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Assessment of the Sequence Dependency for the Binding of 2-Aminonaphthyridine to the Guanine Bulge

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Abstract—We have studied the sequence dependent binding of 2-amino-1,8-naphthyridine derivative **1** to a single guanine bulge. The free energy changes for the binding to a guanine bulge with different sequence contexts (5′X_Y3′/3′X′GY′5′) were determined by a curve fitting of the thermal denaturation profile of DNA in the presence and absence of **1**. The data showed that (i) the binding of **1** to a guanine bulge is stronger for those flanking the G–C base pair than A–T base pair, (ii) the guanine 3′ side to **1** in the complex is especially effective for the complex stabilization, and (iii) the increase of T_m in the presence of **1** is not a good estimate for the sequence dependent binding. The most efficient **1**-binding was observed for the sequence of G_G/CGC. Molecular modeling simulations suggested that stacking interaction between the 3′ side guanine and **1** is the molecular basis for the strong binding to G_G/CGC.

Introduction

A bulge is the site where one or more extra nucleotide bases remain unpaired in the duplex of DNA and RNA.[1–3] The bulged site in DNA is not only a crucial structural element for the recognition by DNA repair proteins,[4,5] but also exhibits a remarkable reactivity toward the drug interactions. Ethidium bromide[6] and other intercalators,[7–10] intercalate into the site of a nucleotide bulge more strongly than does into the standard Watson–Crick base pairs. Thermodynamic stabilization of a nucleotide bulge is thought to be a molecular basis of the mutagenic properties of many intercalating agents.[11] One-electron oxidation of duplex DNA by external oxidants proceeds preferentially at a guanine bulge compared with the normal guanine in a G–C base pair.[12–14] Covalent modification of guanine by a DNA alkylating intercalator occurred selectively near the bulge possibly due to a preferential drug binding to a bulged site.[15] Besides these biological aspects, the site of a nucleotide bulge provides an important clue for the mutation detection with small molecular probes.[16,17]

We have recently demonstrated that DNA intercalators possessing hydrogen-bonding groups fully complementary to those of nucleotide bases are a unique class of compounds that bind to a bulge structure and, hence, stabilize the duplex with high base selectivity.[12,16,18–20] 2-Amino-1,8-naphthyridine derivative **1** having an alignment of hydrogen bonding groups in a donor–donor–acceptor orientation selectively binds to a guanine bulge in duplex DNA (Fig. 1a). In contrast to ethidium bromide,[6] **1** strongly stabilized a guanine bulge duplex, but only weakly did other bulges and a fully matched duplex.[12] We have evaluated the drug binding by thermal denaturation of a guanine bulge-containing duplex. Thus, the melting temperature (T_m) of the duplexes increased with increasing the degree of **1** binding. Structure–activity relationship (SAR) studies for **1** binding to a guanine bulge showed that (1) an alignment of hydrogen bonding groups fully complementary to that of a guanine is essential for a strong binding and (2) at least two condensed aromatic rings are necessary for the efficient binding. These observations suggest that stacking of the hydrogen-bonded pair produced from **1** and a bulged guanine with the flanking base pairs significantly contributes in energy to stabilize the complex. We here report the overall free energy change for the 1-binding to the guanine bulge with

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different flanking sequences. The data showed that the binding of **1** to a guanine bulge was the most efficient for the sequence of G_G/CGC, where the bound **1** was stacked by both 5′ and 3′ side guanine in the complex. Furthermore, the 3′ side guanine to the intercalator in the complex is especially effective for the stabilization of the complex.

Results and Discussion

Sequence Dependence for the stabilization of a guanine bulge by 1

The sequence dependence for the stabilization of a guanine bulge by **1** binding was examined for the duplexes with ten different flanking sequences (Fig. 1b). These duplexes are shown by a general formula of 5′X_Y3′/3′X′GY′5′ (Table 1). For the duplex containing cytosine in the position of X and/or Y (e.g., C_G/GGC, C_C/GGG, and G_C/CGG), the site of a guanine bulge can not be defined due to an unavoidable migration of the bulge immediately next to the designated position (e.g., C_G/GGC↔_CG/GGC). Therefore, the data for these sequences are average of all possible bulge structures. In the study reported here, naphthyridine derivative **1** possessing two-methylene linker was used. This derivative showed an improved thermal stability and stronger binding compared to the three methylene derivative reported previously.[12] All of the thermal denaturation curves of guanine bulge-containing duplexes showed a sigmoidal shape (Fig. 2). Melting temperatures of these duplexes were determined as the first derivative of the curve (Table 2). As expected, thermal stability of the guanine bulge-containing duplexes strongly depends on the base pair flanking the guanine bulge.[21] Oligomers having G–C and/or C–G base pairs at the both 5′ and 3′ side of a guanine bulge showed a pronounced stability compared with those having at least one A-T base pair flanking to a guanine bulge. The lowest T_m of 17.6 °C under the conditions was observed for oligomer A_A/TGT, where the bulged guanine was located between two thymine bases.

