DNA-binding characterization of a novel anti-tumour benzo[*a*]phenazine derivative NC-182: spectroscopic and viscometric studies

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NC-182 is a novel anti-tumour compound having a benzo[*a*]phenazine ring. Fluorescence, absorption and c.d. spectroscopy, as well as viscometric titrations, were systematically performed to investigate the interaction mode of this drug with DNA and its effect on DNA conformation, based on comparative measurements with distamycin (DNA minor-groove binder) and daunomycin (DNA-base intercalator). NC-182 was found to be a potent intercalator of DNA, especially the B-form DNA, although no specificity was observed against the base-pair. The binding of NC-182 to B-DNA behaves biphasically, depending on the molar ratio (*r*) of drug to DNA: NC-182 acts to render the B-form structure rigid at relatively low *r* value and to promote the transformation of B- to non-B forms at high *r* values. It was also shown that NC-182 promotes the unwinding of Z-form DNA to B-form. Viscometric, u.v. 'melting' and c.d. experiments further showed that (1) the DNA duplex structure is thermally stabilized by intercalation with NC-182 and (2) the intercalation of NC-182 into a poly(dA)·2poly(dT) DNA structure thermally stabilizes the triplex structure, resulting in a melting point close to that of the duplex structure; the melting curves of triplex and duplex structures coincide at $r > 0.06$. These observations make a significant contribution to our understanding of the biological properties of this novel benzo[*a*]phenazine derivative, a new anti-tumour agent against multidrug-resistant and sensitive tumours.

INTRODUCTION

N-β-Dimethylaminoethyl-9-carboxy-5-hydroxy-10-methoxybenzo[*a*]phenazine-6-carboamide compounds (Figure 1) are novel anti-tumour agents against multidrug-resistant and sensitive tumours (Nakaike et al., 1989; Tsuruo et al., 1990). They exhibit potent anti-tumour activities for a number of *in vivo* tumour systems, and NC-190 is presently undergoing Phase I clinical trials. In contrast with the physiological and pharmacological effects of these compounds, the nature of the mode of interaction with DNA, which is essentially associated with its anti-tumour activity, is far from being fully understood, although the biological activity of NC-190 is believed to be due to its ability to bind to DNA, which results in the inhibition of DNA synthesis (Nakaike et al., 1992) and DNA topoisomerase activity (Tsuruo et al., 1990).

The elucidation of how these compounds interact with DNA and/or how they recognize a particular DNA base-pair is essential as the first step for understanding the molecular basis of their anti-tumour activity and is of special importance for further development of clinically useful drugs. The present paper deals with the DNA-binding characterization of NC-182, a ester form of NC-190, by systematic optical and viscometric measurements and by comparison of measured results with those of distamycin (a typical DNA minor groove binder) as well as daunomycin and ethidium (typical intercalators into DNA base-pairs); the chemical structures of these are also shown in Figure 1. The present study clearly shows that NC-182 is a potent intercalator with nearly the same affinity as daunomycin, and the degree of binding of DNA with NC-182 is dependent on the conformation and not on the base sequence/base-pair. The significant stabilization of the DNA triplex structure by intercalation with NC-182 is noteworthy.

MATERIALS AND METHODS

Materials

Poly(dA–dT), poly(dG–dC), poly(dG-m^{5}dC), poly(dA)·poly(dT), poly(dT), poly(rA)·poly(rU) and calf thymus DNA were purchased from P-L Biochemicals, and distamycin A, daunomycin, ethidium bromide and actinomycin D were purchased from Sigma Chemical Co. and used without further purification. NC-182 and NC-190 were provided by Taisho Pharmaceutical Co. All other chemicals used were of analytical grade, and the sample solutions were prepared with doubly distilled and deionized water.

The measurements were carried out three times and each spectrum reported is an average of them. Polynucleotide concentrations were determined using the standard absorption values reported by the supplier (P-L Biochemicals) or in the literature (Rao et al., 1988) and are expressed in terms of mol of phosphate/litre. The concentrations of drugs were also spectrophotometrically determined by using the reported molar absorption coefficients (Rao et al., 1988; Chen et al., 1993; Remeta et al., 1993). On the other hand, the molar absorption coefficients of NC-182 and NC-190 were spectrophotometrically determined to be (ε_{500}) 5533 $M^{-1}\cdot cm^{-1}$ and (ε_{483}) 5525 $M^{-1}\cdot cm^{-1}$ respectively, using the gravimetrically prepared solution of 10 mM sodium cacodylate buffer containing 5% dimethylformamide (pH 6.4, 25 °C) for NC-182 or of 10 mM boric acid (pH 9.6, 25 °C) for NC-190, and they were used for the determination of concentration. Measurements covering a comprehensive range of drug/nucleotide ratio (*r*) were performed at either constant nucleotide or constant drug concentration, where *r* was defined as the molar ratio of total amount of drug to DNA phosphate; for the measurement of association constants, the molar ratio (*R*) of drug to nucleotide base-pair was used for clarity of interpretation.

