Side Chain-Dependent Binding of Antitumor Indoloquinoxaline Derivatives to DNA: Comparative Spectroscopic and Viscometric Measurements

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NCA0424 (1) and its side chain positional isomer, NCA0465 (2), are indoloquinoxaline derivatives with potent antitumor activity. To investigate the effect of side chain position for binding with DNA, the interactions of 2 with various B-form DNAs were studied by spectroscopic (circular dichroism (CD), fluorescence and UV) and viscosity measurements and were compared with those of 1. The binding preference for the base sequence was different in each case. The CD spectra showed that 2 formed an asymmetric binding of indoloquinoxaline ring with adenine in DNA, whereas such a base selectivity was not found with 1. The binding features are discussed based on association constants and thermodynamic parameters, indicating the importance of the side chain position for binding specificity for DNA.

Key words indoloquinoxaline; DNA; interaction; viscosity; spectroscopy; thermal parameter

NCA0424, [2-methyl-2-[(6-methyl-6H-indolo[2,3-*b*]quinoxaline-4-yl)methylamino]-1,3-propanediol, **1**] shows antitumor activity against in various *in vitro* and *in vivo* tumor models.[1)] Generally, the antiviral/antitumor activities of numerous aromatic compounds are revealed through interaction with DNA, in addition to inhibitory effects on topoisomerases and other processes such as DNA replicative or repair enzymes. Compound **1** belongs to this category, since it exhibits not only inhibitory activity against topoisomerase II,[1)] but also potent binding to DNA.[2)]

As a part of work on clarifying the binding features of antitumor or carcinogenic aromatic compounds to DNA, we have been investigating their interactions by spectroscopic methods such as UV, fluorescence, and circular dichroism (CD), together with viscosity measurements.[3)] Previously, we reported the appearance of a CD band at 340—390 nm in the binding of **1** to DNA.[2)] Since this induced CD (ICD) band was not observed in the interaction of the indoloquinoxaline ring itself with DNA, it suggested the importance of the side chain of **1** for the specific interaction with DNA.

In order to discuss the structure–activity relationships of indoloquinoxaline derivatives at the molecular level, it is important to examine whether the binding features of **1** to DNA stem from the position of the side chain attached to the aromatic ring. Compound **2** (NCA0465, Fig. 1) is a positional isomer of the side chain in **1** and also shows potent antitumor activity, although its spectrum of activity is different from **1**.[4)] Thus, interaction of **2** with DNA was examined by spectroscopic and viscometric methods and compared with **1**.[2)] Herein, we report **2**–DNA interactions and discuss the possible relationship of the side chain position with the binding preference to DNA.

Experimental

Materials Poly(dA-dT)$_2$, poly(dG-dC)$_2$, poly(dG-dT)·poly(dA-dC), poly(dA)·poly(dT), and calf thymus (CT) DNA were purchased from P-L Biochemicals. Distamycin A and ethidium bromide were from Sigma Chem-

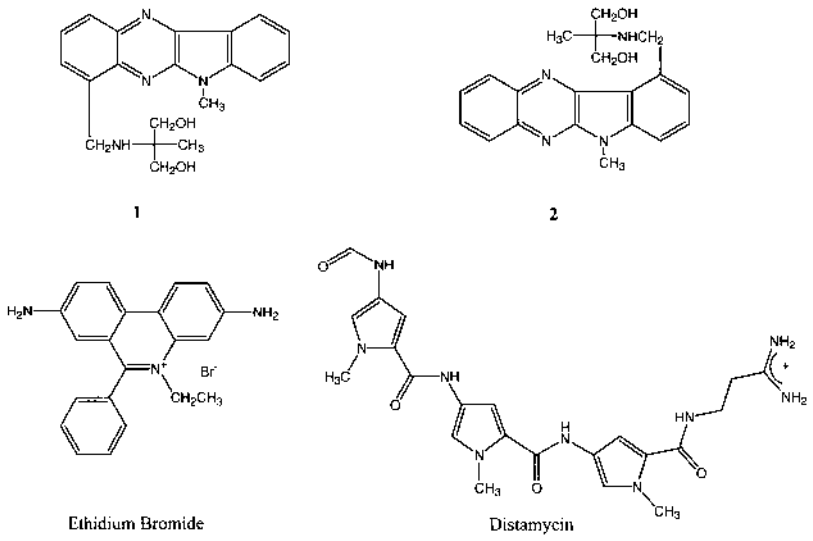

Fig. 1. Chemical Structures of **1**, **2**, Ethidium Bromide (EtBr), and Distamycin (Dist.)