In the presence of **1**, the denaturation curves of the guanine bulge-containing duplexes were shifted to a higher temperature range with keeping the sigmoidal shape unchanged. Increase of T_m (ΔT_m) was 11.8 and 11.2 °C for G_G/CGC and A_G/TGC, respectively, whereas ΔT_m obtained for other oligomers was about in the same range (6.7–8.3 °C). Thus, a sequence dependence of the stabilization of a guanine bulge by **1** could not be clearly deduced form the analysis of ΔT_m.

Estimation of the free energy change

Since the binding of naphthyridine to the guanine bulge was intrinsically weak ($\sim 10^4$ M^{-1}), isothermal titration calorimetry and other techniques determining an accurate free energy change for the binding are not applicable to the **1** binding to the G bulge. We have estimated the free energy change for the binding of the naphthyridine to the G bulge by subtracting the free energy change for the formation of bulge-containing duplex from the overall free energy change obtained in the presence of the drug and two single stranded DNAs. Free energy changes for each process were obtained by nonlinear least-square method to fit the thermal denaturation curves to a two-state model with ΔH, ΔS, and extinction coefficients of single and double stranded

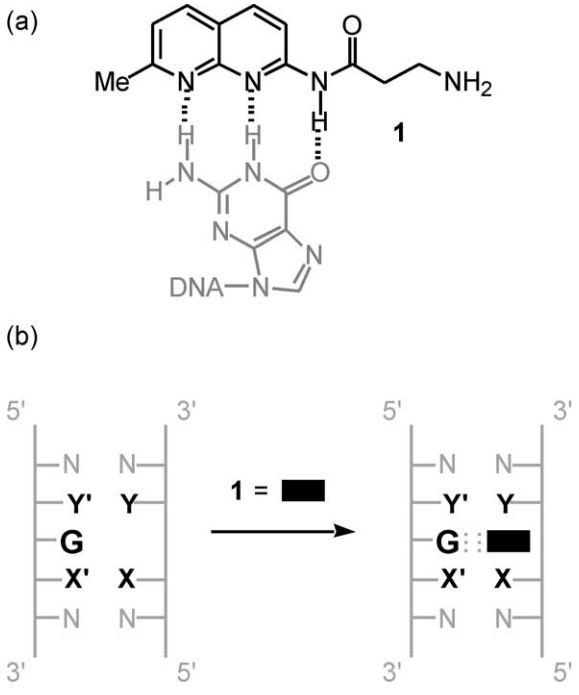

Figure 1. (a) An illustration of a hydrogen bonding pair of **1** with guanine. (b) A schematic representation of the binding of **1** to a guanine-bulge containing duplex in a sequence of 5′X_Y3′/3′X′GY′5′.

Table 1. Oligomers used in the studies

A_A/TGT:	5′-TCCA **A_A** CAAC-3′ 3′-AGGT **TGT** GTTG-5′
A_G/TGC:	5′-TCCA **A_G** CAAC-3′ 3′-AGGT **TGC** GTTG-5′
C_C/GGG:	5′-TCCA **C_C** GAAC-3′ 3′-AGGT **GGG** CTTG-5′
C_G/GGC:	5′-TCCA **C_G** CAAC-3′ 3′-AGGT **GGC** GTTG-5′
G_A/CGT:	5′-TCCA **G_A** CAAC-3′ 3′-AGGT **CGT** GTTG-5′
G_C/CGG:	5′-TCCA **G_C** GAAC-3′ 3′-AGGT **CGG** CTTG-5′
G_G/CGC:	5′-TCCA **G_G** CAAC-3′ 3′-AGGT **CGC** GTTG-5′
G_T/CGA:	5′-TCCA **G_T** CAAC-3′ 3′-AGGT **CGA** GTTG-5′
T_G/AGC:	5′-TCCA **T_G** CAAC-3′ 3′-AGGT **AGC** GTTG-5′
T_T/AGA:	5′-TCCA **T_T** CAAC-3′ 3′-AGGT **AGA** GTTG-5′

DNAs as variable parameters.[22,23] Since the binding of naphthyridine to the G bulge is concentration dependent, the above estimated free energy is a function of the fraction of the drug–DNA complex in the total DNA and drug concentrations. To obtain the actual free energy change for the binding of naphthyridine to the G bulge, the fraction of the complex under the given drug concentration should be determined.

