SANGUINARINE: A MONOFUNCTIONAL INTERCALATING ALKALOID

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1. Introduction

Sanguinarine (fig.1), a benzophenanthridine alkaloid, has been reported to possess anti-tumour and anti-microbial activity [1]. It inhibits both photosynthetic phosphorylation associated with ferricyanide reduction and cyclic photophosphorylation catalyzed by phenazine methosulphate [2]. The alkaloid increases the intrinsic viscosity of DNA and uncouples respiration and oxidative phosphorylation in rat liver mitochondria [3]. These physical and biochemical effects can be attributed to the formation of a complex of sanguinarine with DNA. We report here a quantitative aspect of the binding of sanguinarine to calf thymus DNA from the measurements of absorption spectrophotometry, equilibrium dialysis, melting behaviour, viscosity, circular dichroism and spectrofluorimetric titration. We also compare our experimental data with ethidium bromide under identical experimental conditions and conclude that sanguinarine (like ethidium) binds by monofunctional mode of intercalation.

2. Materials and methods

Highly polymerized calf thymus DNA (type I) and ethidium bromide were purchased from Sigma

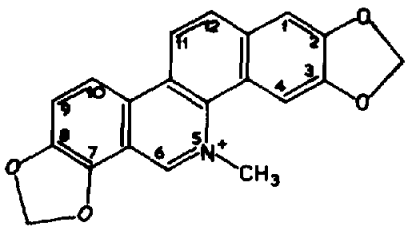

Fig.1. Chemical structure of sanguinarine.

(St Louis MO). Chemically pure sanguinarine chloride was kindly supplied by Dr S. B. Mahato of this Institute. The concentrations of the DNA solutions are stated in terms of nucleotide phosphorus calculated from an extinction coefficient of 6600 $M^{-1} . cm^{-1}$ at 260 nm [4]. The drug concentrations were determined spectrophotometrically by using the following extinction coefficients: ethidium, 5680 $M^{-1} . cm^{-1}$ at 480 nm [5]; sanguinarine, 15 140 $M^{-1} . cm^{-1}$ at 325 nm [6]. All binding experiments were conducted in 10 mM Tris–HCl (pH 7.4) + 100 mM NaCl (THS buffer) at 22°C. For thermal melting experiments, 10 mM Tris–HCl (pH 7.4) + 10 mM NaCl (0.1 THS buffer) was used. Glass-distilled deionized water and analytical grade reagents were used throughout.

The absorption spectra of drugs mixed with or without DNA were obtained using the Zeiss automatic recording spectrophotometer 'Specord UV–Vis' against an appropriately prepared reference sample. Fluorescence measurements were recorded in an Aminco Bowman spectrophotofluorometer. The values of emission maxima reported here were taken from uncorrected emission spectra. Fluorescence titration of DNA with ethidium and sanguinarine was performed with the excitation 510 nm and 330 nm, respectively. The emission wavelengths were 610 nm and 420 nm, respectively. More detailed studies on the fluorescence of sanguinarine will be reported elsewhere. Spectrophotometric titration, equilibrium dialysis and spectrofluorimetric titration of the drug/DNA mixture were carried out following [4,5,7–10]. Thermal melting profiles were determined using a Zeiss spectrophotometer VSU 2 fitted with a thermostatic-controlled bath type U1 as in [4]. The viscometric measurements of DNA were performed with a type 75 Cannon Manning semi-microviscometer at 22 ± 0.2°C. A quantitative analysis of the viscosimetric measurements using various published proce-

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dures or equations are described in section 3. The sheared DNA used for viscosimetric studies was prepared by using an MSE sonicator as in [4]. These gave calf thymus DNA with an intrinsic viscosity (η_o) of 2.1 dl/g from which the M_r-value was estimated to be ~3.5×10^5. Circular dichroism (CD) spectra were recorded in a JASCO spectropolarimeter model J-20 A with: λ expansion, 10 nm/cm; time constant, 16; scan speed, 2 cm/min; cell length, 10 mm).