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ical Co. and were used without further purification. The HCl salts of **1** and **2** were provided by Taisho Pharmaceutical Co. All other chemicals used were of analytical grade. Sample solutions were prepared with doubly distilled and deionized water.

Sample Preparation Solutions of B-form DNA were prepared in 300 μM sodium cacodylate buffer (pH 7.0). Measurements were carried out four times and each spectrum reported is an average. DNA concentrations were determined using the standard molar extinction values (M^{-1} cm^{-1}) reported in the appended documents from P-L Biochemicals or in the literature[5] and were expressed in terms of moles of phosphate per liter. The molar extinction coefficients per base of the oligomers were calculated according to the method of Cantor *et al.*[6] The molar extinction coefficients of **1** and **2** were both determined to be $\varepsilon_{418}=3536\ M^{-1}\,cm^{-1}$.

CD Measurements CD spectra were recorded on a JASCO J-820 spectropolarimeter with a DP-500N data processor using 5-mm path-length cells. Sample temperature in the cuvette was regulated by a circulating water bath and was kept at 25 °C. Measurements covering a comprehensive range of r were performed at 100 μM DNA concentration, where r is defined as the molar ratio of drug to DNA base pair. The molar ellipticity $[\theta]$ ($deg\cdot cm^2\cdot dmol^{-1}$) was calculated from the equation, $[\theta]=100\times\theta_{obsd}/lC_p$, where θ_{obsd} is the observed ellipticity in degrees, C_p is the DNA concentration in terms of phosphate, and l is the path-length in centimeters.

Provided that one binding mode (or constant proportion of a number of modes) is present over a range of drug concentrations, the equilibrium binding constant (K) between the drug and DNA can be evaluated from the intensity change of the CD band according to the following equation[7]:

$$\frac{\Delta C_{ik}}{\Delta\rho_{ik}}=\left(\frac{d}{\alpha}\right)\left[\frac{\Delta(C_{ik}/\rho_{ik})}{\Delta\rho_{ik}}\right]+\alpha \tag{1}$$

$$K=\frac{\alpha\rho}{\left(d-\alpha\rho\right)\left(C_{tot}-\alpha\rho\right)} \tag{2}$$

where ΔC_{ik} is the difference between the ith and kth concentrations of drug, $\Delta\rho_{ik}$ is the difference between the ith and kth CD intensities, $\Delta(C_{ik}/\rho_{ik})$ is the difference between the C_i/ρ_i and C_k/ρ_k values, d is the concentration of binding sites on the DNA ($d=C_M/n$, where C_M=the concentration of DNA, n=the effective site size), $\alpha\rho$ ($=C_b$) is the bound concentration of drug, and C_{tot} is the total concentration of drug. For a constant DNA concentration, a plot of $\Delta C_{ik}/\Delta\rho_{ik}$ *versus* $\Delta(C_{ik}/\rho_{ik})/\Delta\rho_{ik}$ gives a straight line with slope d/α and intercept α. The n and K values are obtained from the above equation by least-squares linear regression analysis.

Fluorescence Measurements Fluorescence spectra were measured on a JASCO FP-770F spectrometer (Nihon Bunko) using a Hg–Xe arc lamp, and a 5-mm path length was employed. The temperature of the sample solution was kept at 25 °C by circulating thermostatically regulated water. The emission spectra (350—650 nm) excited at 358 nm (for **1**) or 361 nm (for **2**) were measured as a function of DNA concentration, where the concentration of compound was fixed at 50 μM.

UV Measurements The UV absorption spectrum of compound was measured as a function of DNA concentration on a JASCO UVIDEC-610 spectrometer using 5-mm path-length cells, where the compound was adjusted to 50 μM concentration. The temperature was controlled by a circulating water bath.

