DNA Interactions

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Ligand-Assisted Complex Formation of Two DNA Hairpin Loops**

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Hairpin loops of RNA can hybridize with other single-stranded and hairpin-loop regions of RNA to provide structural features such as pseudoknots and loop–loop interactions for building up higher-order structures.[1] In fact, tertiary structures of tRNA, rRNA, and riboswitches[2] consist of a number of these interactions. A loop–loop kissing interaction at the palindromic 5′-GUGCAC-3′ sequence in the purine-rich stem loop of the HIV-1 genome initiates dimerization of the RNA genome, eventually leading to the formation of an extended dimer.[3] Besides the biological significance, loop–loop interactions have been used for the construction of nanoscaled structures of RNA.[4] Despite the many examples of loop–loop interactions in RNA structures, interactions of DNA hairpin loops have attracted only limited attention.[5] Hybridization of DNA fragments with hairpin DNA that leads to hairpin opening has been used as basis for molecular beacons[6] that detect genetic mutations and as fuel to drive DNA-based nanomachines.[7]

We herein report our attempt to induce the connection of two DNA hairpin loops with the assistance of small-molecular ligands (Figure 1). A tetrameric form of *N*-methoxycarbonyl-1,8-naphthyridine (NCT, Scheme 1a) brought two hairpin loops that consist of a $d(CGG)_3$ sequence together to produce a ligand-assisted complex of two DNA hairpin loops, the formation of which was confirmed by double-labeling experiments using native polyacrylamide gel electrophoresis (PAGE). The data described herein provide new insights into the ligand-assisted construction of higher-order structures of DNA.

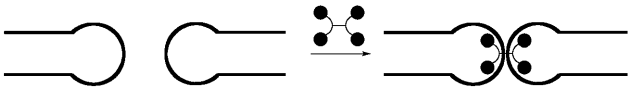
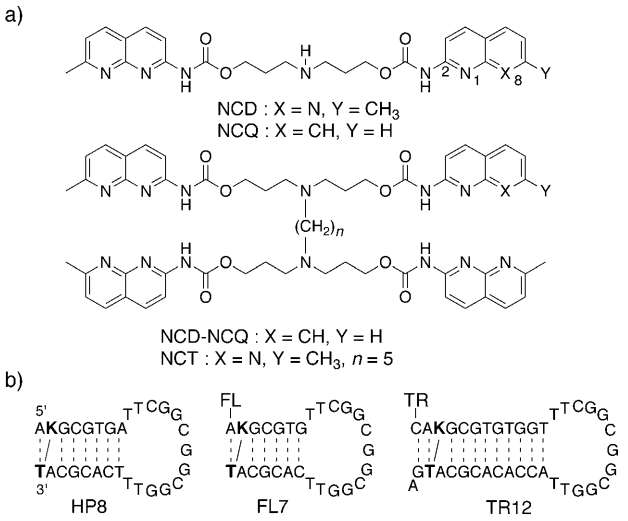


Figure 1. Illustration of formation of a ligand-assisted complex from two DNA hairpin loops.



Scheme 1. a) Structures of ligands. b) Sequences and secondary structures of hairpin DNAs (HP8, FL7, and TR12). A cross-link between K and T is shown in bold face. FL: FAM; TR: Texas Red.

NCT is a dimeric form of the naphthyridine carbamate dimer (NCD, Scheme 1a), which was observed strongly to bind to the CGG/CGG triad in the stem region of the hairpin secondary structure of $d(CGG)_n$ repeats.[8] NMR spectroscopic analysis of the complex of NCD and the CGG/CGG triad showed that naphthyridine formed hydrogen bonds with the guanine bases in the CGG/CGG motif. The cytosines that were left unpaired because of the invasion of hydrogen-bonded guanine by naphthyridine were forced to flip out of the π stack.[9] On the basis of these observations, we reported that NCD could induce hybridization of two single-stranded DNAs by binding to the CGG site of each strand.[8b] While the hairpin loop is much more constrained than the normal extended single-stranded form in terms of the degree of structural and conformational freedom, we anticipated that NCT may have a chance to bind simultaneously to two hairpin loops that consist of the $d(CGG)_n$ repeat to give a ligand-assisted complex of two DNA hairpin loops. Because the loop–loop interaction of hairpin DNA is reported to lead to an extended dimer when the stem length is too short,[7c] we used cross-linking of the stem region by photocycloaddition of cyanovinylcarbazole nucleoside (K)[10] to the thymine in the opposite strand to suppress the equilibrium with the extended dimer.