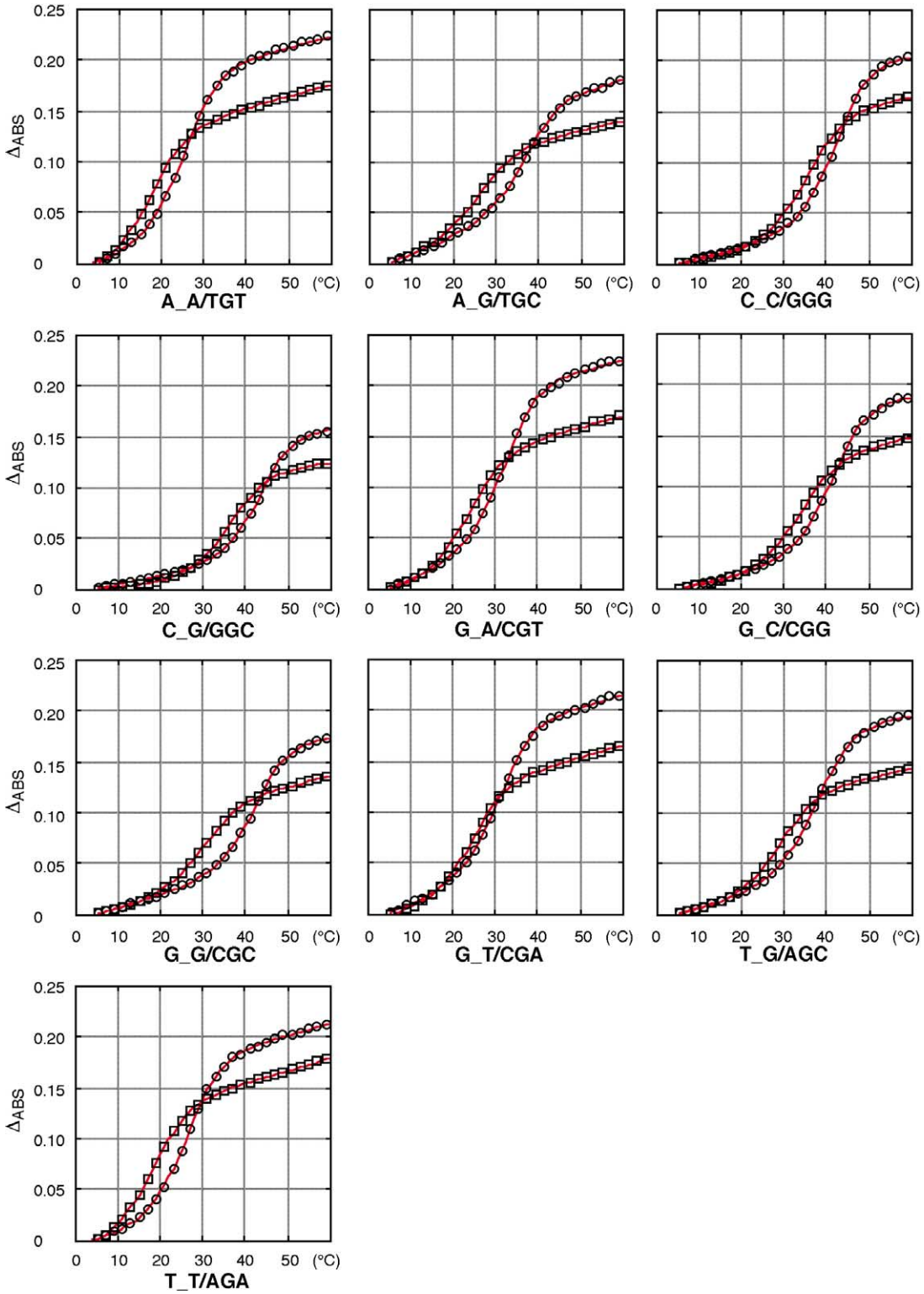

Figure 2. Thermal denaturation profiles of guanine bulge-containing duplexes (4.77 μM) in the absence (open square) and presence (open circle) of **1** (200 μM). The changes of the absorbance (Δ_{ABS}) defined as $Abs_{(T°C)}-Abs_{(4°C)}$, were plotted against temperature. The absorbance at 260 nm was measured in 10 mM sodium cacodylate buffer (pH 7.0) containing 0.1 M NaCl. Temperature was increased from 4 to 70 °C at a rate of 1 °C/min. The absorbance was measured with an interval of 1 °C. Measurements were carried out at least three times. The average of three data sets of denaturation profiles was used for the plots. The calculated curves obtained for each measured profile were shown with a solid red line. Data points are shown in every 2 °C between 4 to 60 °C for clarity.

Table 2. Melting temperature of guanine bulge-containing duplexes in the absence and presence of **1**

Duplex	T_m (−)[a,b]	T_m (+)[a,b]	ΔT_m[c]
A_A/TGT	17.6±0.1	25.3±0.2	7.7
A_G/TGC	25.6±0.2	36.8±0.0	11.2
C_C/GGG	35.3±0.1	42.1±0.2	6.8
