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The heterodimer of 2-amino-1,8-naphthyridine and 3-aminoisoquinoline binds to the CTG/CTG triad via hydrogen bonding

Shuhei Sakurabayashi, Takeshi Yamada, Kazuhiko Nakatani *

Department of Regulatory Bioorganic Chemistry, The Institute of Scientific and Industrial Research (SANKEN), Osaka University, 8-1 Mihogaoka, Ibaraki 567-0047, Japan

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ABSTRACT

Myotonic dystrophy type 1 (DM1) is caused by the aberrant expansion of CTG repeats within the DMPK gene. This study investigated the potential binding of "X-linker-Y" type molecules to the CTG/CTG motif present in CTG repeats, using heterocyclic units X and Y capable of forming complementary hydrogen bonds with nucleobases. Among the tested molecules, the heterodimer of 2-amino-1,8-naphthyridine (X) and 3-aminoisoquinoline (Y) showed significant binding to the CTG/CTG motif. NMR analysis suggested hydrogen-bonded interactions between 3-aminoisoquinoline and thymine.

Molecules that recognize specific sequences or structural motifs of DNA have been considered as potential therapeutics and molecular probes in chemical biology research.[1,2] Oligonucleotide therapeutics are one of the important classes of molecules targeting nucleic acids with high sequence selectivity, but the issues related to intracellular transport and toxicity remain to be addressed. Small molecules that show superior drug transport properties to oligonucleotides draw recent attention as a new modality treating nucleic acids, but the relatively low sequence selectivity for the targets compared to oligonucleotides is a key issue to realize small molecule drugs targeting nucleic acids.

To date, our group has developed small molecules that selectively bind to repeat DNA sequences involved in neurological disorders. The molecule **NA** consisting of two heterocycles 2-acylamino-1,8-naphthyridine (NP) and 8-azaquinolone (AQ) connected by a linker binds to the CAG/CAG triad in the hairpin secondary structure of the CAG repeat.[3,4] Aberrant expansion of the CAG repeat in the *HTT* gene causes Huntington's disease.[5,6] The molecule **NCD**, a homodimer of NP, binds to the CGG/CGG triad in the expanded CGG repeat DNA,[7–9] of which aberrant expansion in the *FMR1* gene causes Fragile X syndrome.[5,6] The heterocycles composing these ligands can form complementary hydrogen bonds (H-bonds) with nucleotide bases; e.g., NP forms three hydrogen bonds with guanine, and AQ does two hydrogen bonds with adenine, allowing these molecules to recognize nucleobase via Watson-Crick (WC) type base pairing. The formation of the hydrogen-bonded complex of **NA** with CAG/CAG and **NCD** with CGG/CGG has been confirmed by NMR spectroscopic analysis.[4,9] Furthermore, **NCD** binding to the three contiguous CGG/CGG triads was supported by NMR analysis using ^{15}N-labeled **NCD**.[10] Remarkably, the **NA** induced repeat contraction of the expanded CAG repeat DNA in a striatum of HD mouse model,[11] indicating the potential of repeat DNA binding small molecules as drugs for the radical treatment of repeat expansion diseases.

Despite the success of the recognition of guanine and adenine by NP and AQ, respectively, the development of molecules targeting the CTG/CTG triad found in the hairpin structure of aberrantly expanded CTG repeats causing myotonic dystrophy type 1 (DM1),[12–14] remain challenging.[15–18] (Fig. 1A) This difficulty is mainly due to the low affinity in the binding to The A-D-A/D-A-D type hydrogen bond, which suffers from secondary electrostatic repulsions,[19] and is not energetically advantageous compared with the A-D/D-A type hydrogen bonding. We have studied several expeditious molecules that were designed to target the T:T mismatch in the CTG/CTG triad by optimizing the hydrogen-bonding face with thymine, but some molecules did not show the expected affinity or others bound to not only CTG/CTG but also CCG/CCG triads.[17] Here, we report our alternative approach in targeting the CTG/CTG triad using a combination of WC-type hydrogen bonding and stacking interactions with the adjacent bases resulting in the development of a new molecule **iQN**.