Firstly, ligand-assisted interactions of two DNA hairpin loops were investigated by native PAGE analysis of a cross-linked hairpin DNA (HP8) that has an 8 bp stem and a

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$TT(CGG)_3TT$ loop sequence (Scheme 1b). Addition of NCT (3 μM) to HP8 (2 μM) produced a new band on the gel (lane 2, band B), that migrated more slowly than HP8 (band A). Upon increasing the concentration (lanes 3–5), the intensity of both bands A and B decreased with the simultaneous appearance of a new band that migrated very slowly on the gel (band C), which became the predominant band at 12 μM of NCT (lane 5). Further increase of NCT resulted in a gradual decrease of band C (lanes 6 and 7) without formation of any other significant bands.

Next, we examined the formation of bands B and C with structurally closely related compounds. The formation of bands B and C was sensitive to the chemical structure of the ligand (Figure 2b). With NCD (12 μM), we could clearly

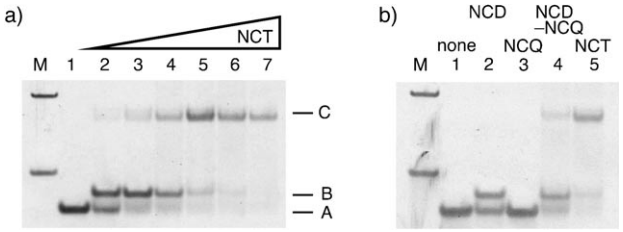

Figure 2. a) Native PAGE analysis of HP8 (2 μM) incubated with the indicated concentrations of NCT. Lane M: DNA marker (20 and 40 bp); lanes 1–7: NCT at 0, 3, 6, 9, 12, 15, and 18 μM, respectively. b) Native PAGE analysis of HP8 (2 μM) incubated with ligand (12 μM). Lane M: DNA marker (20 and 40 bp); lane 1: no ligand; lane 2: NCD; lane 3: NCQ; lane 4: NCD–NCQ; lane 5: NCT.

detect the formation of band B (lane 2), but not band C. In contrast, naphthyridine-quinoline hybrid (NCQ), where one of the two 2-amino-1,8-naphthyridine heterocycles in NCD was replaced by 2-aminoquinoline, did not induce the formation of band B (lane 3). A marked substitution effect of naphthyridine by quinoline was also observed in the formation of band C. Thus, NCD–NCQ, where one of four naphthyridines was replaced by quinoline, could induce the formation of band B, but only lead to weak formation of band C (lane 4). In separate experiments, we confirmed that a higher concentration of NCD (20 μM) did not induce the formation of band C (Figure S7). The 2-amino-1,8-naphthyridine is fully complementary to guanine in terms of hydrogen-bond formation, but substitution of the nitrogen (N8) in 1,8-naphthyridine by the carbon (C8H) in quinoline disturbs the hydrogen bonding to a guanine.[11] With these results, it was clear that 1) the minimal structure necessary for the formation of band B was NCD, and 2) the formation of band C required two NCD moieties in one molecule. The remarkable substitution effect suggests that the hydrogen-bonding interaction of 2-amino-1,8-naphthyridine with a guanine base in the loop sequence is indispensable for the formation of both bands B and C.

NCT consists of two NCD molecules and a linker that connects them. The efficiency for the formation of band C could depend on the linker length. Three NCT variants NCT*n* (NCT6, NCT7, and NCT8), where *n* is 6, 7, and 8, having a linker that is one, two, and three methylene groups longer

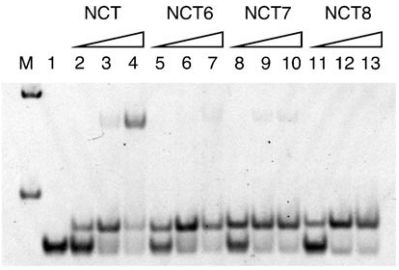

Figure 3. Native PAGE analysis of HP8 (2 μM) incubated with NCT*n*. Lane M, DNA marker (20 and 40 bp); lane 1: HP8; lanes 2–4: NCT at 5, 10, 20 μM; lanes 5–7: NCT6 at 5, 10, 20 μM; lanes 8–10: NCT7 at 5, 10, 20 μM; lanes 11–13: NCT8 at 5, 10, 20 μM.

than NCT were synthesized and examined for the interaction with HP8 (Figure 3). All NCT variants showed the formation of band B with comparable efficiency. In marked contrast, the formation of band C was most efficient for NCT ($n=5$) among all together four NCT variants. Careful inspection of the gel image revealed that the efficiency for the formation of band C decreased as the linker length increased. Thus, a faint band C was detected in lanes 7 and 10, but not in lane 13. These results show that the equilibrium between ligand-bound complexes of HP8 that produce the bands B and C is sensitively affected by the linker length.