C_G/GGC	36.5±0.1	43.2±0.1	6.7
G_A/CGT	23.9±0.0	31.7±0.1	7.8
G_C/CGG	34.0±0.4	41.1±0.4	7.1
G_G/CGC	30.1±0.3	41.9±0.1	11.8
G_T/CGA	24.4±0.2	30.1±0.1	6.7
T_G/AGC	28.9±0.1	37.2±0.2	8.3
T_T/AGA	18.3±0.2	26.3±0.1	8.0

[a] $T_m(-)$ and $T_m(+)$ are melting temperatures (°C) of a duplex (4.77 μM) in the absence and presence of **1** (200 μM), respectively, in 10 mM sodium cacodylate buffer (pH 7.0) containing 0.1 M NaCl. Temperature was increased at a rate of 1 °C/min.
[b] Measurement for each duplex was carried out at least three times. The average of three denaturation profiles was analyzed by a curve fitting.
[c] $\Delta T_m = T_m(+) - T_m(-)$.

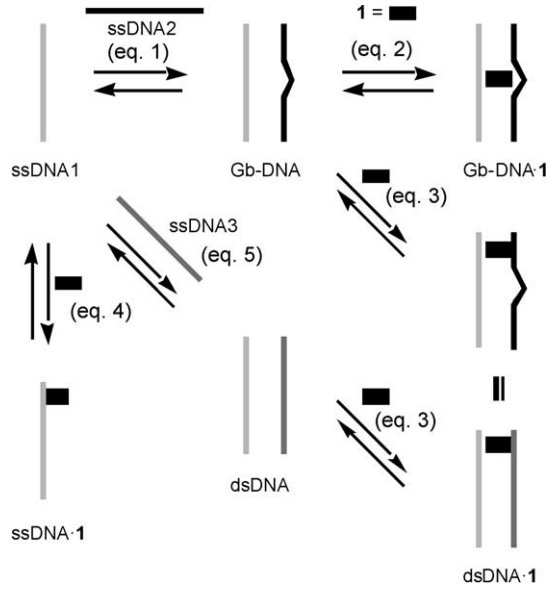

Figure 3. Illustrations of the binding of **1** to a single stranded DNA (ssDNA), a fully matched double stranded DNA (dsDNA), and a guanine bulge-containing duplex (Gb-DNA). Hybridizations of ssDNA1 with ssDNA2 and ssDNA1 with ssDNA3 produces Gb-DNA and dsDNA, respectively. ssDNA·**1** represents the complex of **1** with ssDNA1, ssDNA2, and ssDNA3.