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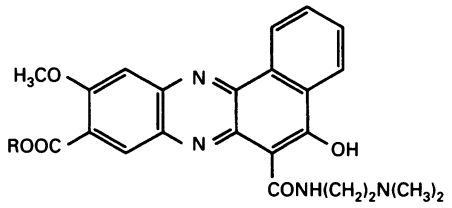
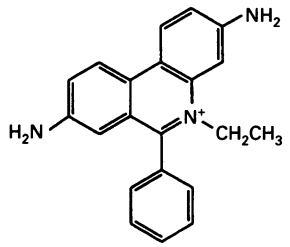
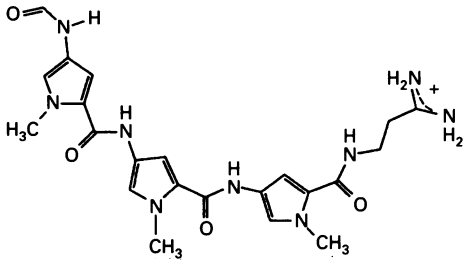


Benzo[*a*]phenazine derivatives

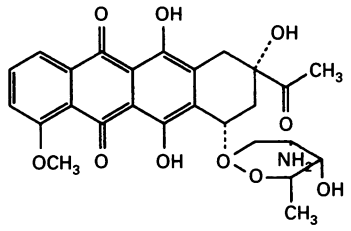
NC-190 : R = Na
NC-182 : R = CH_3



Ethidium



Distamycin A



Daunomycin

Figure 1 Chemical structures of DNA-binding drugs used in the present work

Fluorescence measurements

Drug binding to nucleic acid was monitored by spectrofluorometric quantitation of the displacement of ethidium from DNA (Cain et al., 1978). Fluorescence measurements (excitation at 546 nm; emission at 600 nm) were performed on a Jasco FP-770F spectrofluorimeter with a 10-mm-pathlength cell at 25 °C in 10 mM cacodylate buffer, pH 6.4, containing 50 mM NaCl and 1 mM $MgCl_2$. Because of solubility requirements, 10 mM sodium cacodylate buffer, pH 7.9, containing 50 mM NaCl and 1 mM $MgCl_2$, was used for the comparative experiment with NC-190 (excitation at 570 nm; emission at 600 nm). The excitation wavelength was selected so as to ensure better quantum yield of ethidium and to minimize the change in absorption due to binding with DNA. The addition of 50 mM NaCl compensates for the screening effect due to light absorption by the DNA. The concentrations of DNA and ethidium used were 2 μM and 1.26 μM respectively, and the concentration of drug was varied as a function of r. The concentration of drug necessary to reduce the initial fluorescence of DNA-bound ethidium by 50%, defined as C_{50}, was measured from the ethidium-displacement curve as an indicator of the binding ability of drug with DNA.

Absorption measurements

In order to obtain the binding parameters of drugs with DNA by the Scatchard (1949) plot, absorption measurements were performed on a Jasco 610A spectrometer using 1-cm-pathlength cells. The temperature was controlled by a circulating water bath. The concentration of drug was adjusted to 15 μM in 10 mM sodium cacodylate buffer containing 5% dimethylformamide, pH 6.4, and DNA concentration (1.5–40 μM in base-pairs) was varied as a function of R at 25 °C.

UV melting curves of poly(dA–dT) and poly(dA)·poly(dT) DNA were measured as a function of r, where the A_{260} was monitored using 20 μM DNA dissolved in 10 mM sodium cacodylate buffer, pH 6.4; for the measurement of poly(dA)·2poly(dT) triplex structure, $MgCl_2$ was added to the buffer to make a 2 mM concentration (Scaria and Shafer, 1991; Durand et al., 1992; Park and Breslauer, 1992). The heating rate of sample solution was set to 0.5 °C/min. Under these conditions, the melting point of poly(dG–dC) is too high and thus cannot be analysed by this method.

C.d. measurements

C.d. spectra were recorded on a Jasco J500A spectropolarimeter with DP-500N data processor using 1-cm-pathlength cells. Sample temperature in the cuvette was regulated by a circulating water bath. The temperature was kept at 20 °C unless otherwise stated. A 30 μM concentration of DNA solution was prepared in 10 mM sodium cacodylate buffer, pH 6.4, containing 50 mM NaCl, 1 mM $MgCl_2$ and 5% methanol, and the c.d. spectral change was monitored as a function of r. The molar ellipticity $[\theta]$ (degrees·cm^2·dmol^{-1}) was calculated from the equation:

$$[\theta] = 100 \times \theta_{obs.}/lC$$

where θ is the measured ellipticity in degrees, C is the DNA concentration in terms of phosphate, and l is the pathlength in cm.

In the case of poly(dA)·2poly(dT), 30 μM DNA dissolved in 10 mM sodium cacodylate buffer, pH 6.4, was used. The duplex and triplex structures of poly(dA)·2poly(dT) was prepared in the absence or presence of 2 mM $MgCl_2$ respectively.

Viscometric measurements

Viscometric measurements were conducted using a Cannon–Manning semi-microdilution viscometer (no. 75). DNA (200 μM) was prepared in 10 mM sodium cacodylate buffer, pH 6.4, containing 5% dimethylformamide. For the sample preparation of poly(dG-m^{5}dC) Z-form structure and poly(dA)·2poly(dT) triplex structure, the buffer containing 50 mM NaCl and 1 mM $MgCl_2$ and that containing 2 mM $MgCl_2$ were used respectively. The temperature was set at 35 °C. A 330 μl sample solution was placed in a viscometer, and the titration was conducted as a function of r.

RESULTS AND DISCUSSION

Fluorometric displacement of DNA-bound ethidium by drug

It is known that the fluorescence of ethidium is markedly enhanced by DNA intercalation (Le Pecq and Paoletti, 1967; Le Pecq et al., 1974), and this permits the fluorometric quantification of ethidium displaced from DNA intercalation sites by competition due to an added drug (Cain et al., 1978; Denny et al., 1979; Lee et al., 1982; Kimura, 1992). Since NC-182 binding to ethidium-bound poly(dA–dT) quenches the fluorescence intensity of ethidium, as is exemplified in Figure 2(a), the quenching degree as a function of r is used to obtain a displacement curve shown in Figure 2(b). From respective drug-dependent curves, the values of C_{50}, which correspond to micromolar drug concentrations necessary to displace 50% of DNA-bound ethidium, could be obtained and used as an indicator for deducing the DNA–drug binding ability. The C_{50} values obtained are summarized in Table 1.

