Design, synthesis and DNA-binding capacity of a new peptidic bifunctional intercalating agent

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A lysyl-lysine bifunctional derivative of 9-aminoacridine has been synthesized and its DNA-binding capacity established by electron-paramagnetic-resonance study. For this purpose the binding parameters of a spin-labelled aminoacridine probe were estimated and the affinities of the lysylacridinyl-lysyl-diamino-octane dimer and of 9-aminoacridine could be evaluated by competitive assays. The competition study provided quantitative results concerning the dissociation constant (K_D) of the dimer. The obtained value was closely similar to the K_D of 9-aminoacridine determined by the same method and to the K_D previously reported for the anti-tumour and antibiotic bifunctional intercalator quinomycins.

The quinoxaline antibiotics, quinomycins (Keller-Schierlein *et al.*, 1959; Dell *et al.*, 1975; Martin *et al.*, 1975) and triostins (Otsuka & Shoji, 1966; Otsuka *et al.*, 1976), are powerful antimicrobial and anti-tumour agents (Katagiri *et al.*, 1975). It is generally agreed that their biological activities are linked to their ability to interact with DNA, and in fact they have been shown to act as bifunctional intercalating agents (Waring & Wakelin, 1974; Wakelin & Waring, 1976). Detailed mechanisms have been proposed for the binding of echinomycin to DNA (Wakelin & Waring, 1976; Ughetto & Waring, 1977). It was reported that the quinoxaline chromophores involved in the intercalation process bound only very weakly to DNA, when studied as monomers. It was also established that the distance between the two quinoxaline moieties was about 1 nm (10 Å) (Cheung *et al.*, 1978) corresponding to the neighbour-exclusion binding mode for intercalative binding to DNA (Crothers, 1971; Wakelin & Waring, 1976).

We decided to design a nucleic-acid-binding intercalator based on the above considerations. In this system, 9-aminoacridine was chosen as the intercalative agent because of its well-known antibacterial activity, which has been attributed to its binding by bacterial DNA (Albert, 1966; Blake & Peacocke, 1968). On the other hand, the high experimental anti-leukaemic activity of some simple

derivatives of 9-aminoacridine has been described (Denny & Cain, 1978).

In our model (Fig. 1) both chromophores were covalently linked to one of the side chains of the dipeptide lysyl-lysine. The other N(ε) atom remained free in view to form an electrostatic binding to negatively charged phosphate groups on the nucleotide.

Dimerization was obtained by covalent linkage to diamino-octane. The length of the chain was determined in part by model building so that the distance between the two chromophores was about 1 nm (10 Å) as in echinomycin. This aliphatic moiety could fit the molecule into the narrow groove of the helix, whereas the peptide portion could provide numerous hydrogen bondings with the deoxyribose phosphate backbone.

Particular attention has been paid with respect to symmetry in the design of our model. The role of twofold symmetry as an important element in the recognition of the DNA helix and in the inhibition of the RNA polymerase reaction has been pointed out for actinomycin D (Sobell *et al.*, 1971) and for regulatory proteins (Francklin, 1974; Adhya *et al.*, 1974). Apparent twofold symmetry of α-amanitin (Plancke *et al.*, 1980), echinomycin (Wakelin & Waring, 1976) and triostin (Otsuka *et al.*, 1976) appears to play a similar role.

The binding affinity of the dimer to DNA was compared with that of 9-aminoacridine. As an approach to the quantitative analysis of the phenomenon, we have examined the competitive bind-

Abbreviations used: Boc, t-butoxycarbonyl; Z, benzyl-oxy-carbonyl.

Fig. 1. Perspective drawing views of echinomycin and bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane

ing between 9-aminoacridine and a spin-labelled aminoacridine derivative on the one hand, and the competition between the dimer and the same spin-carrying probe spin-labelled 9-aminoacridine on the other.

Experimental procedure

Materials

The purity of all the compounds was tested by t.l.c. on 5 cm $\times$ 10 cm glass plates precoated with silica gel (0.25 mm thick, F₂₅₄; Merck); the solvent system was chloroform/methanol (4:1, v/v) in a saturated NH₃ atmosphere. Melting-point determinations were performed on a Tottoli melting point apparatus and were uncorrected. I.r. spectra were recorded with a Perkin-Elmer 177 i.r. spectrometer by using KBr pellets. The final bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane was purified by preparative h.p.l.c. on a Prep 500 Waters chromatograph with a Nucleosil C18 support and a methanol/water mixture (4:1, v/v) at a flow rate of 250 ml/min and at a pressure of 17.25 MPa (2500 lbf/in²). Field-desorption mass spectrum of a bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane sample was recorded on a Varian MAT 311A apparatus.