We considered five equilibriums for the thermodynamic analysis of **1** binding to a guanine bulge (Fig. 3). These include (i) a formation of DNA containing a guanine bulge (Gb-DNA) from two single stranded DNAs (ssDNA), (ii) a specific binding of **1** to the guanine bulge producing a complex (Gb-DNA·1), (iii) a formation of a complex (dsDNA + 1) by a nonspecific binding of **1** to a double stranded DNA (dsDNA), (iv) a nonspecific binding of **1** to ssDNA producing a complex (ssDNA + 1), and (v) a formation of dsDNA. We assumed that a nonspecific binding of **1** to a duplex region of Gb-DNA is indistinguishable in the energy from that to a fully complementary duplex. These five equilibriums could be described with the free energy changes of ΔG_{bulge}, $\Delta G_{specific}$, $\Delta G_{duplex+1}$, $\Delta G_{single+1}$, and ΔG_{duplex} for each equilibration of eqs 1–5, respectively.

$$\text{ssDNA1} + \text{ssDNA2} \rightarrow \text{Gb-DNA} + \Delta G_{bulge} \quad (1)$$

$$\text{Gb-DNA} + \mathbf{1} \rightarrow \text{Gb-DNA}\cdot\mathbf{1} + \Delta G_{specific} \quad (2)$$

$$\text{dsDNA} + \mathbf{1} \rightarrow \text{dsDNA}\cdot\mathbf{1} + \Delta G_{duplex+1} \quad (3)$$

$$\text{ssDNA} + \mathbf{1} \rightarrow \text{ssDNA}\cdot\mathbf{1} + \Delta G_{single+1} \quad (4)$$

$$\text{ssDNA1} + \text{ssDNA3} \rightarrow \text{dsDNA} + \Delta G_{duplex} \quad (5)$$

The overall free energy change ($\Delta G_{bulge\text{-}overall}$) for the formation of Gb-DNA·**1** from two ssDNAs and **1** is described with a combination of ΔG_{bulge}, $\Delta G_{specific}$, $\Delta G_{duplex+1}$, and $\Delta G_{single+1}$ by eq 6,

$$\Delta G_{bulge\text{-}overall} = \Delta G_{bulge} + \alpha\cdot\Delta G_{specific} + \beta\cdot\Delta G_{duplex+1} - \gamma\cdot\Delta G_{single+1} \quad (6)$$

$$\alpha = [\text{Gb-DNA}\cdot\mathbf{1}]\cdot[\text{Gb-DNA}_0]^{-1} \quad (7)$$

where α indicates a molar fraction of the complex Gb-DNA·**1** to the initial concentration of Gb-DNA produced from two ssDNAs ([Gb-DNA$_0$]), whereas β and γ are molar fractions of the complexes dsDNA·**1** and ssDNA·**1** to the concentrations of Gb-DNA and ssDNA, respectively. This equation shows that the overall energy change reduces, as the nonspecific binding of **1** to ssDNA becomes stronger. The overall free energy gain by the binding of **1** to dsDNA in the presence of **1** ($\Delta G_{duplex\text{-}overall}$) could be described with the free energy changes regarding the nonspecific bindings of **1** to ssDNA and dsDNA by eq 8.

$$\Delta G_{duplex\text{-}overall} = \Delta G_{duplex} + \beta\cdot\Delta G_{duplex+1} - \gamma\cdot\Delta G_{single+1} \quad (8)$$

With a combination of eqs 6 and 8, the free energy change for the specific binding of **1** to a guanine bulge ($\Delta G_{specific}$) could be described with the experimentally obtainable energy terms by eq 9,

$$\alpha\cdot\Delta G_{specific} = \Delta G_{bulge\text{-}overall} - \Delta G_{bulge} - (\Delta G_{duplex\text{-}overall} - \Delta G_{duplex}) \quad (9)$$

Since the nonspecific binding of **1** to a double stranded region is much weaker than the specific biding of **1** to a

guanine bulge, we assumed that the initial concentration of bulge-containing duplex [Gb-DNA$_0$] is equal to a sum of concentrations of Gb-DNA in a free state and a complex Gb-DNA·**1**. It is important to note that under these estimations the deviation of the calculated data from the actual value will be larger as weaker the specific binding. The concentrations of Gb-DNA in a free and a complex state are described with α and [Gb-DNA$_0$] by eqs 10 and 11.