It is known that daunomycin is a typical intercalator (Arcamone, 1981) showing no specific base-pair preference, and distamycin is an A–T-specific minor-groove binder (Zimmer and Wahnert, 1986). When the C_{50} value of NC-182 is compared with those of daunomycin and distamycin, it is suggested that NC-182 interacts with DNA and has no base-specificity; its DNA-binding ability is nearly the same as that of daunomycin. Interestingly, the binding of NC-182 to poly(rA)·poly(rU) is obviously unfavourable, indicating the DNA-specific binding of this drug; a similar result was also obtained from c.d. measurements (discussed below). From comparison of C_{50} values of NC-182 and NC-190 it is reasonable to consider that the interaction of NC-182 with DNA is mainly due to van der Waals forces, whereas an electrostatic force disturbs the DNA binding of the drug chromophore. Since the interaction of the benzo[a]phenazine chromophore with nucleic bases seems to occur in the same manner for both NC-182 and NC-190, the following investigations were focused on the interaction of NC-182 with DNA.

Table 1 C_{50} values of NC-182 (pH 6.4, a) and NC-190 (pH 7.9, b), together with those of daunomycin and distamycin, obtained from ethidium-displacement curves

(a)

	C_{50} (μM)		
	NC-182	Daunomycin	Distamycin
Poly(dA–dT)	0.62 ± 0.03	0.54 ± 0.01	0.13 ± 0.02
Poly(dG–dC)	1.07 ± 0.10	0.57 ± 0.13	84.7 ± 9.8
Calf thymus DNA	0.38 ± 0.04	0.4 – 2.1*	3.0 ± 0.4
Poly(rA)·poly(rU)	3.19 ± 0.38	–	–

(b)

	C_{50} (μM)	
	NC-190	Daunomycin
Poly(dA–dT)	9*	0.38 ± 0.15
Poly(dG–dC)	6–9*	0.50 ± 0.08

* Because of large fluctuations of experimental data, the S.D. values could not be determined exactly.

Absorption binding studies

The overall association constants for binding of drug to DNA were determined from changes of the absorption spectra. The absorption spectra of NC-182 and daunomycin in the absence

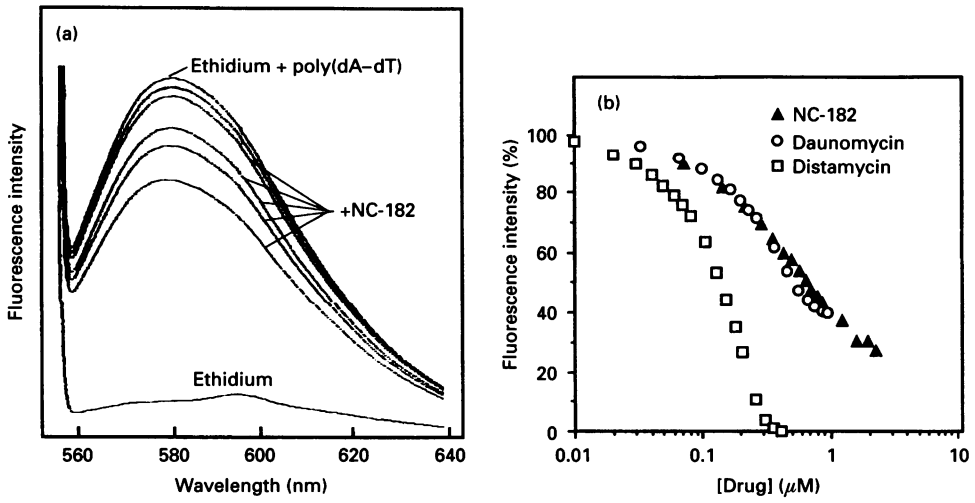


Figure 2 (a) Decrease of fluorescence emission spectrum of ethidium intercalated into poly(dA–dT) with respect to the concentration of NC-182, and (b) ethidium-displacement curves for NC-182, daunomycin and distamycin

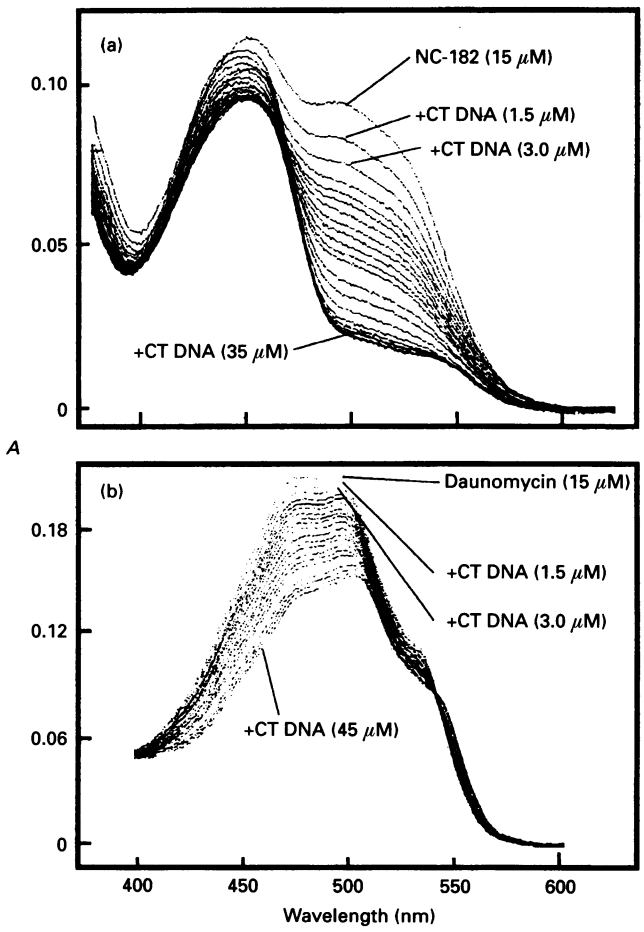


Figure 3 Absorption spectra for NC-182 (a) and daunomycin (b) in the presence of varying amounts of calf thymus (CT) DNA

