DNA Cleavage

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Binding of Naphthyridine Carbamate Dimer to the $(CGG)_n$ Repeat Results in the Disruption of the G–C Base Pairing

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The expansion of the $(CGG)_n$ trinucleotide repeat in the FMR1 gene causes the neurological disorder fragile X syndrome.[1–3] The molecular basis for the $(CGG)_n$ expansion involves the formation of a metastable hairpin structure[4–6] consisting of continued 5′-CGG-3′/5′-CGG-3′ triads, in which a G–G mismatch is flanked by two G–C base pairs. We recently reported the remarkable binding of naphthyridine azaquinolone (**NA**) to the 5′-CAG-3′/5′-CAG-3′ triad in the hairpin form of the $(CAG)_n$ repeat, where the A–A mismatch was flanked by G–C base pairs. The ligand-bound structure was intriguing because 1) two **NA** molecules were bound to a single CAG/CAG triad which 2) induced the cytosine—which was hydrogen-bonded to the guanine—to flip out from the base stack (Figure 1).[7] The **NA**-immobilized sensor was useful for the rapid diagnosis of the $(CAG)_n$ repeat length. We have reported a series of ligands binding to a G–G mismatch,[8–10] and therefore the remarkable structure of **NA** bound to the CAG/CAG triad prompted us to investigate the mode of ligand binding to the CGG/CGG triad and, hence, the possibility of the cytosine flipping out in the ligand-bound complex. Herein, we report that naphthyridine carbamate dimer (**NC**)[11] binds to a single CGG/CGG triad with exclusively 2:1 **NC**/triad stoichiometry. The binding of **NC** to the CGG/CGG triad induced the disruption of the guanine–cytosine base pairing in the triad, and made the cytosine susceptible to the subsequent chemical cleavage reaction initiated by addition of hydroxylamine.

Among the ligands that we synthesized for binding to the G–G mismatch, **NC** showed a marked preference in binding to the CGG/CGG triad. The binding of **NC** to the CGG/CGG triad in the 13-mer duplex increased the melting temperature (T_m) by 23.1 °C, whereas the increase in T_m (ΔT_m) was only 6.7 °C for the GGC/GGC triad (Table 1). A survey of the effect of the flanking sequence suggested that the strong and selective **NC** binding to the CGG/CGG triad is most likely a consequence of the interaction of **NC** not only with the G–G mismatch, but also with the cytosine on the 5′ side and/or the guanine on the 3′ side.

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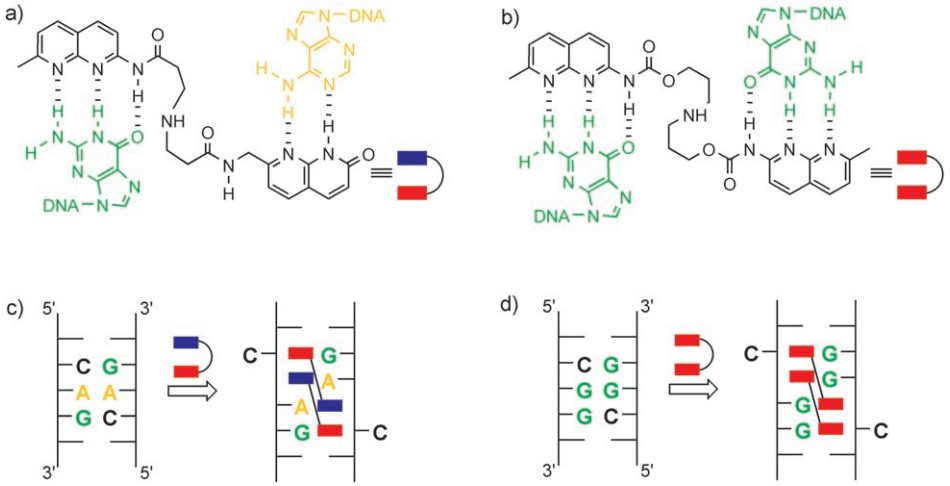


Figure 1. Hydrogen-bonding between a) **NA** and the G–A mismatch and b) **NC** and the G–G mismatch. Schematic illustrations of c) the NMR-confirmed **NA**-CAG/CAG triad complex and d) the proposed **NC** binding to the CGG/CGG triad. Red rectangles: 2-amino-1,8-naphthyridine; blue rectangles: 8-azaquinolone.