Since the molecular motif (X-linker-Y) consisting of two heterocycles connected by a linker worked well for the recognition of the CAG/CAG and CGG/CGG triads, we designed molecules for the recognition of the CTG/CTG triad using NP as a heterocycle for G-recognition and 3-acylamino-isoquinoline (iQ), 2-acylamino-pyridine (Py), and 2-acylamino-

* Corresponding author.
E-mail address: nakatani@sanken.osaka-u.ac.jp (K. Nakatani).

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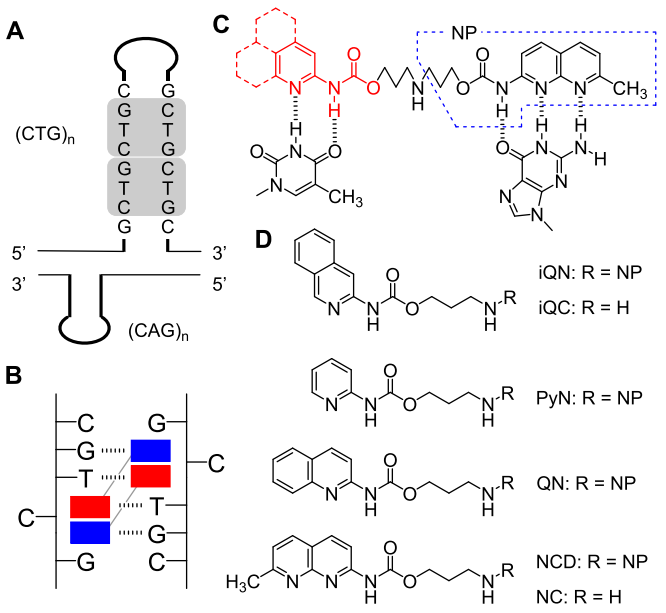

Fig. 1. (A) Illustration of the slip-out hairpin structure of $d(CTG)_n/d(CAG)_n$ triads in a double-stranded DNA. (B) Hypothetical mode of small molecule binding to the CTG/CTG site based on our previous results. (C) Feasible hydrogen-bonding patterns between guanine and 2-acylamino-1,8-naphthyride (NP), and thymine and ring-expanded 2-acylamino-pyridine derivatives. (D) Chemical structures of six molecules used in this study.

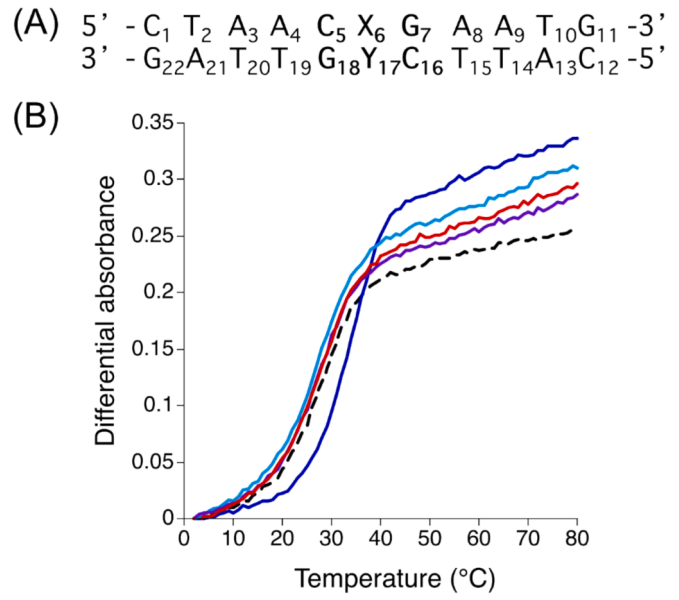

Fig. 2. (A) DNA sequence and numbering used in the experiment. The residues represented by X and Y correspond to Adenine, Thymine, Guanine, or Cytosine. (B) UV-melting profiles of the 5 μM DNA containing CTG/CTG triad (X = Y = Thymine in Fig. 2A) in the presence or absence of 50 μM ligand in 10 mM sodium phosphate buffer (pH 7.0) and 100 mM NaCl. Key: DNA only (black dotted line, entry 1 in Table 1); DNA with **iQN** (blue, entry 2), **PyN** (purple, entry 3), **NCD** (light blue, entry 4), and **QN** (red, entry 5).