and presence of calf thymus DNA are shown in Figure 3(a) and Figure 3(b) respectively; similar spectral behaviour was also observed for poly(dA–dT) and poly(dG–dC). In proportion to the concentration of DNA added, the concomitant hyper- and hypo-chromic effects were observed in the region around 450 and 500 nm for NC-182 respectively. The hypochromic band ($\lambda_{max.}$ 500 nm in the absence of DNA) showed a small shift towards the lower wavelength ($\lambda_{max.}$ 507 nm in the presence of 35 μM calf thymus DNA) without showing any isosbestic point. This result would be indicative of the intercalative binding of NC-182 into DNA with the multiple binding modes. In contrast, the respective absorptions of daunomycin at around 480 and 550 nm showed conversely the hypo- and hyper-chromic effects, a typical intercalative behaviour with a shift of absorption band towards longer wavelength and an isosbestic point (Wilson et al., 1988). This characteristic difference could be primarily due to the different electronic states of both drugs, and may reflect their different binding modes with DNA. The Scatchard (1949) plot of R'/C against R', where C is concentration of free drug and R' is the molar ratio of DNA-bound drug (C_b) to DNA base-pair, showed non-linear curves, as exemplified in Figure 4. Thus the results were analysed using the following two-classes-of-sites model (Complex I and II), where two independent non-co-operative binding sites are postulated (Mais et al., 1974; Rosenthal, 1967):

$$\frac{R'}{C} = \frac{n_1 K_1}{1+K_1 C} + \frac{n_2 K_2}{1+K_2 C} \quad (1)$$

where n_1 and n_2 are the numbers of primary and secondary sites with corresponding association constants K_1 and K_2. The concentration of free drug is defined as $C = C_t - C_b$, where C_t represents the total concentration of drug. The concentration C_b was calculated from hypochromic data at 500 nm as described previously (De Fontane et al., 1977; Rao et al., 1988; Aich et al., 1992).

Concerning the DNA binding of aromatic amines such as ethidium, generally, it has been considered that there are two different binding modes, the intercalative (Complex I) and electrostatic (Complex II) bindings, which are dominant at low and high concentrations of drug, respectively (Saenger, 1984).

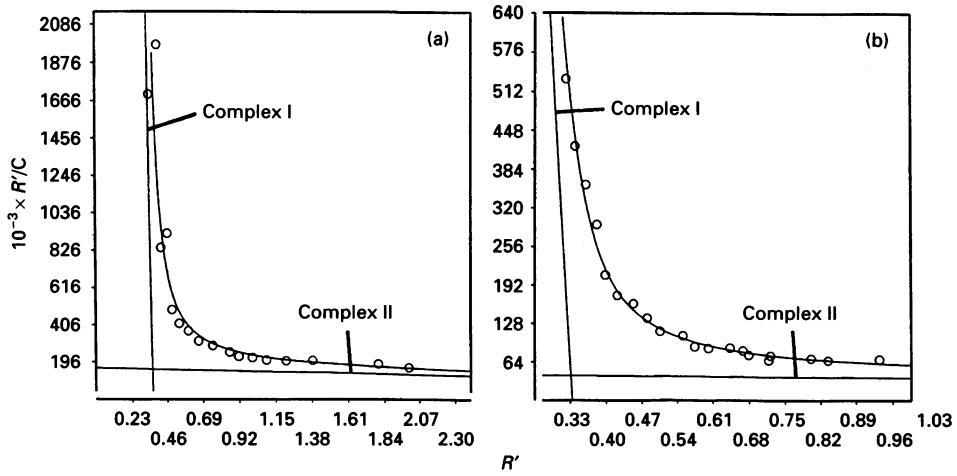


Figure 4 Binding curves generated from two lines of Complexes I and II for NC-182 (a) and daunomycin (b)

Open circles represent experimental values.

Table 2 Binding constants and their estimated S.D. values for NC-182-DNA (complex I), together with those for daunomycin–calf thymus DNA

	$10^{-7} \times K_1$ (M^{-1})	n_1	$10^{-7} \times k_{app.}$ (M^{-1})
Poly(dA–dT)	3.11 ± 1.15	0.397 ± 0.069	1.18 ± 0.28
Poly(dG–dC)	4.97 ± 1.88	0.456 ± 0.058	2.38 ± 1.07
Calf thymus DNA	3.80 ± 1.35	0.351 ± 0.031	1.32 ± 0.44
Calf thymus DNA + daunomycin	2.06 ± 0.93	0.316 ± 0.024	0.651 ± 0.291

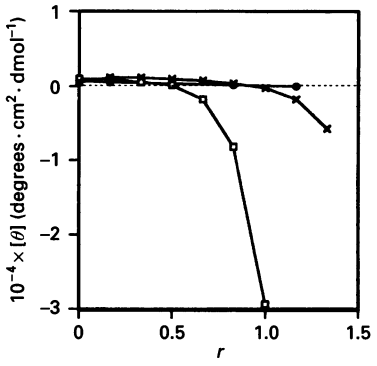


Figure 6 Variation profiles of [θ] as a function of NC-182 concentration at 270 nm for poly(dG–dC) (×), 260 nm for poly(dA–dT) (□) and 270 nm for calf thymus DNA (●)

The former and latter bindings are expressed by the first and second terms on the right-hand side of eqn. (1) respectively. The R/C versus C values were fitted to the equation using a non-linear regression program giving the best-fit values for binding parameters; the fitting curve calculated from these parameters shows that the experimental data lie within experimental error (Figure 4). The obtained values for n_1, K_1 and $K_{app.}$ of Complex I are listed in Table 2, where $K_{app.}$ represents the apparent binding constant and is calculated from:

$$K_{app.} = n_1 K_1$$

The values of Table 2 clearly show that NC-182 and daunomycin have nearly the same binding ability with DNA and no base-specificity; this is in agreement with the C_{50} values from fluorescence measurements. On the other hand, the binding parameters (n_2 and K_2) in the case of Complex II showed large deviations in the range of $n_2 = 2$–60 and $K_2 = (1\text{–}2) \times 10^3\ M^{-1}$, which would be in part due to non-specific electrostatic interaction.