Synthesis of 4-(9-acridinylamino)-2,2,6,6-tetramethyl-1-piperidinyloxy (spin-labelled 9-aminoacridine)

Spin-labelled 9-aminoacridine was obtained by the action of 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy on 9-chloroacridine (Sinha *et al.*, 1976) (m.p. 190°C).

Synthesis of NN'-bis(L-lysyl- ϵ -acridinyl-L-lysyl)-1,8-diamino-octane

NN'-Bis(α -Boc- ϵ -Z-L-lysyl)-1,8-diamino-octane (I). N- α -Boc- ϵ -Z-L-Lysine (7.6 g; 20 mmol) and 1,8-diamino-octane (1.4 g; 10 mmol) were dissolved in dry methylene chloride (100 ml). The solution was cooled to 0°C and NN'-dicyclohexylcarbodi-imide (4.4 g; 24 mmol) and 1-hydroxy-1H-benzotriazole (4.4 g; 33 mmol) added. The mixture was stirred for 3 h at 0°C and then overnight at room temperature. The precipitate of dicyclohexylurea was filtered off, the methylene chloride solution washed successively with 0.1 M-HCl, water and 0.1 M-NaHCO₃ and then dried over anhydrous Na₂SO₄. The organic solvent was evaporated to dryness affording compound I [5.5 g, 64% yield, m.p. 210°C, *R*_F 0.64 (chloroform/methanol, 4:1, v/v)].

NN'-Bis(ϵ -Z-lysyl)-1,8-diamino-octane (II). *NN'*-Bis(α -Boc- ϵ -Z-L-lysyl)-1,8-diamino-octane (I) (5.5 g; 6.3 mmol) was dissolved in trifluoroacetic acid (20 ml) and left at room temperature for 1 h. The solution was evaporated to dryness *in vacuo* (25°C). The residue was taken up with 1 M-NaHCO₃. Crude precipitate was filtered off and washed with diethyl ether to yield compound II [3.5 g, 83%, m.p. 125°C, R_F 0.35; i.r.: ν = 3360 (NH), 2950, 2880 (CH₂), 1700, 1600 (CO) cm⁻¹].

NN'-Bis(α -Z- ϵ -Boc-L-lysyl- ϵ -Z-L-lysyl)-1,8-diamino-octane (III). *NN'*-Bis(ϵ -Z-lysyl)-1,8-diamino-octane (II) (3.5 g; 5.2 mmol) was stirred with α -Z- ϵ -Boc-L-lysine *N*-hydroxysuccinimide ester (4.5 g; 10.6 mmol) in dimethylformamide (20 ml) for 12 h. After removal of the solvent, the residue was taken up with ethyl acetate, giving compound III [4 g, 55%, m.p. 137°C, R_F 0.69; i.r.: ν = 3400, 3360 (NH), 2950, 2880 (CH₂), 1700, 1650 (CO) cm⁻¹].

NN'-Bis(α -Z-L-lysyl- ϵ -Z-L-lysyl)-1,8-diamino-octane (IV). *NN'*-Bis(α -Z- ϵ -Boc-L-lysyl- ϵ -Z-L-lysyl)-1,8-diamino-octane (III) (4 g; 2.8 mmol) was left at room temperature in trifluoroacetic acid (10 ml) for 1 h. The acidic solution was evaporated *in vacuo* (25°C) and the residue taken up with 1 M-NaHCO₃. The crude precipitate was recrystallized from diethyl ether, yielding compound IV [2.5 g, 73%, m.p. 130°C, R_F 0.07; i.r.: ν = 3600 (NH) 1700, 1640 (CO) cm⁻¹].

NN'-Bis(L-lysyl- ϵ -acridinyl-L-lysyl)-1,8-diamino-octane. *NN'*-Bis(α -Z-L-lysyl- ϵ -Z-L-lysyl)-1,8-diamino-octane (IV) (1.2 g; 1 mmol) was heated with 9-chloroacridine (0.44 g; 2 mol) and NaHCO₃ (1 g; 3 mol) in ethanol (20 ml) under reflux for 12 h. After cooling and filtration, the filtrate was refluxed in cyclohexene (10 ml) in the presence of palladium black (0.5 g) for 24 h. The catalyst was removed by filtration and the solvent evaporated to dryness *in vacuo* to give a yellow powder. The purification of the crude product was achieved through column chromatography on Sephadex LH20, with methanol as eluent.

The pure dimer, bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane, crystallized in yellow crystals [0.6 g, 59%, m.p. 188°C, R_F 0.0 in chloroform/methanol, R_F 0.72 in butanol/pyridine/acetic acid/water (15:10:3:12, by vol.); i.r.: ν = 3340, 3320 (NH), 1720, 1650 (CO) cm⁻¹, M^+ = 1010].