3. Results

The effect of progressive increasing concentration of DNA on the absorption spectra of sanguinarine is shown in fig.2. The spectral changes involved essentially a progressive red shift and hypochromicity in the complex until saturation was recorded at sanguinarine/DNA ratio of 0.16 (fig.2). A maximum red shift of 15–17 nm was observed at a sanguinarine/DNA ratio of 0.16 or lower. No significant change in the absorption pattern and, more particularly, no blue shift resulted at a very high value of sanguinarine/DNA ratio. The drugs obeyed Beer's law in the concentration range used.

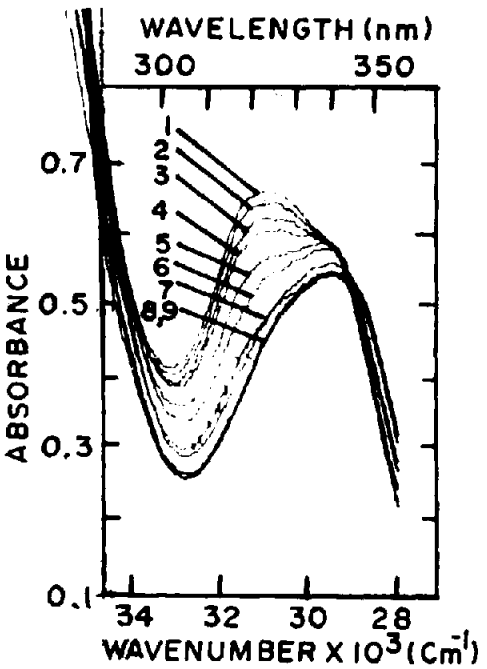

Fig.2. Influence of calf thymus DNA on the absorption spectrum of sanguinarine in THS buffer. Curve (1) sanguinarine (43 μM) treated with varying concentrations of DNA as shown by curves (2–9) denoting 10.1, 30.2, 50.3, 100.6, 151.5, 201.2, 301.8 and 430 μM DNA, respectively.

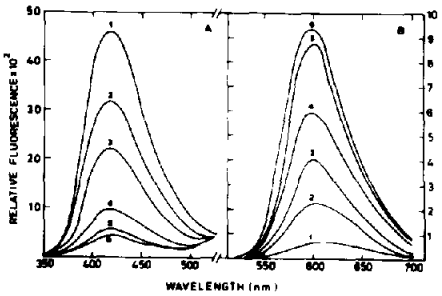

Fig.3. Fluorescence spectra resulting from the interaction of (A) sanguinarine and (B) ethidium with calf thymus DNA: (A) curve (1) sanguinarine (32 μM) treated with varying concentrations of DNA as shown by curves (2–6) denoting 29.7, 59.5, 149.1, 211.2 and 448 μM DNA, respectively; (B) curve (1), ethidium (30 μM) is treated with varying concentrations of DNA as shown by curves 2–6 denoting 24, 55, 87, 144 and 450 μM DNA, respectively.

The strong enhancement of fluorescence spectra of ethidium with increasing concentration of DNA is shown in fig.3B. A different spectral distribution was found for sanguinarine–DNA complex. Fig.3A shows the progressive quenching effect of DNA on the fluorescence of sanguinarine at 420 nm with increasing concentration of DNA.