C.d. binding studies

C.d. spectra were measured to monitor the conformational changes of B-, Z- and A-form duplexes and triplex DNA structures in the presence of NC-182; no c.d. band was observed in NC-182 alone.

(1) B-form DNA

C.d. spectra of B-form poly(dG–dC), poly(dA–dT) and calf thymus DNA in the absence and presence of NC-182 are shown in Figure 5. The formation of a complex between the NC-182 and DNA was confirmed from the observation of a band emerging from the 320–450 nm region. The c.d. bands specific to poly(dG–dC) and poly(dA–dT) B-form structures changed with increasing concentrations of NC-182. The positive band at ~ 280 nm was slightly increased in the range of $r = 0 - 0.3 \sim 0.7$ and then decreased drastically at $r > 1.0$ (Figure 6). Contrastly, the negative band at ~ 250 nm decreased in the range of $r =$ 0–0.3 ~ 0.7 and then increased linearly with the concentration of NC-182 at $r > 1.0$. In the former phase of $r = 0$–0.3 [for poly(dA–dT)] or 0–0.7 [for poly(dG–dC)], NC-182 is thought to render the B-form structure rigid, because the degree of c.d. ellipticity is directly dependent on the extent of stacking into

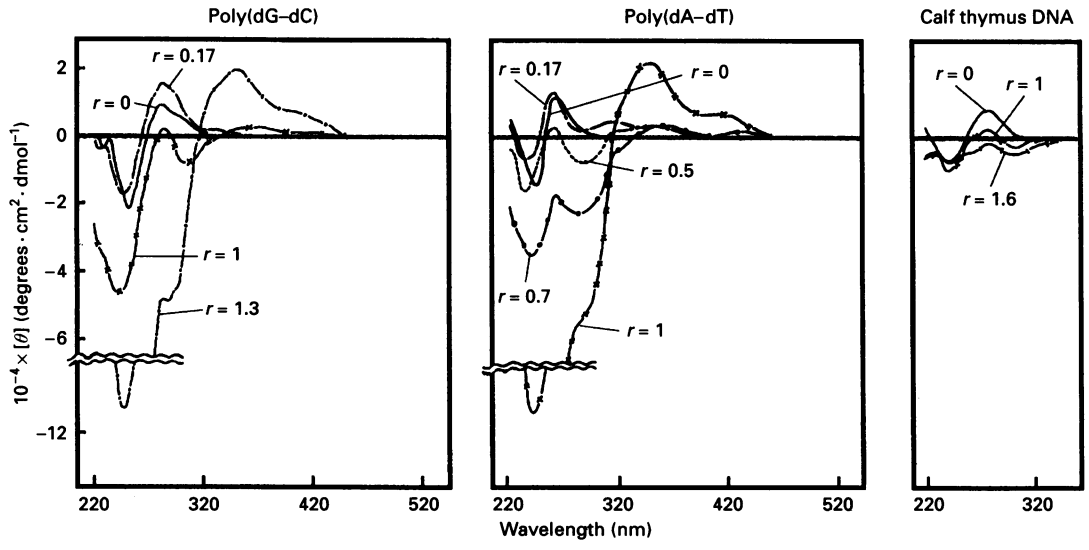


Figure 5 C.d. spectra for B-form poly(dG–dC), poly(dA–dT) and calf thymus DNA as a function of NC-182 concentration

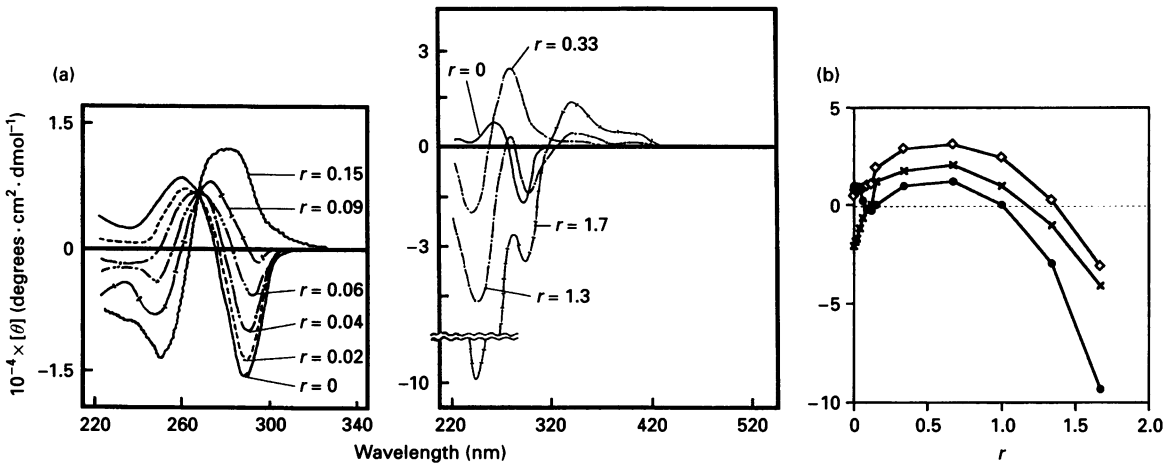


Figure 7 (a) Circular dichroism spectra for poly(dG–m^{5}dC) Z-form structure as a function of NC-182 concentration and (b) variation profiles of [θ] as a function of NC-182 concentration at 260 nm (●), 275 nm (◇) and 290 nm (×) for poly(dG–m^{5}dC)