The association constant (K) between the compound and DNA was obtained from the slope of Eadie–Hofstee plot[8] by least squares linear analysis:

$$\Delta A=-\frac{1}{K}\frac{\Delta A}{[DNA]}+\Delta A_C \tag{3}$$

where ΔA is the difference of absorbance between the compound alone and in the presence of DNA at the concentration of [DNA], and ΔA_c is the difference of absorbance between the compound alone and fully complexed with DNA. The thermodynamic parameters [enthalpy change ($\Delta H°$), entropy change ($\Delta S°$) and Gibbs free energy change ($\Delta G°$)] of interactions were obtained from the following equations:

$$\Delta G°=-\boldsymbol{R}T\ln K \tag{4}$$

$$\Delta G°=\Delta H°-T\Delta S° \tag{5}$$

$$\ln K=-\Delta H°/\boldsymbol{R}T+\Delta S°/\boldsymbol{R} \tag{6}$$

where $\boldsymbol{R}$ is the gas constant ($1.987\ cal\,mol^{-1}\cdot deg^{-1}$), and T is the temperature in Kelvin. $\Delta H°$ was evaluated by least-squares from the slope of a van't Hoff plot (equation 6), and $\Delta G°$ and $\Delta S°$ were from Eqs. 4 and 5, respectively.

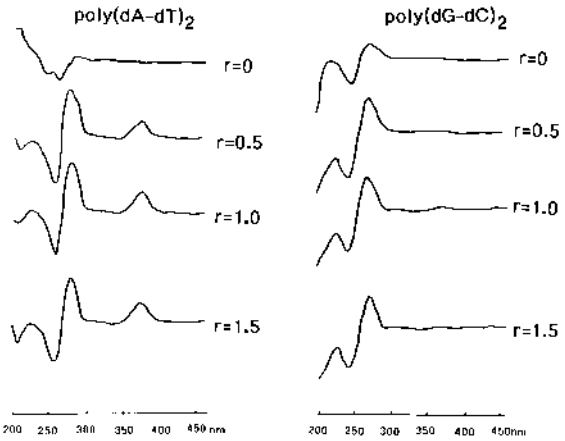

Fig. 2. CD Spectra for B-Form Poly(dA-dT)$_2$ and Poly(dG-dC)$_2$ as a Function of Concentration of **2**

Viscometric Measurements Viscometric measurements were conducted using a Cannon–Manning semimicrodilution viscometer (No. 75). The temperature was kept at 35 °C. A sample solution of 330 μl was placed in a viscometer and the relative velocity ($\eta/\eta o$) of 400 μM DNA concentration with respect to distilled water was measured as a function of the concentration of compound.

Results and Discussion

CD Spectra CD spectral changes of various B-form DNAs were measured as a function of r (the molar ratio of **2** to DNA base pairs). The changes for poly(dG-dC) and poly(dA-dT) duplexes are shown in Fig. 2. Similar to the case of **1**, the CD spectra of DNAs were changed by **2**. The enlargement of the concomitant positive (*ca.* 275 nm) and negative (*ca.* 255 nm) ellipticities, which are a typical characteristic of B-form DNA, was observed in the range of $0<r<0.5$, and the change was saturated at $r\geqq0.5$. The former phase reflects the reinforcement of the B-form structure of DNA by the interaction with **2**. The saturation at $r=ca.\ 0.5$ may correspond to 1 : 2 stoichiometry of **2** with respect to DNA base pairs.