Table 1: ΔT_m [°C] and ΔT_m values [°C] for the 13-mer duplexes containing a G–G mismatch in a different flanking sequence.[a]

5′-GCTAA XGZ AATGA-3′
3′-CGATT YGW TTACT-5′

5′-XGZ-3′/5′-WGY-3′	$T_m(-)$[b]	$T_m(+)$[c]	ΔT_m[d]
CGG/CGG	34.1 (0.9)	57.2 (0.4)	23.1 (0.4)
CGC/GGG	38.6 (0.1)	49.3 (0.8)	10.7 (0.8)
GGC/GGC	40.4 (0.3)	47.1 (1.4)	6.7 (1.4)
CGA/TGG	31.8 (0.2)	44.4 (0.3)	12.6 (0.3)
CGT/AGG	33.6 (0.2)	44.0 (0.5)	10.4 (0.5)
GGA/TGC	34.2 (0.3)	43.4 (0.4)	9.2 (0.4)
AGT/AGT	28.7 (0.4)	41.9 (1.0)	13.2 (1.0)
AGG/CGT	31.8 (0.6)	39.9 (0.8)	8.1 (0.8)
GGT/AGC	33.7 (0.4)	39.3 (0.3)	5.6 (0.3)
TGA/TGA	17.8 (0.4)	35.9 (1.0)	18.1 (1.0)

[a] The UV melting curve was measured for a duplex (4.5 μM) in a sodium cacodylate buffer (10 mM, pH 7.0) containing NaCl (100 mM). The temperature was increased at a rate of 1 K min^{-1}. All measurements were made three times, and standard deviations are shown in parentheses. [b] T_m values of oligomers. [c] T_m values of oligomers in the presence of **NC** (100 μM). [d] ΔT_m was calculated as the difference between $T_m(+)$ and $T_m(-)$.

To determine the stoichiometry of the **NC**-CGG/CGG complex, cold-spray ionization time-of-flight mass spectrometry (CSI-TOF MS)[12] of the 11-mer self-complementary duplex containing the CGG/CGG triad was carried out (Figure 2). In the absence of **NC**, three ions derived from the oligomer were observed. One ion corresponded to the 3− ion of a single-stranded form ($[ss]^{3-}$; m/z: found 1118.13, calcd 1117.19). Duplexes were detected as a 5− ion ($[G–G]^{5-}$; m/z: found 1342.32, calcd 1340.83) and a 4− ion ($[G–G]^{4-}$; m/z: found 1678.07, calcd 1676.29). Upon addition of **NC** to the duplex with a 1:1 molar ratio, the intensity of the ions corresponding to $[ss]^{3-}$, $[G–G]^{5-}$, and $[G–G]^{4-}$ became weak, with the concomitant appearance of a new ion corresponding to the 5− ion of a 2:1 complex of **NC** and the duplex ($[G–G+2\,\mathbf{NC}]^{5-}$; m/z: found 1544.13, calcd 1542.13). A 1:1 complex was not detected. In the presence of two equivalents of **NC**, ions derived from the free duplex disappeared and only $[G–G+2\,\mathbf{NC}]^{5-}$ was detected. At an increased concentration of **NC**, the ion corresponding to $[G–G+2\,\mathbf{NC}]^{5-}$ was still predominant. These results clearly show that the binding of **NC** to the duplex containing the CGG/CGG triad proceeded exclusively with a 2:1 stoichiometry. The binding of **NC** to the CGG/CGG triad was further characterized by UV absorption titration experiments (see the Supporting Information). The crossover point of the Job's plot obtained from the titration was at 66%, which confirmed that the binding stoichiometry of **NC** to the CGG/CGG triad was 2:1, as determined by CSI-TOF MS (see the Supporting Information).

The high sequence preference and the 2:1 stoichiometry for **NC** binding are the same characteristic features that we observed for the binding of **NA** to the CAG/CAG triad.[7] Encouraged by these facts, we investigated whether the