quinoline (Q) for T-recognition to provide **iQN**, **PyN**, and **QN**, respectively (Fig. 1B–D). The synthesis of **QN** was previously reported,[20] so we newly prepared **iQN** and **PyN** (Scheme S1). Two reference molecules having partial **iQN** structure (**iQC** and **NC**, Fig. 1D) were also prepared (Scheme S1). Since the complementary H-bond between NP and guanine was validated by previous NMR studies (Fig. 1C right), we investigated whether the iQ, Py, and Q moieties, having a donor–acceptor (D-A) type hydrogen bonding surface, can form a hydrogen bonding pair with thymine (Fig. 1C left). The hydrogen bonding energy of 2-acylaminopyridine and thymine was estimated to be –15.31 kcal/mol by QM calculations (geometry optimization: X3LYP/6-31G**, energy calculation: LMP2/cc-pVTZ(−f) and cc-pVQZ(−g)) using the Jaguar module in Maestro (SCHRÖDINGER Suite)[21] and found to be compatible with –14.69 kcal/mol of the adenine–thymine pair (Fig. S1A, B). We also used **NCD** as a candidate molecule as NP has a complementary hydrogen bonding surface with thymine as well. The binding of these molecules was studied by thermal melting (T_m), circular dichroism (CD) spectrum, isothermal titration calorimetry (ITC), and NMR measurements.

The binding ability of the prepared molecules toward CTG/CTG was studied by measuring the T_m of a double-stranded DNA (dsDNA) containing a CTG/CTG triad. The DNA sequence used in the experiments is shown in Fig. 2A (X = Y = thymine, hereafter **TT1**). The difference of T_m values (ΔT_m) in the absence and presence of the ligand is summarized in Table 1. In the presence of 50 μM **iQN**, the T_m value was increased by 5.8 °C (Fig. 2B blue line, Table 1 entry 2), while **PyN**, **QN**, and **NCD** showed no significant increase in the T_m values (Fig. 2B, Table 1 entries 3, 4, and 5). **NC** and **iQC**, which have the partial structure of **iQN**, showed also negligible increase in T_m values (Table 1 entries 6 and 7). Summarizing these results, **iQN** was the only molecule showing a significant increase in T_m value of **TT1** among the compounds we investigated, indicating that both **NC** and **iQC** moieties in **iQN** are likely to be indispensable for the binding to the CTG/CTG triad. Next, T_m measurements of DNA duplexes containing three CXG/CYG triads (X = Y = adenine, guanine, and cytosine) were performed in the presence of 50 μM **iQN**. The ΔT_m values were 2.8 °C for CAG/CAG, (Table 2 entry 1), 3.9 °C for CGG/CGG (Table 2 entry 2), 2.5 °C for CCG/CCG (Table 2 entry 3). For fully-matched CCG/CGG and CAG/CTG, the ΔT_m values were 0.0 °C and −0.2 °C (Table 2 entry4 and entry 5). Considering that the T_m value of CGG/CGG in the absence of ligands (28.2 °C) is slightly higher than that of CTG/CTG (27.8 °C), the 3.9 °C of the T_m increase for CGG/CGG suggests that **iQN** binds to the CGG/CGG motif to a similar extent as to CTG/CTG. It has been reported that NA, which contains NP

Table 1
T_m values for the **TT1** in the absence or presence of compounds. UV-melting temperatures for the 5 μM DNA in the absence or the presence of each compound were measured in 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. All measurements were made three times, and standard deviations (SDs) are shown in the parentheses.

Entry	molecule	T_m (°C)	ΔT_m (°C)
1	–	27.8 (0.3)	–
2	**iQN**	33.6 (0.0)	5.8
3	**PyN**	27.8 (0.0)	0.0
4	**NCD**	27.9 (0.1)	0.1
5	**QN**	27.7 (0.3)	–0.1
6	**iQC**	28.1 (0.1)	0.3
7	**NC**	28.0 (0.1)	0.2

Table 2
T_m values for the dsDNA (sequence is shown in Figure 1A) in the absence or presence of **iQN**. UV-melting temperatures for the 5 μM DNA in the absence or the presence of **iQN** were measured in 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. All measurements were made three times, and standard deviations (SDs) are shown in the parentheses. (a) For entry 6, dsDNA having TTT-ATA motif instead of CXG-CYG motif was used.