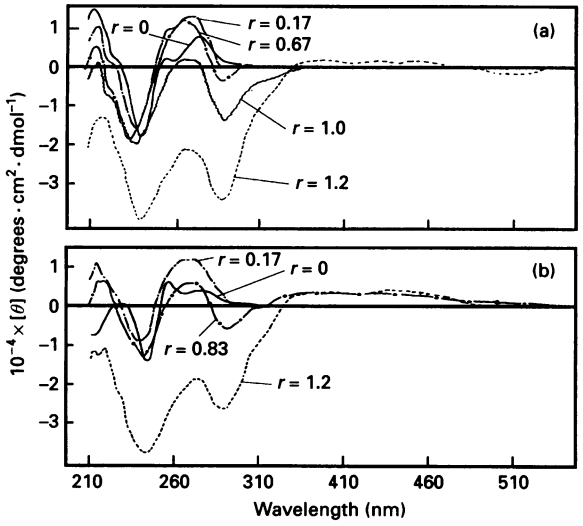


Figure 8 C.d. spectra for poly(dA)·2poly(dT) of the B-form duplex structure (a) in the absence of $MgCl_2$ and of the triples structure (b) in the presence of $MgCl_2$, as a function of NC-182 concentration

DNA bases (Lerman, 1961). The spectral change at $r > 1.0$ can be interpreted in terms of the transformation of the B-form to a non-B-form structure of DNA, which is characterized by an intense negative Cotton band (Zimmer et al., 1982). Since such a spectral change was not observed for daunomycin, this was thought to be NC-182 specific; a similar c.d. change was also observed for Trp-P-1, a mutagenic tryptophan pyrolysate (Inohara et al., 1993). In contrast with these polynucleotides, no significant spectral changes were observed for calf thymus DNA. This result is in conflict with those obtained by fluorescence and absorption measurements, where nearly the same binding affinity of NC-182 to these DNAs was suggested. Although a satisfactory explanation for this discrepancy is not given here, Figure 5 may reflect the essentially different binding modes (including intercalation and electrostatic interactions) between poly(dG–dC) or poly(dA–dT) consisting of alternating purine–pyrimidine sequences and calf thymus DNA without the alternating sequence.

(2) Z-form DNA

The c.d. spectral changes in the left-handed Z-form poly(dG-m^{5}dC) upon interaction with NC-182 are shown in Figure 7(a).

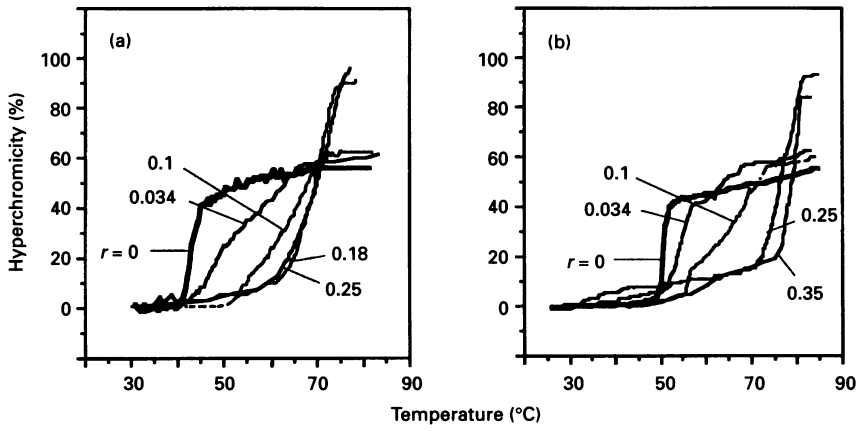
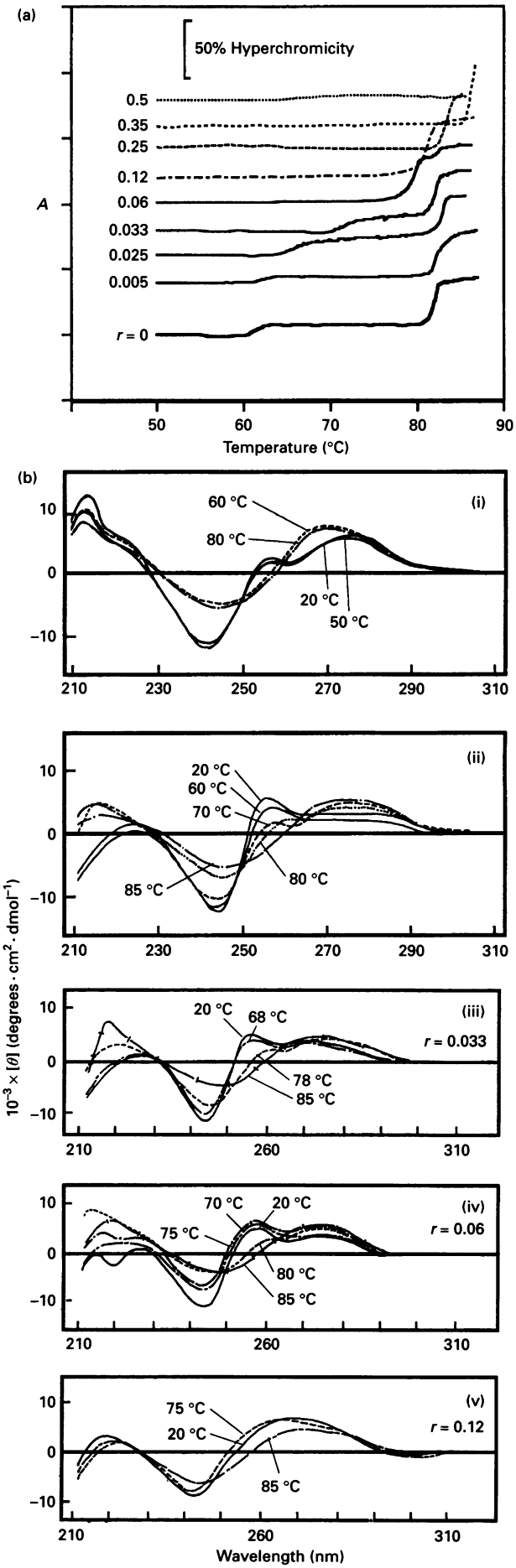