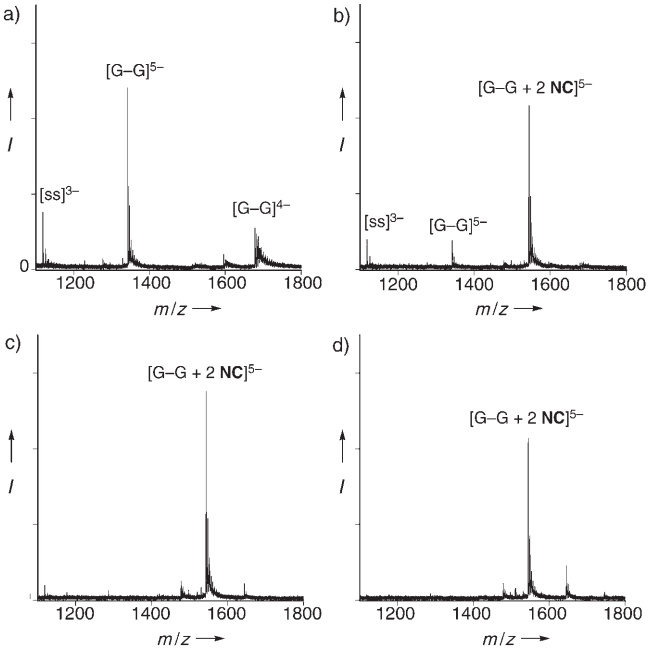


Figure 2. CSI-TOF MS of 11-mer self-complementary duplex 5′-d(TCAA CGG TTGA)-3′/5′-d(TCAA CGG TTGA)-3′ containing the CGG/CGG triad. Samples contained 20 μM duplex in 50% aqueous methanol and 100 mM ammonium acetate. The sample solution was cooled at −10°C during injection at a flow rate of 0.5 mL h^{-1}. a) Duplex only; b)–d) duplex with 20, 40, and 60 μM **NC**, respectively.

cytosine in the CGG/CGG triad was free from the G–C base pairing upon **NC** binding. We examined the reaction of **NC**-bound CGG/CGG triad with hydroxylamine. Cytosines in the single-stranded regions and in the mismatched base pairs efficiently reacted with two molecules of hydroxylamine at the C4 and C6 positions, whereas those in the G–C base pair in a duplex were not susceptible to the addition of hydroxylamine. The cytosine modified with hydroxylamine underwent degradation on heating with piperidine, eventually leading to strand cleavage.[13]

The reaction of 11-mer self-complementary 5′-d(TCAA CGG TTGA)-3′ (**1**) with hydroxylamine was monitored by reversed-phase HPLC (Figure 3). The duplex of **1** (13 μM) was not reactive toward hydroxylamine (2.8 M, pH 6.0) at 0 °C for 12 h, whereas a new product **2** was produced in the presence of **NC** (40 μM). A prolonged reaction time (≈24 h) resulted in 70% consumption of **1** without the formation of any major by-products (see the Supporting Information), which suggests that both strands of **1** duplex reacted with hydroxylamine and produced **2**. MALDI-TOF MS showed that product **2** was the adduct of **1** with two molecules of hydroxylamine (*m*/*z*: found 3406.06, calcd 3403.62; see Scheme 1 and the Supporting Information).[13] After isolation by HPLC, product **2** was treated with piperidine at 90 °C for 30 min to give products **3** and **4**. The

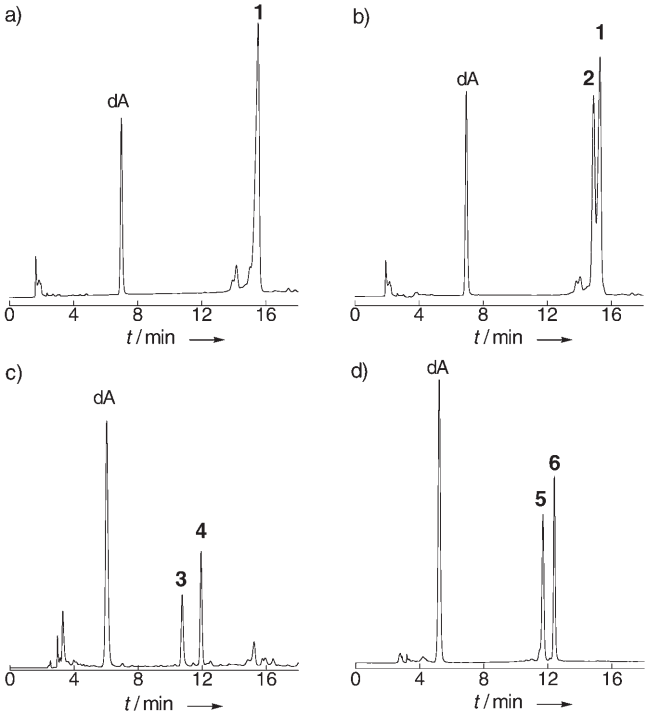
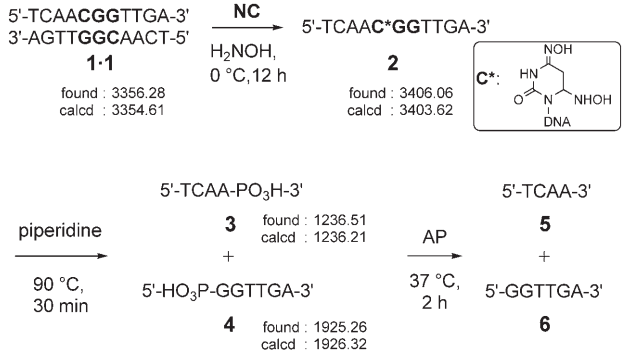