Entry	X-Y	T_m (°C) (–iQN)	T_m (°C) (+iQN)	ΔT_m (°C)
1	A-A	21.8 (0.2)	24.6 (0.1)	2.8
2	G-G	28.2 (0.3)	32.1 (0.1)	3.9
3	C-C	21.6 (0.2)	24.1 (0.0)	2.5
4	C-G	44.8 (0.5)	44.8 (0.1)	0.0
5	A-T	38.5 (0.4)	38.2 (0.2)	–0.2
6	T-T[(a)]	16.6 (0.2)	20.1 (0.3)	3.5

moiety, binds to G:G mismatches to some extent. Similarly, **iQN** contains NP moiety, so it can bind to the CGG/CGG motif. Furthermore, since iQ (H-bond surface: D-A) can form hydrogen bonds with guanine (H-bond surface: A-D-D), the formation of G:iQ base pairs may contribute to the binding of **iQN** to the CGG/CGG motif. The lower ΔT_m values observed for CAG/CAG, CCG/CCG, and fully-matched CCG/CGG and CAG/CTG compared to those of CTG/CTG and CGG/CGG indicate that **iQN** preferentially binds to dsDNA containing CTG/CTG or CGG/CGG motifs rather than CAG/CAG, CCG/CCG, and fully-matched dsDNA. To investigate the effect of guanines adjacent to T:T mismatches, we also measured the T_m of dsDNA containing the TTT/ATA motif. **iQN** increased its T_m value by 3.5 °C, suggesting that **iQN** moderately binds to T:T mismatches having no adjacent guanines. However, Considering that the T_m value of TTT/ATA in the absence of ligand was much lower than CTG/CTG, **iQN** binds more favorably to T:T mismatches with adjacent guanines, such as CTG/CTG than other to T:T mismatches.

iQN binding was also confirmed by CD spectrum and ITC measurements. The UV–Vis spectrum of **iQN** and CD spectrum of CXG/CXG triad in the absence or presence of **iQN** is shown in Fig. S2. Induced CD bands were observed at 238 nm and 340 nm (Red arrows in Fig. S2B), suggesting that non-chiral small molecule **iQN** was incorporated into the chiral environment of the DNA duplex. The induced CD bands were observed at the red-shifted absorption range of **iQN**, indicating that heterocycles of **iQN** is likely stacked within the duplex DNA. Other DNAs did not show any induced CD band (Fig. S2B), supporting the superior binding of **iQN** to CTG/CTG motif over other CXG/CXG motifs including. While T_m measurements did not clearly show the binding preference between CTG/CTG and CGG/CGG motifs, CD measurements supported the superior binding of **iQN** to the CTG/CTG motif to the CGG/CGG. The reason why **iQN** did not show induced CD for sequences other than the CTG triad can be attributed to (1) the weak binding of **iQN** to sequences other than CTG/CTG and (2) the lack of site-specificity in **iQN** binding. It is likely that the binding is based on hydrophobic interactions arising from the high hydrophobicity of the isoquinoline moiety, rather than hydrogen bonding. As a result, the structure of the complex did not converge uniformly, not showing the apparent induced CD bands in other CXG triad but for CTG triad. We then measured CD spectra of d(CTG)$_9$ (5 μM) in the absence and presence of **iQN** to confirm the binding ability of **iQN** to the CTG repeat (Fig. S3). As in the case of the CTG/CTG triad, the induced CD band was observed in a concentration-dependent manner. These results demonstrate that **iQN** can bind to the CTG repeat, as well as the CTG/CTG triad. While it is ambiguous whether the binding to the CTG repeats exhibits positive or negative cooperativity, repeat binding molecules typically show negative cooperativity when interacting with consecutive repeat units,[10,15] as expected from the nearest-neighbor exclusion principle. Therefore, when **iQN** binds to CTG repeats, it is expected to skip one or more repeat units, rather than bind in a consecutive manner.