$$[\text{Gb-DNA}\cdot\mathbf{1}] = \alpha\cdot[\text{Gb-DNA}_0] \tag{10}$$

$$[\text{Gb-DNA}_0] = [\text{Gb-DNA}] + [\text{Gb-DNA}\cdot\mathbf{1}] \tag{11}$$

$$[\text{Gb-DNA}] = (1-\alpha)\cdot[\text{Gb-DNA}_0]$$

An association constant for the binding of **1** to a guanine bulge ($K_{specific}$) could be described by eq 12,

$$\begin{aligned} K_{specific} &= [\text{Gb-DNA}\cdot\mathbf{1}]\cdot[\text{Gb-DNA}]^{-1}\cdot[\mathbf{1}]^{-1} \\ &= \alpha\cdot(1-\alpha)^{-1}\cdot([\mathbf{1}_0]-\alpha\cdot[\text{Gb-DNA}_0])^{-1} \end{aligned} \tag{12}$$

where [**1**$_0$] is an initial concentration of **1**. Thus, a free energy change for the **1** binding to a guanine bulge is described as a function of α with experimentally obtainable energy terms by eq 13.

$$\begin{aligned} \Delta G_{specific} =& \{\Delta G_{bulge\text{-}overall} - \Delta G_{bulge} \\ & - (\Delta G_{duplex\text{-}overall} - \Delta G_{duplex})\}\cdot\alpha^{-1} \\ =& -RT\cdot\ln\{\alpha\cdot(1-\alpha)^{-1}\cdot([\mathbf{1}_0] \\ & - \alpha\cdot[\text{Gb-DNA}_0])^{-1}\} \end{aligned} \tag{13}$$

ΔG_{bulge} and $\Delta G_{bulge\text{-}overall}$ were obtained by a curve-fitting analysis of a guanine bulge-containing duplex in the absence and presence of **1**, respectively, at the initial concentration of [**1**$_0$]. ΔG_{duplex} and $\Delta G_{duplex\text{-}overall}$ were similarly obtained by a curve-fitting analysis of a fully matched duplex in the absence and presence of **1**, respectively. With the values of the free energy changes thus obtained, α and $\Delta G_{specific}$ for the specific binding of **1** to a guanine bulge at the temperature of T could be obtained by solving the eq 13.

Sequence dependence for the binding of **1** to the Guanine bulge. Curve fittings of the thermal denaturation profiles shown in Figure 2 to a two-state model provides ΔG_{bulge} and $\Delta G_{bulge\text{-}overall}$ for the formation of the guanine bulge−**1** complex with ten flanking sequences. ΔG_{duplex} and $\Delta G_{duplex\text{-}overall}$ were obtained from the similar analysis of a fully matched duplex of d(TCC AGG CAA C)/d(GTT GCC TGG A) and were −16.2 and −16.7 kcal/mol at 277.15 K, respectively. Accordingly, the contribution of the non-specific binding of **1** to ssDNA and dsDNA in the overall free energy change is determined to be −0.5 kcal/mol. Under the experimental conditions, we estimated that the initial concentration of guanine bulge-containing duplex ([Gb-DNA$_0$]) is equal to the concentration of a single stranded DNA (4.77×10^{-6} M) and the initial drug concentration ([**1**$_0$]) is 2.00×10^{-4} M. With these data in hand, $\Delta G_{specific}$ and $K_{specific}$ at 277.15 K were calculated (Table 3). The flanking sequences we examined could be divided into three groups regarding the magnitude of the free energy gain for the **1** binding. As we expected, the strongest binding of **1** was observed for the sequence of G_G/CGC with the free energy change ($\Delta G_{specific}$) of −5.3±0.3 kcal/mol among other guanine bulge containing sequences. The apparent equilibrium association constant ($K_{specific}$) for **1** binding to G_G/CGC was $15.2\pm4.0\times10^3$ M^{-1}, that is in a good agreement with 3.4×10^4 M^{-1} previously obtained by DNase I footprint titration within an experimental variability.[12] The marked difference of $\Delta G_{specific}$ between G_G/CGC and A_A/TGT by 0.8 kcal/mol showed that the strong binding of **1** to the sequence is due to the stacking of **1** by two flanking guanine bases, but is not due to the stacking by two purines. The second group of the sequences is those containing G base 3′ side to **1** in the complex. The guanine base immediately next to the 3′ side of **1** in the complex (Y=G) has strong effects on the stabilization of the complex. Substitution of the 3′ side G in G_G/CGC to A in G_A/CGT, C in G_C/CGG, and T in G_T/CGA resulted in a loss of the free energy change for the complex formation by ca. 0.7 kcal/mol. A strong stabilization of **1** by the 3′ side guanine is in a

Table 3. Free energy changes and association constants for the binding of **1**[a]