To gain further insight into the interaction between NCT and the hairpin loop, hairpin DNAs with different loop sequences were examined (Figure 4a). The $d(CGG)_3$

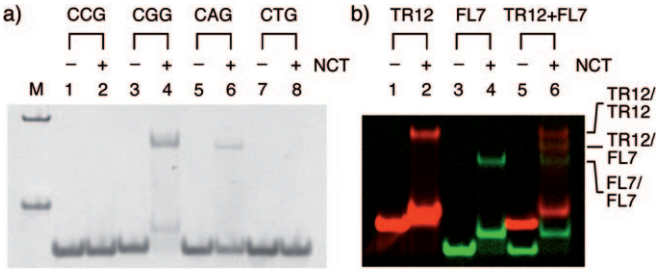

Figure 4. a) Sequence-dependent interaction with NCT. Hairpin ODNs (2 μM) containing repeat sequences were incubated with NCT (12 μM). Lane M: DNA marker (20 and 40 bp); lanes 1 and 2: $d(CCG)_3$; lanes 3 and 4: $d(CGG)_3$; lanes 5 and 6: $d(CAG)_3$; lanes 7 and 8: $d(CTG)_3$. b) Identification of the ligand-assisted complex of two DNA hairpin loops by double-labeling experiments. TR12 and FL7 (2 μM) were incubated with NCT (12 μM). Lanes 1 and 2: TR12; lanes 3 and 4: FL7; lanes 5 and 6: TR12 and FL7.

sequence in the hairpin loop of HP8 was replaced by $d(CCG)_3$, $d(CAG)_3$, and $d(CTG)_3$. All other sequences were kept unchanged for the four hairpin DNAs. The formation of bands B and C was not observed for the hairpin loop that consists of CCG and CTG repeats (lanes 2 and 8, respectively). The formation of a faint band C was detected for the hairpin DNA that contains the $d(CAG)_3$ loop sequence (lane 6). In separate experiments, we confirmed that 1) the TT sequence that flanks the CGG repeat has no significant effect on the formation of band C and that 2) NCT binding to the

hairpin loop that has a smaller loop size of $(CGG)_2$ produced the band C (Figure S5 and S6). In addition to the results obtained with ligands that consist of different heterocycles (Figure 2b), these results clearly showed that bands B and C were caused by the NCT binding selectively to the CGG loop sequence, and were fully consistent with our earlier study that NCD strongly binds to the CGG repeat and weakly to the CAG repeat, but not to the CTG and CCG repeats.[12] Band B was the bimolecular complex of HP8 and NCT, in which NCT binds to the interhairpin CGG/CGG site, whereas band C was most likely the complex of two HP8 and NCT, in which NCT bridged over two hairpin loops. The apparent dissociation constant K_d of NCT bound to the hairpin DNA for the band B complex was determined to be about 150 nM by surface plasmon resonance (SPR) assay using the CM5 sensor holding hairpin DNA on the surface (BIAcore, Figure S12). SPR analysis for the complex that corresponds to band C was disturbed by an unexpected large signal that most likely originates from the increased NCT concentration. It is likely that the NCT binding between two hairpin loops resulted in the change of surface structure. It is worth noting that the formation of band C was also observed for the non-cross-linked hairpin DNA that has a $d(CGG)_3$ loop sequence, as well as for the cross-linked HP8 (Figure S13 and S14), although the non-cross-linked hairpin showed an unidentified band on PAGE analysis.