Figure 9 U.v. melting curves at 260 nm for poly(dA–dT) (a) and poly(dA)·poly(dT) (b) as a function of molar ratio with NC-182



This Figure clearly shows that NC-182 promotes the unwinding of the Z-form DNA to the B-form. Upon the addition of NC-182 to the DNA solution, the c.d. spectrum characteristic of Z-form DNA (a negative band at ~ 280 nm and a positive band near 260 nm) was transformed into the B-form spectrum in the range of $0 \leqslant r \leqslant 0.15$; a similar conversion from Z- into B-form DNA has been observed in the interaction with ethidium (Walker et al., 1985) and daunomycin (Chaires, 1983). After the completion of conversion from the Z-form into the B-form at about $r = 0.33$, the B-form structure was further transformed into the non-B-form structure (Fig. 7b) in a similar manner to that shown in Figure 5.

(3) A-form RNA

No notable c.d. spectral change was observed for the A-form poly(rA)·poly(rU) in the absence and presence of NC-182 (results not shown). On the basis of results of the ethidium-displacement experiment (Table 1), it is supposed that the binding of NC-182 to poly(rA)·poly(rU) RNA is not as strong as that to B-form DNA, indicating the DNA-selective binding of this drug.

(4) Triplex DNA

The c.d. spectrum of poly(dA)·2poly(dT) shows typical triplex and duplex structures in the presence and absence of $MgCl_2$, respectively (Scaria and Shafer, 1991; Durand et al., 1992; Park and Breslauer, 1992) (Figure 8). The c.d. spectra of the duplex and triplex structures are significantly altered in the presence of NC-182. In particular, the positive bands in the region of 250–300 nm increase their ellipticities in the range of $0 \leqslant r \leqslant 0.17$, with the concomitant disappearance of c.d. bands differentiating the duplex structure from a triplex one. When $t > 0.2$, the positive bands begin to decrease in proportion to r, and changes in their c.d. spectra are similar to those of B-form poly(dA–dT) or poly(dG–dC) in the range of $0.2 \leqslant r \leqslant 1.2$, with the exception of the c.d. spectra of poly(dA)·2poly(dT) being characteristically saturated at $r = 1.2$. The result clearly shows that NC-182 also interacts with the triplex DNA and modifies its tertiary structure.

Effect of NC-182 on DNA thermal stability

Figure 9 shows the change of u.v. melting curves (at 260 nm) of poly(dA–dT) and poly(dA)·poly(dT) with NC-182 concentration. The increase of the melting temperature (T_m) of poly(dA–dT) was observed in proportion to NC-182 in the concentration range of $0 < r \leqslant 0.18$. T_m increased from 40 °C ($r = 0$) to 70 °C ($r = 0.18$) and saturated at $r > 0.18$. The saturation value of $r = 0.18$ is in good agreement with $n_1 = 0.397$ given in Table 2. A similar increase in T_m was also observed for the poly(dA)·poly(dT), where about 30 °C was raised in the range of 0 ($T_m = 50$ °C) $< r <$ 0.35 ($T_m = 80$ °C). The melting curve at $r = 0.1$ shows a biphasic transition of T_m. This phenomenon may be due to the difference in conformation between the drug-bound and -unbound DNAs, which reflects the unsaturated DNA binding of the drug, as has been frequently observed in the T_m experiment of DNA (Breslauer et al., 1987; Pemeta et al., 1993).

The u.v. melting profiles of poly(dA)·2poly(dT) triplex structure, together with its c.d. spectra at different temperatures, are shown in Figure 10. The triplex structure (Figure 10a, $r = 0$) of

Figure 10 (a) Thermal denaturation profiles of poly(dA)·2poly(dT) triplex structure with NC-182 concentration and (b) influence of temperature on the c.d. spectra of poly(dA)·2poly(dT) in the absence (i) or presence (ii) of $MgCl_2$ and in the triplex structure–NC-182 complexes (iii–v)

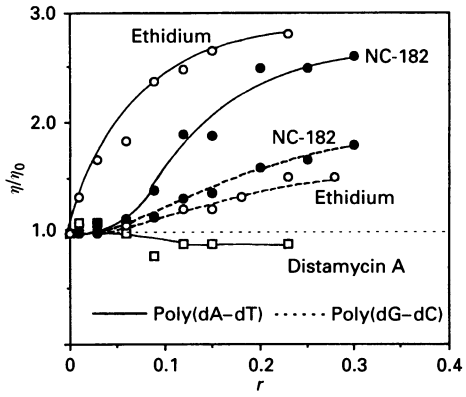


Figure 11 Viscometric titrations of B-form poly(dA–dT) (——) and poly(dG–dC) (------) with drugs