Isothermal titration calorimetry (ITC) measurements were performed to investigate the thermodynamic aspects of **iQN** binding to the CTG/CTG motif. The generated heat by the titration of **iQN** to the **TT1** is shown in Fig. S4A. The integrated heat plot showed one sigmoidal curve and was fitted with a one-set-of-site model, providing the apparent K_D value of 1.9 μM (SD: 0.1) with ΔG of –7.8 kcal/mol (SD: 0.0), ΔH of –37.8 kcal/mol (SD: 3.3), and $-T\Delta S$ of +29.2 kcal/mol at 25 °C (SD: 1.0). The binding stoichiometry calculated by the ITC data was 1.90 (SD: 0.06), showing that two **iQN** molecules bind to one CTG/CTG motif. The significant exothermic heat upon the titration of **iQN** to the CTG/CTG triad indicates the enthalpy-driven binding of **iQN**, supporting the formation of hydrogen bonds between **TT1** and **iQN**. Note that guanine, which has an A-D-D type hydrogen bonding surface, could form two hydrogen bonds with iQ (D-A) as well as thymine (D-A-D), but the binding of **iQN** to the CGG/CGG triad produced no significant heat in the ITC measurements (Fig. S4B), again supporting the selectivity.

We then analyzed the binding of **iQN** by NMR spectroscopy. First, the 1D ^{1}H NMR titration experiment was performed to validate the **iQN** binding to the CTG/CTG motif (Fig. 3). The assignment of free **TT1** imino protons (shown as [**TT1**]:[**iQN**] = 1:0, Fig. 3) is conducted based on the 2D NOESY spectra measured in 90 % H_2O and 10 % D_2O at 20 °C (Fig. 4, black). Three types of imino protons were observed: (1) thymine imino protons in WC A:T base pairs (13–14 ppm), (2) guanine imino protons in WC G:C base pairs (12–13 ppm), and (3) thymine imino protons in a T:T mismatch (10–11 ppm). It is known that the imino proton signal of non-canonical base pairs, including T:T mismatch, appears at 10 to 12 ppm.[22] Imino protons in terminal guanines (G11, G22: marked by stars in 1:0) and a T:T mismatch (T6 and T17) could not be distinguished due to insufficient NOEs with other residues. Upon the addition of **iQN**, new resonances (Fig. 3, marked by blue dotted lines) appeared with concomitant decreased intensities of the free **TT1** signals (Fig. 3, marked by red dotted lines). A small change in their chemical shifts and line shape upon **iQN** addition indicates that **iQN** binding is in a slow to intermediate exchange regime with free **TT1** within the NMR timescale. The changes in signal intensities are almost saturated when two equivalents of **iQN** are added to **TT1**, consistent with the binding stoichiometry of 1:2 suggested by ITC results. Measurements of the complex in 100 % D_2O confirmed that all signals in this region (10–14 ppm) are derived from exchangeable protons (Fig. S5). The multiple signals in the 10–12 ppm range, typically associated with imino protons of pseudo-WC base pairs like G:NP, and A:AQ,[4,9] suggest the formation of H-bonds between NP and guanine, and iQ and thymine. The strong NOEs observed for these signals in the NOESY spectra (Fig. 4, red) indicate the proximity of H-bond donors, further validating H-bond formations between the nucleobases and the ligand.

We then measured TOCSY spectra to investigate the H5 and H6 chemical shifts of cytosines (Fig. 5). We can selectively monitor cytosine aromatic protons in TOCSY spectra because only cytosine has J-coupled aromatic protons (H5 and H6) among four DNA bases. In the absence of **iQN**, **TT1** showed four peaks corresponding to C1, C5, C12, and C16 residues (Fig. 5, black). The H5 and H6 chemical shifts of these cytosines showed good accordance with canonical B-form DNA.[23] Upon the addition of **iQN**, the C5 and C16 peaks disappeared from the canonical region and appeared in the lower field region, while the chemical shifts of terminal C1 and C12 remained almost unchanged. (Fig. 5, red). This kind of downfield shift of cytosine is commonly observed upon exposure to aqueous solvents, i.e., flip-out of the base from the stacked base-paired region in the binding of **NA** to the CAG/CAG and **NCD** to the