To confirm that band C represents the NCT-assisted complex of two hairpin loops, double-labeling experiments using two hairpin DNAs that have the same $d(CGG)_3$ loop sequence but being differentiated by the stem length and fluorescence labels were conducted (Figure 4b). One hairpin DNA with a 7 bp stem (FL7) was labeled by the dye FAM, whereas the other having a 12 bp stem (TR12) was labeled with Texas Red (Scheme 1b). Upon treatment of TR12 and FL7 separately with NCT, the formation of both bands B and C that have the characteristic fluorescence signals of Texas Red and FAM was detected at different positions on the gel (see lanes 2 and 4). Upon treatment of a mixture of TR12 and FL7 with NCT (lane 6), the formation of two bands B, as observed in lanes 2 and 4, was detected, whereas three bands were found in the region of the band C in the gel. Among the three bands, the two bands that show the slowest and the fastest mobilities were identified as band C produced from TR12 and FL7, respectively, by comparing the mobility on the gel (see band C in lanes 2 and 6, and lanes 4 and 6) and the fluorescence signal. The third band exhibited an intermediate mobility with an orange color in the fluorescence image, thus showing that the band contained both Texas Red and FAM labels. Therefore, the band was identified as the hetero loop complex (TR12/FL7) that consists of TR12 and FL7, whereas the other two bands were identified as the homo loop complexes TR12/TR12 and FL7/FL7. While the precise mode of the NCT binding remains to be clarified by spectroscopic methods, we hypothesized that each NCD moiety in NCT did bind to one hairpin loop, because NCD and NCQ induced the formation of band B but did not lead to the formation of band C. The preference for a short linker between two NCD moieties for the formation of band C suggested that the direct interactions between nucleobases in the hairpin loops might also be involved.

In conclusion, we have shown that NCT assisted the formation of the complex of two hairpin DNAs that have a $d(CGG)_3$ loop sequence. The binding of NCT to the hairpin loop produced two complexes that were detected as the bands B and C. The equilibrium between these two complexes was sensitively affected by the structure of the heterocycles and the linker that connects the heterocycles. The ligand-assisted formation of complexes of two DNA hairpin loops described herein provides a new way to the design of higher-order structures of DNA. Preliminary experiments showed that the NCT-assisted complex formation is not limited to the DNA hairpins but applicable to RNA hairpins. The next challenge is to find ligands that assist the formation of complexes of two different loop sequences, which would broaden the scope of ligand-assisted formation of nucleic acid structures.

Experimental Section

Synthesis of NCT: A 50% glutaraldehyde solution saturated with NaCl was extracted with $CHCl_3$. The organic phase was evaporated to dryness to give glutaraldehyde as a viscous liquid. Glutaraldehyde (8 mg, 0.08 mmol) and $NaBH_3CN$ (18.7 mg, 0.30 mmol) were added to a solution of NCD (80 mg, 0.16 mmol) in MeOH (3 mL), adjusted to pH 6 with acetic acid. The mixture was stirred at room temperature for 10 h, then poured into $CHCl_3$ and washed with saturated sodium hydrogen carbonate and brine. The organic layer was dried over $MgSO_4$ and evaporated in vacuo. The crude residue was purified by chromatography on silica gel ($CHCl_3$/MeOH, 15:1) then gel permeation chromatography (GPC) to give NCT (20 mg, 23%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): $\delta = 8.11$ (d, 4H, $J = 9.1$ Hz), 8.07 (d, 4H, $J = 8.7$ Hz), 8.00 (d, 4H, $J = 8.2$ Hz), 7.23 (d, 4H, $J = 8.2$ Hz), 4.24 (t, 8H, $J = 6.4$ Hz), 2.64 (s, 12H), 2.65–2.58 (8H), 2.50 (t, 4H, $J = 6.4$ Hz), 1.87 (t, 8H, $J = 6.4$ Hz), 1.49 (m, 4H), 1.39 ppm (m, 2H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 163.8$, 155.8, 155.4, 155.2, 140.1, 138.6, 122.3, 119.2, 114.2, 64.3, 49.4, 49.2, 27.7, 27.1, 25.0 ppm; HRMS (ESI, positive-ion mode, MeOH): calcd for $C_{57}H_{66}N_{14}O_8$+Na^+: 1097.5086 $[M+Na]^+$; found: 1097.5083.

Polyacrylamide gel electrophoretic mobility shift assays: Hairpin oligodeoxynucleotides (ODNs; 2 μM) were incubated in 10 mM sodium cacodylate buffer (pH 7.0), 100 mM NaCl, and 10% glycerol at room temperature for 15 min. The samples were loaded onto 12% (19:1) native polyacrylamide gels in TBE buffer, and were run for 10 min at 100 V and 45 min at 250 V and 4 °C. The polyacrylamide gels were stained with SYBR Gold and visualized.