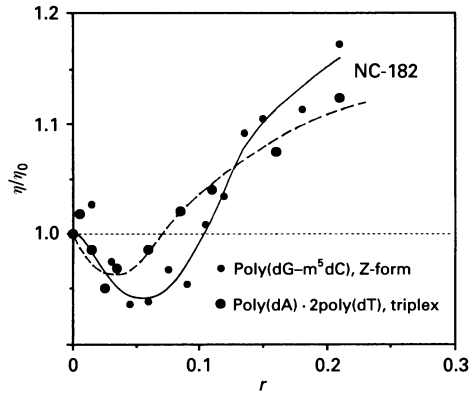


Figure 12 Viscometric titrations of NC-182 for Z-form poly(dG–m^{5}dC) duplex structure (●) and poly(dA)·2poly(dT) triplex structure (●)

poly(dA)·2poly(dT) in the presence of $MgCl_2$ shows a two-state transition curve at 60 °C and 80 °C (Park and Breslauer, 1992). Since the triplex structure of poly(dA)·2poly(dT) is thought to consist of the Hoogsteen-type binding of the third poly(dT) to the Watson–Crick-type poly(dA)·poly(dT), the former and latter T_m values may be due to the transitions of poly(dA)·2poly(dT) triplex structure ⇒ poly(dA)·poly(dT) duplex structure + poly(dT) and of poly(dA)·poly(dT) duplex structure ⇒ poly(dA) + poly(dT) respectively. This interpretation is also supported by the c.d. spectra at different temperatures. In Figure 10(b) the c.d. spectrum (ii) of poly(dA)·2poly(dT) triplex structure at 20 °C shows two characteristic positive bands at 227 and 255 nm, while the former band shifts to 217 nm and the intensity of the latter band is decreased in the case of duplex structure (i) (Scaria and Shafer, 1991; Durand et al., 1992; Park and Breslauer, 1992). On the other hand, the spectrum of the triplex structure is transformed into that of the duplex structure at temperatures between 60 and 80 °C, and into that of a random coil at more than 80 °C.

On the other hand, the addition of NC-182 affects the melting process of triplex poly(dA)·2poly(dT) significantly. The u.v. melting profiles of the triplex as a function of r are shown in Figure 10(a). Comparison of these profiles reveals that the binding with NC-182 thermally stabilizes the triplex structure of poly(dA)·2poly(dT) ($\Delta T_m = +20$ °C between $r = 0$ and $r = 0.12$), but destabilizes the duplex structure ($\Delta T_m = -3$ °C). This is in contrast with the result of netropsin, a typical DNA groove binder (Park and Breslauer, 1992), where the drug stabilizes the duplex structure, but destabilizes the triplex structure. This suggests that the u.v. melting measurement of the DNA triplex structure is a useful approach for determining the type of drug binding. Similar stabilization of the triplex structure has also been observed for an intercalator, ethidium bromide (Scaria and Shafer, 1991).

The comparison of thermal-denaturation profiles with their c.d. spectra [Figure 10b(iii)] at $T = 68$ °C (triplex structure), 78 °C (duplex structure) and 85 °C (random coil) indicates that as a result of the stabilization of the poly(dA)·2poly(dT) triplex structure by binding with NC-182, its melting point approaches that of the duplex structure; the melting curves of the DNA triplex and duplex structures coincide at $r = 0.06$. The u.v. melting profile at $r > 0.06$ reveals that the two-state melting process of the poly(dA)·2poly(dT) triplex structure is fused together into a one-step melting process in which the c.d. spectrum of the pre-melting state at 75 °C no longer shows the typical triplex structure. This may indicate that the NC-182 binding becomes saturated at $r = 0.06$ and the co-existence of the drug of $r > 0.06$ causes the structural change of the triplex structure into the single one, accompanied by the melting process.

Viscometric titrations

The viscometric titrations of drugs for B-form poly(dA–dT) and poly(dG–dC) are shown in Figure 11. The viscosity of DNA was significantly increased by ethidium, but not by distamycin. The increase of DNA viscosity is primarily due to the lengthening of DNA structure by the intercalation of drug (Scaria and Shafer, 1991). As judged from the η/η_0 (where η is the specific viscosity of a polymer–drug complex and η_0 is the specific viscosity of polymer alone) profiles measured as a function of r, the binding of NC-182 is clearly intercalative for poly(dA–dT) and poly(dG–dC). The increasement of η/η_0 value produced by drug binding is most significant for B-form DNA, and is within the range typically observed for intercalators (1.9 ± 0.3) (Waring, 1981; Wilson and Jones, 1981). However, the increase for poly(dG–dC) is small compared with that for poly(dA–dT). This reflects different unwinding angles of the two DNA duplexes, which is caused by the intercalation of the drug, and would be resulted from different stiffness of their tertiary structures.

Viscometric titrations of NC-182 for Z-form poly(dG–m^{5}dC) duplex structure and poly(dA)·2poly(dT) triplex structure are shown in Figure 12. The η/η_0 values for both structures increased at the NC-182 concentration of $r > 0.1$, suggesting the intercalation of this drug into these DNAs. On the other hand, the phenomenon commonly observed in these titration curves is that the viscosity decreases in the early stage of titration ($0 < r < 0.1$) and increases beyond this concentration range. From c.d. measurements of the Z-form DNA duplex structure and from melting profiles of DNA triplex structure, it is reasonable to consider that the initial decrease of the η/η_0 values corresponds to the transformation of the initial structures to the B-form DNA, and the subsequent increase results from the intercalation of NC-182 into the B-form DNA.

Conclusion

Systematic spectroscopic and viscometric studies on the DNA–NC-182 inter-reaction have demonstrated the following. (1) NC-182 is a potent DNA intercalator with nearly the same binding ability as daunomycin and has no base specificity. (2) The mode of interaction of NC-182 with DNA depends on the concentration

of the drug, where the intercalative and electrostatic bindings are dominant at low and high concentrations of the drug respectively. (3) The binding of NC-182 to B-form DNA functions biphasically depending on the molar ratio (*r*) of drug to DNA, i.e. rendering the B-form DNA structure rigid at low *r* values and the transformation of the B-form to a non-B-form at high *r* values. (4) NC-182 promotes the unwinding of the Z-form DNA to a B-form. (5) The thermal stabilization of the DNA duplex structure is increased by interaction with NC-182. (6) The triplex structure of poly(dA)·2poly(dT) is thermally stabilized by binding with NC-182. These observations are important for understanding the biological property of this novel benzo[*a*]phenazine derivative as a new anti-tumour agent against multidrug-resistant and sensitive tumours.

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