Figure 3. HPLC profiles for the hydroxylamine-induced cleavage at the cytosine moiety in the CGG/CGG triad. The 11-mer self-complementary oligomer 5′-d(TCAA CGG TTGA)-3′ (**1**) (13 μM as a duplex) in NaCl (100 mM) was treated with hydroxylamine (2.8 M, pH 6.0) in the a) absence and b) presence of **NC** (40 μM) at 0 °C for 12 h. c) Product **2** was isolated and treated with piperidine at 90 °C for 30 min. d) Products **3** and **4** were treated with alkaline phosphatase at 37 °C for 2 h. Deoxyadenosine (dA) was added as an internal standard.



Scheme 1. Hydroxylamine-induced cleavage at cytosine in the **NC**-bound CGG/CGG triad. AP = alkaline phosphatase.

MALDI-TOF mass spectra showed that **3** was the oligomer 5′-d(TCAA)-PO_3H-3′ (*m*/*z*: found 1236.51, calcd 1236.21), whereas **4** was the oligomer 5′-HO_3P-d(GGTTGA)-3′ (*m*/*z*: found 1925.26, calcd 1926.32). The phosphorylated termini of **3** and **4** were removed by treatment with alkaline phosphatase to give 5′-d(TCAA)-3′ (**5**) and 5′-d(GGTTGA)-3′ (**6**), respectively. The oligomers **5** and **6** showed the same retention times as authentic oligomers, as observed by co-injection in reversed-phase HPLC. These HPLC analyses clarified that the cytosine in the CGG/CGG triad in **1** duplex became susceptible to the hydroxylamine upon binding with **NC**. The **1** duplex contained two kinds of cytosine, one in the CGG/CGG triad and the other in 5′-TCA-3′/5′-TGA-3′. The cytosine in the latter sequence was insensitive to hydroxylamine, regardless of the presence of **NC**. These results showed that the binding of **NC** to the CGG/CGG triad made the cytosine free from the base pairing to the guanine moiety in the opposite strand.

CSI-TOF MS measurements suggested that the 2:1 **NC**-CGG/CGG complex, which was confirmed for an oligomer duplex, was also produced in the $(CGG)_n$ repeat. CSI-TOF MS of $d(CGG)_{10}$ with **NC** showed the ions corresponding to $[d(CGG)_{10}+6\mathbf{NC}]^{6-}$ and $[d(CGG)_{10}+6\mathbf{NC}]^{7-}$, which suggests the formation of the 2:1 **NC**-CGG/CGG triad complex in the $d(CGG)_{10}$ repeat (see the Supporting Information). In addition, a large conformational change of $d(CGG)_{10}$ was observed in CD measurements upon binding with **NC** (see the Supporting Information), which supports the formation of a hairpin structure, as observed in the binding of **NA** to $d(CAG)_{10}$.[7]

In summary, the data presented herein have shown that 1) **NC** is the first molecule to bind to the CGG/CGG triad, and 2) the cytosine in the triad is free from hydrogen bonding to the guanine moiety upon **NC** binding. It is suggested that the 2:1 **NC**-CGG/CGG complex is produced in the hairpin structure of the $d(CGG)_n$ repeat, which is proposed to form in the replication leading to the repeat expansion. From the structural viewpoint, the **NC**-bound CGG repeats are particularly interesting. Base flipping as a result of the disruption of base pairing is one of the mechanisms for the repair enzyme to recognize the damaged bases and mismatched base pairs.[14–17] Therefore, **NC** could be a useful molecule for studying not only the repeat expansion mechanism, but also

the nucleobase–ligand interactions on the biologically important repeat sequence.

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