5′X_Y3′	3′X′GY′5′	ΔT_m (°C)	ΔG_{bulge}	$\Delta G_{bulge\text{-}overall}$	α	$\Delta G_{specific}$	$K_{specific}$ ($10^3\cdot M^{-1}$)
G_G	CGC	11.8	−12.0±0.2	−16.5±0.3	0.75±0.04	−5.3±0.3	15.2±4.0
A_G	TGC	11.2	−11.5±0.2	−14.4±0.1	0.53±0.02	−4.8±0.2	5.7±0.8
C_G	GGC	6.7	−13.7±0.1	−16.6±0.1	0.51±0.02	−4.7±0.1	5.3±0.4
C_C	GGG	6.8	−12.6±0.1	−15.4±0.2	0.49±0.02	−4.7±0.2	4.9±0.7
T_G	AGC	8.3	−12.0±0.1	−14.7±0.4	0.49±0.06	−4.7±0.4	4.9±1.6
G_C	CGG	7.1	−12.9±0.1	−15.6±0.3	0.48±0.05	−4.6±0.3	4.7±1.0
G_A	CGT	7.8	−10.5±0.2	−13.2±0.5	0.46±0.08	−4.6±0.5	4.3±1.6
T_T	AGA	8.0	−9.5±0.2	−11.9±0.2	0.41±0.03	−4.5±0.2	3.5±0.8
A_A	TGT	7.7	−9.2±0.1	−11.4±0.3	0.40±0.06	−4.5±0.3	3.4±0.9
G_T	CGA	6.7	−11.1±0.2	−13.3±0.1	0.39±0.04	−4.4±0.2	3.2±0.5

[a]All free energies were reported in kcal·mol^{-1} at 277.15 K. $\Delta G_{duplex\text{-}overall} - \Delta G_{duplex} = -0.5$ kcal·mol^{-1}.

good agreement with our previous studies of the sequence selective intercalation of charge neutral intercalators into the sequence of 5′GG3′ and 5′CG3′.[24] The last group of the sequence contains the A–T base pair in the 3′ side of **1** in the complex. The binding of **1** to these sequences was especially weak. On the basis of these data, the strong binding of **1** to G_G/CGC is most likely due to a cooperative stabilization by both 3′ and 5′ side Gs. Substitution of one G in the sequence by other bases dramatically decreased the complex stability.

Another important conclusion derived from the data in Table 3 is that the ΔT_m is not good for an assessment of the drug binding to DNAs.[25] This is especially the case when the given DNA duplexes for the assessment are significantly different in T_m from each other. While the ΔTm obtained for C_G/GGC in the presence of **1** is equal to that obtained for G_T/CGA, the binding of **1** to C_G/GGC is 1.7 fold stronger than the binding to G_T/CGA. This discrepancy of the data between the ΔT_m and $K_{specific}$ is simply due to a very larger difference in T_m of the duplex by 12.1 °C between the two oligomers.

Conclusion

The studies described here showed that the binding of **1** to a guanine bulge is sequence dependent. The molecular basis of the sequence dependence is most likely a strong stacking stabilization by the flanking G–C base pairs. For the strong binding to a guanine bulge flanking A–T base pairs, intercalating agents with much wider aromatic surface is desirable. However, such intercalators may intercalate more strongly into normal base pairs than **1** does, resulting in lowering the specificity to the guanine bulge. In order to circumvent these difficulties, an additional molecular device that binds to the major groove face of the bound guanine is necessary to be incorporated into the next generation of the bulge-targeting intercalators. A guanidium group that can bind to the N7 and O6 of the G from the major groove is conceivable for the auxiliary functional group.

Experimental

Materials

Reagents and solvents were purchased from standard suppliers without further purification. ^{1}H and ^{13}C NMR spectra were measured on a JEOL JNM α-400 (^{1}H spectra at 400 MHz; ^{13}C spectra at 100 MHz) spectrometer. FAB mass spectra were recorded on a JEOL JMS HX-110 spectrometer.