On the other hand, a new CD band was observed in the 340—400 nm region ($\lambda_{max}=ca.$ 370 nm) by the interaction of **2** with poly(dA-dT)$_2$, while such a new band was not observed with poly(dG-dC)$_2$. This is in contrast with **1**, in which such an ICD was observed for both poly(dG-dC)$_2$ and poly(dA-dT)$_2$.[2] Since **2** itself has a UV band ($\lambda_{max}=362$ and 418 nm), but not a CD band, in the region 300—450 nm, this ICD implies the formation of a new asymmetric environment for **2** as a result of the interaction with DNA. Since similar ICDs were also observed for CT-DNA, poly(dA-dC)·poly(dG-dT), and poly(dA)·poly(dT) (data not shown), it suggests that the asymmetric binding of **2** to DNA is A-specific and has no specific preference for the base sequence, although ICD formation with DNA of alternative A and T sequence was most significant. According to a previous paper,[2] the association constants, K (M^{-1}), were estimated from the ICD values at λ_{max} (372 nm) in the range of $0<r<0.5$, and the results are listed in Table 1. **2** has the highest binding preference for the A–T base sequence with the interaction site size being about two base pairs, while **1** shows similar

binding preferences for both A–T and G–C base sequences. As the B-form structures of poly(dG-dC)$_2$ and poly(dA-dT)$_2$ were similarly affected by **2**, these K values indicate the importance of the side chain position for binding preference with the DNA sequence. In the case of **2**, the position of the side chain functions so as to induce asymmetric binding selectivity for adenine bases in DNA.

Fluorescence Spectra The different binding patterns of **2** to poly(dA-dT)$_2$ and poly(dG-dC)$_2$, were also observed by fluorescence spectra. Their changes as a function of poly(dA-dT)$_2$ or poly(dG-dC)$_2$ concentration are shown in Fig. 3. In the poly(dA-dT)$_2$–**2** interaction, the fluorescence intensity was increased in proportion to the DNA concentration, where the intensity was almost linearly or exponentially increased in the range of $0<r<0.5$ or $r>1.0$, respectively. On the other hand, a different change was observed in the poly(dG-dC)$_2$–**2** interaction. Although the change was not so significant, the intensity was linearly increased in the range of $0<r<0.5$, saturated at $0.5<r<1$, and then began to decrease at $r>1$. These spectral differences appear not to be in conflict with the CD results.

It has been reported that Hext33258, an aromatic DNA groove binder,[9] increases its fluorescence intensity with an increase of DNA concentration.[10] Therefore, the interaction of **2** with poly(dA-dT)$_2$ appears to be responsible for the binding to the groove site of the DNA double helical structure, leading to the appearance of the ICD band. On the other hand, the interaction of **2** with poly(dG-dC)$_2$ could be intercalative, since it is generally accepted that the quenching of fluorescence intensity reflects the stacking interaction of chromophore with DNA bases.

UV Spectra In contrast to the CD and fluorescence results, no clear difference was observed in the interactions of **2** with poly(dA-dT)$_2$ and poly(dG-dC)$_2$. Their UV spectra showed similar hypochromicity with an increase of DNA concentration, together with a slight red-shift of the maximum peak (Fig. 4). Since the hypochromicity of chromophore generally results from π-electron transfer by stacking interaction with nucleic bases, the spectral change appears to reflect the contribution of intercalation into the nucleic base pair.

Taking advantage of the linear Eadie–Hofstee plot[8] based on the hypochromic change (insert in Fig. 4), the association constants with poly(dG-dC)$_2$ and poly(dA-dT)$_2$ at several temperatures were measured for **1** and **2**, and their thermal parameters for interaction ($\Delta H°$, $\Delta S°$, $\Delta G°$ at 25 °C) were then estimated from the van't Hoff's plots. The results are given in Table 2. As is obvious from the table, **2** interacts more preferably with poly(dG-dC)$_2$ than poly(dA-dT)$_2$ through a stacking interaction. Compared with the binding features of **1**, no notable difference could be observed for the respective association constants and thermal parameters, although the binding preference of **1** to poly(dG-dC)$_2$ may be notable. The driving forces for bindings of **1** and **2** with poly(dA-dT)$_2$ and poly(dG-dC)$_2$ are almost the same. The associations result from cooperative contributions of negative $\Delta H°$ and positive $\Delta S°$, thus leading to negative $\Delta G°$ energies.

The association constants from the UV data are considerably different from those from ICD data (Table 1). This must be due to the observation of different interaction modes. As already stated, the ICD data should reflect the non-stacking binding of compound to DNA. In the case of **2**, major groove binding is most probable, because the polar group of A base is located at this groove site. Because it can observe the local asymmetric environment of a compound in the interaction with DNA, the ICD data should be much more sensitive than the UV data.