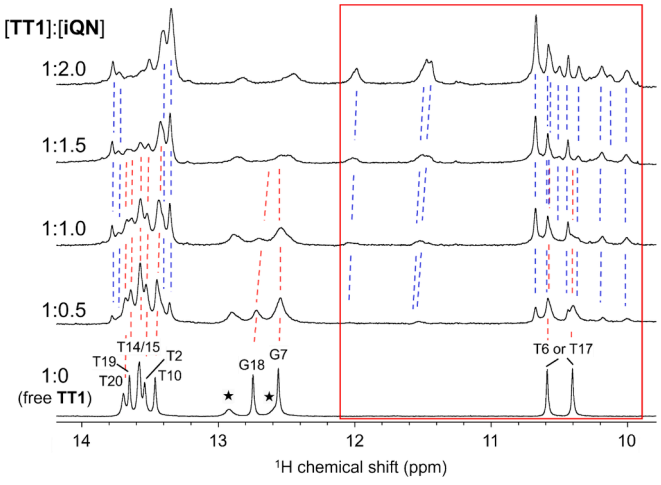

Fig. 3. One-dimensional imino proton spectra of dsDNA containing CTG/CTG triad (**TT1**, X = Y = thymine in Fig. 2A, 200 μM) in the presence of **iQN** with various dsDNA-**iQN** ratios measured at 298 K. The ratio of **TT1** and **iQN** is shown on the left. The assignment of free **TT1** is described in the spectra. Star represents the terminal guanine protons (G11 and G22). The typical chemical shift of the imino protons observed in the ligand-nucleobase base pair is indicated by a red square.

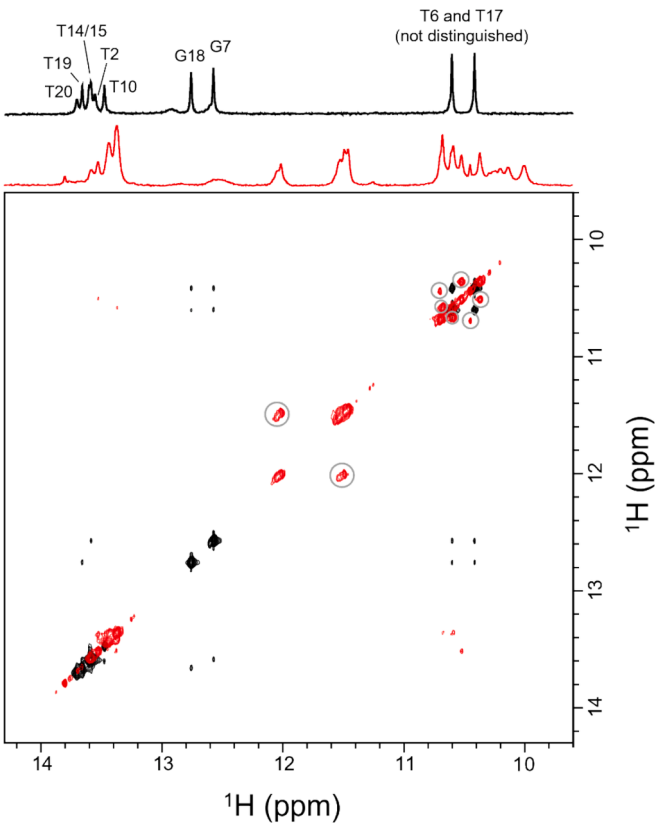

Fig. 4. 2D ^{1}H–^{1}H NOESY spectra of **TT1** in the absence (black) and presence (red) of **iQN**. The spectra were measured with 90 %/10 % of H_2O/D_2O at 298 K with a 700 MHz spectrometer. The Imino proton region is shown expanded here. Strong NOE peaks observed in the **TT1**-**iQN** complex were marked by grey circles.