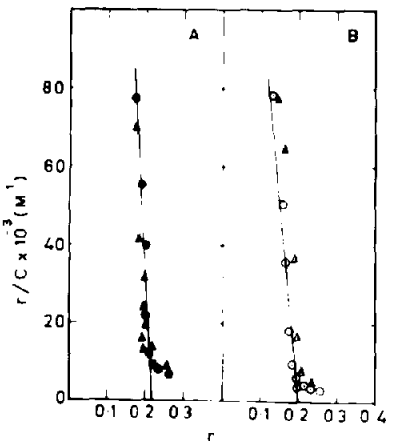

Fig.4. Scatchard plots for the binding of (A) sanguinarine and (B) ethidium to calf thymus DNA: r, ratio of mol ligand bound/mol nucleotides; C, the molar concentration of free ligand in solution; (——) initial slope of binding isotherms; data points are shown for titration of calf thymus DNA by spectrophotometry (●,○) and spectrofluorimetry (▲,△). Sanguinarine and ethidium are 32 and 30 μM, respectively, in each experiment.

Table 1
Fluorescence and equilibrium binding parameters of the interaction of DNA–sanguinarine and DNA–ethidium complexes

Parameters	Methods	Sanguinarine		Ethidium	
		Free	Bound	Free	Bound
Absorption maxima (nm)	Spectrophotometry	325	342	480	520
Emission maxima (nm)	Spectrofluorimetry	420	420	610	600
Relative intensity	Spectrofluorimetry	1	0.09	1	16
k_1 (M^{-1})	Spectrophotometry		1.9×10^6		1.18×10^6
	Equilibrium dialysis		2.0×10^6		1.4×10^6
	Spectrofluorimetry		1.9×10^6		1.55×10^6
n_1 (no. binding sites/nucleotide)	Spectrophotometry		0.22		0.20
	Equilibrium dialysis		0.22		0.20
	Spectrofluorimetry		0.22		0.20

The binding isotherm (Scatchard plot) obtained from spectrophotometric and spectrofluorimetric titration data are shown in fig.4 and quantitative results on binding parameters are presented in table 1. Under the present experimental conditions, the binding of ethidium and sanguinarine could be resolved into two components: one with high average intrinsic binding constant (k_1); the other with a low intrinsic binding which was not evaluated due to insufficient data. The present value of k_1 is in agreement with single binding constant for ethidium derived from absorbance [9] and fluorescence [5,10].

The melting temperatures (T_m) of calf thymus DNA (60 μM) in 0.1 THS buffer in absence and presence of sanguinarine (16 μM) were 68°C and 91°C, respectively and that in presence of ethidium (16.5 μM) was 90.5°C.

The viscosimetric titrations of sonicated calf thymus DNA (450 μM) were done by sanguinarine and ethidium. The experimental values for the ratio of the reduced viscosity of the complex ($\eta = \eta_{sp}/C$, where η_{sp} is the specific viscosity) to that of native DNA (η_o) when plotted against drug/DNA, showed a very steep rise and a saturation at drug/DNA = 0.3 which remained unchanged up to the maximum drug/DNA value of ~0.50. The same ratio when plotted against r calculated experimentally from the measurements of spectrophotometric, spectrofluorimetric and equilibrium studies, showed linearity only up to $r = 0.17$.

For confirmation of the monofunctional intercalative mode of interaction between sanguinarine and DNA, the effect of sanguinarine treatment on viscosity of the sheared and rod-like DNA was studied. The increase of sheared DNA helix length upon intercalation of sanguinarine can be calculated from the experimental results of the intrinsic viscosities according to the approximation [13]:

$$L/L_o \cong (\eta/\eta_o)^{1/3} = 1 + \beta r$$

where:

L and L_o = the contour lengths of the DNA in presence and absence of the drug, respectively;

η and η_o = the corresponding values of intrinsic viscosity (approximated by the reduced viscosity) of the solution;

r = mol ligand bound/mol nucleotides;

β = the slope when L/L_o or $(\eta/\eta_o)^{1/3}$ is plotted against r.