Viscosity The viscosity of DNA was measured as a function of r of **2**, ethidium bromide (EtBr), or distamycin

Table 1. Association Constants[a] from ICDs for the Interaction of **2** with DNAs in Comparison with **1**

DNA	**2**		**1**	
	n[b]	$K\times10^{-7}$ (M^{-1})[c]	n	$K\times10^{-7}$ (M^{-1})
Poly(dG-dC)$_2$	—	—	2.2	8.99
Poly(dA-dC)·poly(dG-dT)	2.2	3.25	2.6	8.69
Poly(dA-dT)$_2$	2.1	8.23	1.3	7.54
Poly(dA-dG)·poly(dC-dT)	1.8	4.09	1.5	6.97
Poly(dA)·poly(dT)	1.5	3.38	1.5	4.61
CT DNA	0.8	3.80	0.7	3.85

a) The data were obtained from Eq. 2. *b*) The value corresponds to the interaction site size (in base pair units). *c*) The experimental errors are all within 10% of the given values.

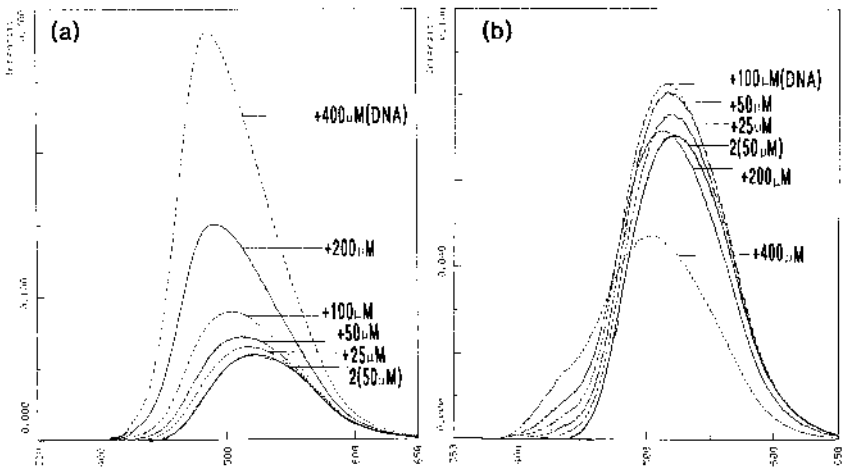

Fig. 3. Fluorescence Emission Spectra of **2** as a Function of Poly(dA-dT)$_2$ (a) or Poly(dG-dC)$_2$ (b) Concentration

The increase or decrease of fluorescence intensity in (a) or (b) was almost saturated at 1.5 mM Poly(dA-dT)$_2$ or at 500 mM Poly(dG-dC)$_2$, respectively.

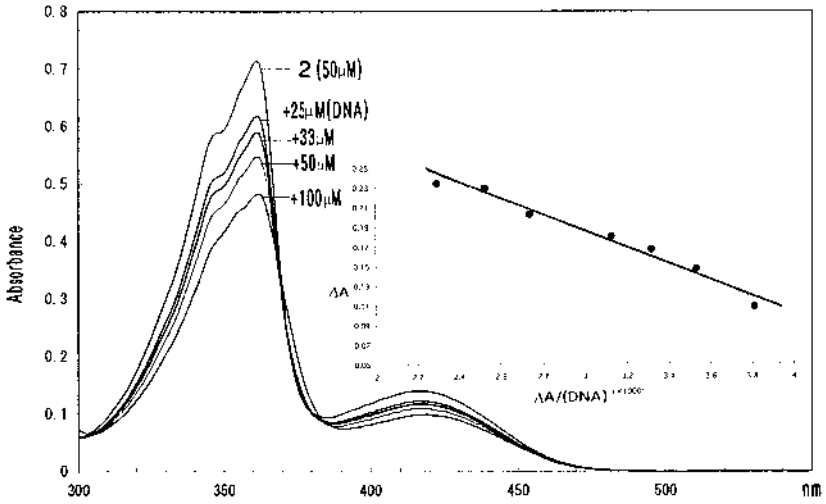

Fig. 4. Hypochromicity of **2** as a Function of Poly(dA-dT)$_2$ Concentration
Insert: Eadie–Hofstee Plot of ΔA *versus* ΔA/[Concentration of Poly(dA-dT)$_2$].