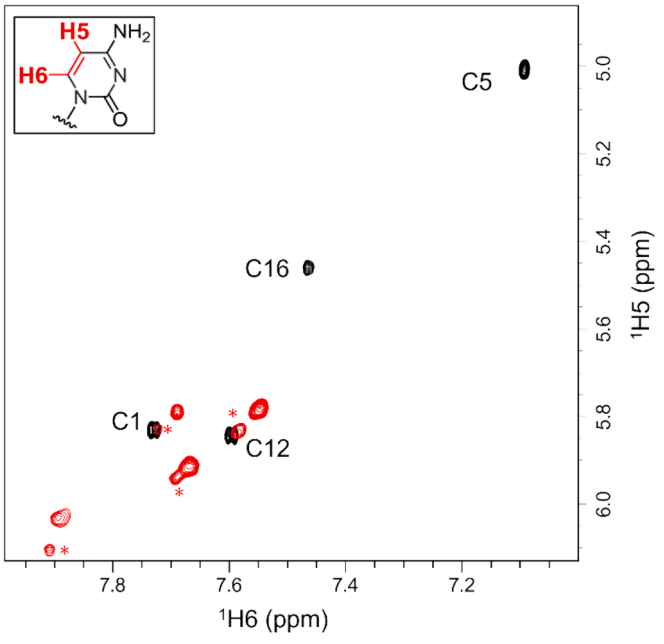

Fig. 5. 2D ^{1}H–^{1}H TOCSY spectra of **TT1** in the absence (black) and presence (red) of **iQN**. The expanded views of the cytosine H5 (vertical) and H6 (horizontal) regions are shown. The spectra were measured with the same condition as Figure 4. The minor peaks observed in the **TT1**-**iQN** complex were marked by red asterisks.

CGG/CGG sequences,[4,9] suggesting that **iQN** binding is also considered to involve a flip-out of C in the CTG/CTG motif. In the TOCSY spectrum, in the presence of **iQN**, a subset of lower intensity peaks was observed near the four major cytosine peaks (shown by asterisks), indicating the presence of a minor ligand-bound conformation. Judging from the peak intensity and chemical shifts, the minor conformation exists approximately 30 % and are close in the structure with the major conformation. Thus, there are at least two ways of binding **iQN** to the CTG/CTG motif. This structural polymorphism hampered signal assignments and further structural determination, but all of the above NMR analysis supports that **iQN** binds to CTG/CTG motif in the manner we hypothesized in Fig. 1B.

Since the thymine recognition units of iQ, Py, and Q, involved in **iQN**, **PyN**, and **QN** share the same hydrogen bonding surface, the difference in the binding of these molecules can be attributed to the additional interaction, which is likely the stacking of the aromatic ring on 2-acylaminopyridine within the bound complex. Fig. S6 illustrates the WC base pairs, the G:NP base pair, and the hypothetical T:iQ, T:Q, and G:iQ base pairs. In thymine-ligand base pairing, the T:iQ base pair is similar to the A:T base pair but features a slightly more extended aromatic ring geometry. Consequently, the stacking interactions with neighboring bases are expected to be stronger than those of the A:T base pair. On the other hand, in the T:Q base pair, where an additional aromatic ring protrudes into the minor groove, stacking interactions are expected to be weaker than the T:iQ base pair. For guanine-ligand base paring, since the G:NP base pair occupies a larger area of the base pair plane than the G:C base pair, strong stacking interactions with the flanking base pairs can be realized. While iQ can assumingly form hydrogen bonds with guanine, but the additional aromatic ring in iQ is expected to cause a steric repulsion with a phosphate backbone. These analyses are supported by the negligible heat generation observed in the ITC experiment of CGG/CGG with **iQN** (Fig. S4B).

In conclusion, we have developed a new CTG/CTG motif binding small molecule **iQN**, a heterodimer of naphthyridine and isoquinoline. T_m studies revealed that both of Np and iQ moiety are required for the binding to the CTG/CTG motif. ITC profiles of **iQN** indicate that the binding toward the CTG/CTG triad is driven by enthalpic factors with an apparent K_D value of 1.9 μM. Also, NMR experiments confirmed the binding of **iQN** recognizes CTG/CTG through hydrogen bonding between nucleobase and ligand, resulting in a flip-out of cytosines. All results suggest that **iQN** recognizes the CTG/CTG motif with hydrogen bonds. Recently, we reported that the linker length of **NCD** can affect the binding efficiency to the CGG/CGG motif.[24] These findings may implicate that the binding affinity and selectivity of **iQN** could be improved by modifying the linker structure in addition to the heterocycle itself to eventually obtain lead compounds for targeting DM1.

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CRediT authorship contribution statement

Shuhei Sakurabayashi: Writing – original draft, Investigation. **Takeshi Yamada:** Writing – review & editing, Investigation. **Kazuhiko Nakatani:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2024.129985.

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