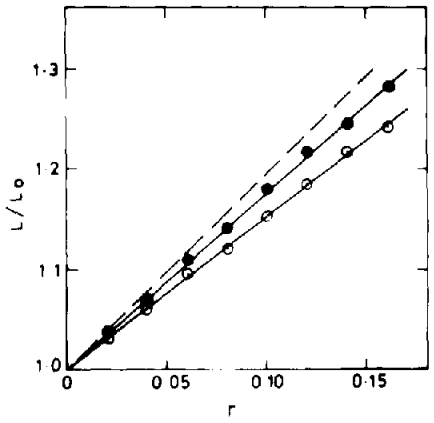

Fig.5. Influence of sanguinarine (•) and ethidium (○) on the relative contour length of sheared rod-like calf thymus DNA. The ordinate represents the fractional increase in helix length and abscissa shows the binding ratio (r). The theoretical lines (– – –) represent a slope of 2, predicted for perfect monofunctional intercalators.

The intercalating model of Lerman [14] predicts that for a perfect monofunctional and a bifunctional intercalator, the β-value should be 2 and 4, respectively. As shown in fig.5 when (L/L_0) is plotted against r, the β-values obtained from the slope were found to be 1.66 for ethidium and 1.8 for sanguinarine. The β-value for ethidium is in agreement with the experimental value of 1.79 [11] and 1.7 [12] reported for calf thymus DNA.

The changes observed in the CD spectrum of DNA in presence of sanguinarine and ethidium are illustrated in fig.6. The ethidium and sanguinarine both affect the CD spectrum of DNA in the same manner and cause large symmetrical increase in ellipticity of both major bands in the CD spectrum of DNA. Since neither the dye nor the alkaloid have an absorption band in this region, it would appear that these changes are the result of similar change in the DNA helical structure caused independently by both sanguinarine and ethidium. This suggests that the mode of sanguinarine binding to DNA is similar to the intercalative binding of ethidium with DNA.

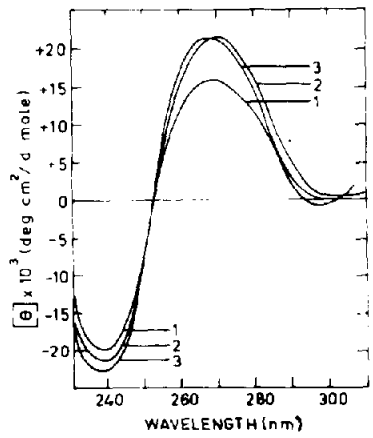

Fig.6. CD spectra of calf thymus DNA (1), DNA–sanguinarine complex (2) and DNA–ethidium complex (3) in THS buffer; DNA was 240 μM, sanguinarine 50.4 μM and ethidium 48 μM.

4. Discussion

The strong interaction of sanguinarine with DNA was evident from the observation of the quenching effect of calf thymus DNA on the absorbance and fluorescence of sanguinarine and of the elevation of T_m and also of the specific changes in viscosity of the sanguinarine treated DNA molecules. In addition to these changes sanguinarine has been shown to alter the CD spectrum of DNA. This finding thus indicates that like ethidium the sanguinarine shows a strong interaction and is believed to involve intercalation of the alkaloid molecule into the adjacent basepair of the DNA [3].

The viscosimetric technique is well established as a method for investigating the extension of the DNA helix associated with intercalation [11–13,15,16]. The slope β in the L/L_0 *vs* r plot (fig.5) is a parameter related to the fractional increase in the contour length of the rod-like DNA molecule induced by intercalative agents. The intercalative model in [14] requires that the helix be extended by an amount equal to the thickness of a basepair (3.4 Å) for each intercalation event. If all bound drugs bind to DNA by intercalation, the β-value should be 2 for monofunctional intercalator and 4 for bifunctional intercalator. Here, the β-value of sanguinarine (1.8) is very close to the theoretical value for monofunctional intercalator, suggesting a perfect monofunctional intercalation for linear rod-like DNA molecules. Moreover, preliminary studies indicate that sanguinarine inhibits the growth and macromolecular synthesis of *Vibrio cholerae* cells at a very low concentration (unpublished). However, the exact mode and mechanism of the anti-microbial and anti-tumour action of sanguinarine are not known.

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