Table 2. Association Constants (K) and Respective Thermodynamic Parameters[a] (at 25 °C) of **2** with Poly(dA-dT) and Poly(dG-dC) Duplexes in Comparison with **1**

	2		**1**	
	Poly(dA-dT)$_2$	Poly(dG-dC)$_2$	Poly(dA-dT)$_2$	Poly(dG-dC)$_2$
K ($\times 10^{-4}$ M^{-1})	0.96±0.11	1.79±0.15	1.92±0.13	3.42±0.19
$\Delta H°$ (cal·M^{-1})	−2060±98	−2748±156	−2532±114	−2425±185
$\Delta S°$ (cal·K^{-1}·M^{-1})	11±1	10±1	11±1	13±2
$\Delta G°$ (cal·M^{1})	−5428±178	−5740±176	−5883±220	−6232±233

a) The data were obtained from Eqs. 3–6.

(Dist) to determine the interaction mode of **2** with DNA. The viscometric titrations of these compounds for poly(dG-dC)$_2$ and poly(dA-dT)$_2$ are shown in Fig. 5. It is known that EtBr increases the viscosity of DNA by intercalation into DNA base pairs,[11] while Dist, a groove binder to DNA, does not affect the viscosity, significantly. In the former binding, it is reported that the increment of the relative viscosity (η/η_0) is most significant for the B-form DNA, and the value is within the range of 1.9±0.3 for the saturated state of a typical intercalator.[12] As judged from the η/η_0 profiles of **2**, its binding mode could be intercalative for both poly(dA-dT)$_2$ and poly(dG-dC)$_2$. Similar to the case of **1**, **2** displayed no notable differences between both types of DNA.

The present results suggested two kind of binding mode for the interaction of **2** with DNA, *i.e.*, groove binding and intercalation. Similar to the case of **1**, these two modes appear to coexist at a relatively dilute concentration of compound/DNA, as judged from the spectroscopic results. Concerning the DNA sequence binding preference, it was shown that there was a difference between **1** and **2**, which depends on the position of the side chain attached to indoloquinoxaline. On the other hand, the viscometric data indicated that intercalation becomes the major binding mode as the concentration of compound/DNA is increased. The potent antitumor activities of **1** and **2** toward various leukemias, fibrosarcomas and melanomas have been observed. In order to effectively understand their biological function, the selective binding of compound to the target position in the DNA sequence is of special important. The present study would be useful of designing i antitumor targeted to a selective position in DNA.

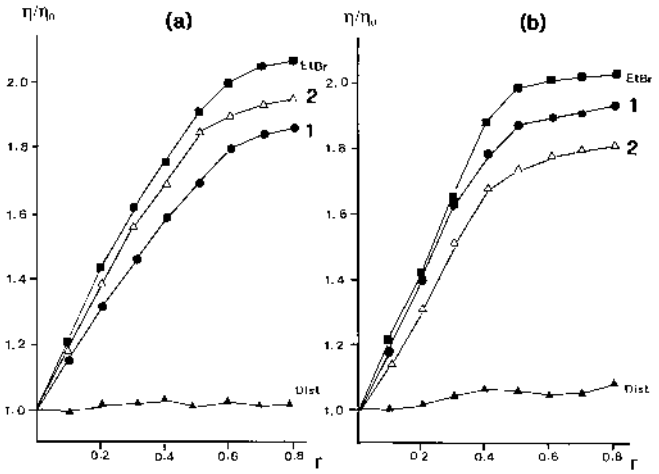

Fig. 5. Viscometric Titrations of B-Form Poly(dA-dT)$_2$ (a) and Poly(dG-dC)$_2$ (b) with **1** (—●—), **2** (—△—), EtBr (—■—) and Dist (—▲—)

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