Gel imaging: Texas Red and FAM labeled hairpin ODNs were PAGE analyzed, and the gel was imaged by using an ImageQuant LAS 4010 (GE Healthcare). FAM-labeled ODNs were excited by Green Epi light (520 nm), and detected by a green fluorescent protein (GFP) detection filter (510DF10/GFP). Texas Red labeled ODNs were excited by Red Epi light (630 nm), and detected by Cy5 detection filter (R670BP/Cy5).

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[1] a) D. P. Giedroc, C. A. Theimer, P. L. Nixon, *J. Mol. Biol.* **2000**, *298*, 167–185; b) R. Brimacombe, *Eur. J. Biochem.* **1995**, *230*, 365–383.

[2] W. Winkler, A. Nahvi, R. R. Breaker, *Nature* **2002**, *419*, 952 – 956.
[3] a) J. C. Paillart, E. Skripkin, B. Ehresmann, C. Ehresmann, R. Marquet, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 5572 – 5577; b) J. C. Paillart, E. Westhof, B. Ehresmann, C. Ehresmann, R. Marquet, *J. Mol. Biol.* **1997**, *270*, 36 – 49.
[4] a) S. Horiya, X. L. Li, G. Kawai, R. Saito, A. Katoh, K. Kobayashi, K. Harada, *Chem. Biol.* **2003**, *10*, 645 – 654; b) A. Chworos, I. Severcan, A. Y. Koyfman, P. Weinkam, E. Oroudjev, H. G. Hansma, L. Jaeger, *Science* **2004**, *306*, 2068 – 2072; c) G. Mayer, M. S. Raddatz, J. D. Grunwald, M. Famulok, *Angew. Chem.* **2007**, *119*, 563 – 566; *Angew. Chem. Int. Ed.* **2007**, *46*, 557 – 560; d) G. Mayer, D. Ackermann, N. Kuhn, M. Famulok, *Angew. Chem.* **2008**, *120*, 985 – 987; *Angew. Chem. Int. Ed.* **2008**, *47*, 971 – 973.
[5] a) J. Völker, N. Makube, G. E. Plum, H. H. Klump, K. J. Breslauer, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 14700 – 14705; b) F. Barbault, D. T. Huynh, J. Paoletti, G. Lanceloti, *J. Biomol. Struct. Dyn.* **2002**, *19*, 649 – 658; c) J. S. Bois, S. Venkataraman, H. M. Choi, A. J. Spakowitz, Z. G. Wang, N. A. Pierce, *Nucleic Acids Res.* **2005**, *33*, 4090 – 4095.
[6] a) S. Tyagi, F. R. Kramer, *Nat. Biotechnol.* **1996**, *14*, 303 – 308; b) S. Tyagi, D. P. Bratu, F. R. Kramer, *Nat. Biotechnol.* **1998**, *16*, 49 – 53; c) W. Tan, K. Wang, T. J. Drake, *Curr. Opin. Chem. Biol.* **2004**, *8*, 547 – 553.
[7] a) B. Yurke, A. J. Turberfield, A. P., Jr. Mills, F. C. Simmel, J. L. Neumann, *Nature* **2000**, *406*, 605 – 608; b) S. J. Green, D. Lubrich, A. J. Turberfield, *Biophys. J.* **2006**, *91*, 2966 – 2975.
[8] a) T. Peng, K. Nakatani, *Angew. Chem.* **2005**, *117*, 7446 – 7449; *Angew. Chem. Int. Ed.* **2005**, *44*, 7280 – 7283; b) T. Peng, C. Dohno, K. Nakatani, *Angew. Chem.* **2006**, *118*, 5751 – 5754; *Angew. Chem. Int. Ed.* **2006**, *45*, 5623 – 5626.
[9] M. Nomura, S. Hagihara, Y. Goto, K. Nakatani, C. Kojima, *Nucleic Acids Symp. Ser.* **2005**, *49*, 213 – 214.
[10] Y. Yoshimura, K. Fujimoto, *Org. Lett.* **2008**, *10*, 3227 – 3230.
[11] a) K. Nakatani, S. Sando, H. Kumasawa, J. Kikuchi, I. Saito, *J. Am. Chem. Soc.* **2001**, *123*, 12650 – 12657; b) S. Hagihara, H. Kumasawa, Y. Goto, G. Hayashi, A. Kobori, I. Saito, K. Nakatani, *Nucleic Acids Res.* **2004**, *32*, 278 – 286.
[12] H. He, M. Hagihara, K. Nakatani, *Chem. Eur. J.* **2009**, *15*, 10641 – 10648.