2,3,4,5,6-Pentafluorophenyl 3-((*tert*-butoxy)carbonylamino)propanoate. To a solution of Boc-β-alanine (3.0 g, 15.7 mmol) in DMF (10 mL) was added pentafluorophenol (2.2 g, 12.0 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.8 g, 14.6 mmol). The mixture was stirred at ambient temperature for 24 h. The solvent was evaporated and the crude product was suspended in $CHCl_3$. The organic layer was washed with H_2O and dried over $MgSO_4$. The solvent was evaporated in vacuo and the crude residue was purified by silica gel column chromatography ($CHCl_3$/hexane = 1/1) to give the title compound (3.8 g, 90%) as white solids: ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 5.12 (br, 1H), 3.51 (q, 2H, J = 6.2 Hz), 2.91 (t, 2H, J = 6.2 Hz), 1.43 (s, 9H); FABMS (NBA), m/e 356 [(M + H)$^+$]; HRMS calcd for $C_{14}H_{15}O_4NF_5$ [(M + H)$^+$] 356.0921, found 356.0931.

3-((*tert*-Butoxy)carbonylamino)-*N*-(7-methylpyridinio[3,2-e]pyridin-2-yl)propanamide (Boc-1). To a solution of the activated ester (449 mg, 1.26 mmol) in dry DMF (4 mL) was added 2-amino-7-methyl-1,8-naphthyridine (200 mg, 1.26 mmol) and *N*,*N*-diisopropylethylamine (162 mg, 1.26 mmol). The mixture was stirred at 40 °C for 24 h. The solvent was evaporated to dryness and the crude residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 50/1) to give Boc-1 (363 mg, 87%) as a pale white solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 8.86 (br, 1H), 8.42 (d, 1H, J = 9.0 Hz), 8.13 (d, 1H, J = 9.0 Hz), 8.00 (d, 1H, J = 8.2 Hz), 7.27 (d, 1H, J = 8.2 Hz), 5.19 (br, 1H), 3.50 (q, 2H, J = 5.9 Hz), 2.74 (s, 3H), 2.70 (t, 2H, J = 5.9 Hz), 1.41 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 171.2, 163.4, 155.9, 154.3, 153.2, 139.2, 136.5, 121.7, 118.6, 114.3, 79.5, 37.5, 36.1, 28.4, 25.5; FABMS (NBA), m/e 331 [(M + H)$^+$]; HRMS calcd for $C_{17}H_{23}O_3N_4$ [(M + H)$^+$] 331.1770, found 331.1771.

3-Amino-*N*-(7-methylpyridino[3,2-e]pyridin-2-yl)propanamide (1). To a solution of Boc-1 (191 mg, 0.58 mmol) in dry $CHCl_3$ (3 mL) was added ethyl acetate containing 4 M HCl (1.5 mL) at 0 °C and the mixture was stirred at room temperature for 0.5 h. The solvent was evaporated to dryness to give the hydrochloride of **1** (quantitative yield) as a white solid. The hydrochloride of **1** was dissolved in H_2O and extracted into $CHCl_3$ by the addition of 28% aqueous ammonia solution. The organic layer was dried over $MgSO_4$ and the solvent was evaporated in vacuo to give **1** (98.8 mg, 74%) as pale white solids: ^{1}H NMR (CD_3OD, 400 MHz) δ = 8.39 (d, 1H, J = 8.9 Hz), 8.25 (d, 1H, J = 8.9 Hz), 8.18 (d, 1H, J = 8.2 Hz), 7.4 (d, 1H, J = 8.2 Hz), 3.02 (t, 2H, J = 6.4 Hz), 2.71 (s, 3H), 2.67 (t, 2H, J = 6.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 173.9, 164.2, 155.5, 140.2, 138.8, 122.8, 119.9, 115.8, 113.6, 40.4, 38.5, 25.0; FABMS (NBA), m/e 231 [(M + H)$^+$]; HRMS calcd for $C_{12}H_{15}ON_4$ [(M + H)$^+$] 231.1246; found 231.1248.

Measurements of the melting temperature of bulge-containing duplexes. Compound **1** (final concentration of 200 μM) was dissolved in a sodium cacodylate buffer (10 mM, pH 7.0) containing bulge duplex (4.77 μM, strand concentration) and NaCl (100 mM). The mixture was heated for 5 min at 50 °C and cooled slowly to make sure that the starting oligomer is in a duplex state. The thermal denaturation profile was recorded on a JASCO V-550DS spectrometer equipped with a Peltier temperature controller. The absorbance of the sample was monitored at 260 nm from 4 to 70 °C with a heating rate of 1 °C/min.

Curve fitting

Three data sets of thermal denaturation profiles obtained by three independent measurements were fitted to a two-state model with a nonlinear least-square program in Sigma Plots (version 2000). The equation used for the fitting was described in the reference.[22]

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