M Na_2HPO_4, pH 7.0 with $[(dTp)_8$·$(dAp)_8] = 2.5 \times 10^{-5}$ M. [b] The difference in T_m between modified and unmodified duplexes. [c] The concentration of distamycin A was 2.5×10^{-5} M. [d] Ethidium bromide (EtBr) was conjugated by its 8-NH_2 position to the 3′-terminal phosphate of the ODNs through a β-alanine linker by the method in ref 11.

(10) The low water solubility of free MPC peptides precluded their use in optical melting experiments.

(11) The ODN−ethidium bromide conjugates used in this study were a gift of Dr. A. Koshkin (Novosibirsk, Russia). Their synthesis is described in the following: Koshkin, A. A.; Ivanova, T. M.; Bulychev, N. V.; Dobrikov, M. I.; Lebedev, A. V. *Bioorg. Khim.* **1993**, *13*, 570.

JA9437417