DNA solutions

Calf thymus DNA was purchased from Serva Feinbiochemica, Heidelberg, Germany, and was used without purification. DNA (200 mg) was dissolved in 1 litre of 0.1 $\times$ SSC buffer (0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0).

The concentration expressed in terms of the nucleotide as a unit of DNA was determined spectrophotometrically at 260 nm by using a molar

absorption coefficient of 6600 M $\cdot$ cm⁻¹ (Mahler *et al.*, 1964).

Methods

E.p.r. measurements were recorded on a Varian E-109 X-band spectrometer with an E-238 cavity operating in the TM₁₁₀ mode. A 100 KHz high-frequency modulation was used with a 20 mW microwave power. The sample solutions were dispensed into a flat quartz cell.

The preparation of drug-DNA complex in solution was performed by stirring a solution of spin-labelled 9-aminoacridine with the DNA solution at room temperature. The mixture could then be studied directly.

To obtain a spectrum of the probe bound to DNA, spin-labelled 9-aminoacridine was stirred in DNA solution at room temperature, the mixture filtered with an Amicon 52 ultrafilter under 345 kPa (50 lbf/in²) of N₂ gas pressure until no more filtrate was expelled. After washings, the paste-like condensed spin-labelled 9-aminoacridine-DNA complex was dissolved in 0.1 $\times$ SSC and, after rapid equilibration, its e.s.r. spectrum was recorded.

Results

The e.p.r. spectrum of spin-labelled 9-aminoacridine in SSC solution at room temperature (Fig. 2) exhibits three sharp peaks separated by 1.6 mT (16 G) corresponding to free rotation of the nitroxyl radical.

Fig. 2. E.p.r. spectra of free spin-labelled 9-aminoacridine probe in SSC solution (—) and of spin-labelled 9-aminoacridine totally intercalated in DNA (---).

The spectrum of totally bound spin-labelled 9-aminoacridine is clearly different (Fig. 2). A broad peak appears at a lower field value than the low-field peak of free spin-labelled 9-aminoacridine, and a broad trough appears at a higher field value than the high-field peak of free spin-labelled 9-aminoacridine.

Spectra of spin-labelled 9-aminoacridine-DNA complexes at equilibrium with increasing concentrations of spin-labelled 9-aminoacridine are given in Fig. 3. They consist of the sum of spectra from bound and unbound species. The total area of each spectrum is proportional to the spin-labelled 9-aminoacridine concentration, whereas the area of the third narrow band can be considered as 33% of the free spin-label concentration.

Binding of spin-labelled 9-aminoacridine to DNA

By this method, e.p.r. recordings allowed the measurement of bound/unbound probe ratios and the binding parameters of spin-labelled 9-aminoacridine could be evaluated ($K_D = 2 \times 10^{-5} \text{ M}$) by using Scatchard (1949) (as shown in Fig. 4) and double-reciprocal Lineweaver-Burk (1934) plots.

Competitive effect

The dissociation constant of bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane ('BLAD') is given by:

$$K_{D_{\text{BLAD}}} = \frac{F_{\text{BLAD}}}{B_{\text{BLAD}}} \times F_{\text{sites}}$$

where F_{BLAD} and B_{BLAD} are the free and bound species of bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane respectively in the presence of spin-labelled 9-aminoacridine ('SLA') as a competitor, and F_{sites} is the free sites concentration.

F_{sites} can be calculated by:

$$F_{\text{sites}} = K_{D_{\text{SLA}}} \frac{B_{\text{SLA}}}{F_{\text{SLA}}}$$

where F_{SLA} and B_{SLA} , free and bound species of spin-labelled 9-aminoacridine, are measured values and $K_{D_{\text{SLA}}}$, the dissociation constant of spin-labelled 9-aminoacridine determined as above.

B_{BLAD} and F_{BLAD} can be evaluated as:

$$B_{\text{BLAD}} = T_{\text{sites}} - F_{\text{sites}} - B_{\text{SLA}}$$

$$F_{\text{BLAD}} = [\text{BLAD}] - B_{\text{BLAD}}$$

where T_{sites} is the total sites concentration and $[\text{BLAD}]$ is the total BLAD concentration.

The competitive effect was obtained when increasing amounts of bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane ($0.5 \times 10^{-5} \text{ M}$ to $1.5 \times 10^{-4} \text{ M}$) were added to a solution of spin-labelled 9-aminoacridine in the presence of DNA and for each bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane concentration,

Fig. 3. E.p.r. spectra of spin-labelled 9-aminoacridine-DNA (200 mg/litre) complexes at equilibrium corresponding to the following concentrations of spin-labelled aminoacridine: $0.5 \times 10^{-4} \text{ M}$ (a); $1 \times 10^{-4} \text{ M}$ (b); $1.5 \times 10^{-4} \text{ M}$ (c)

B_{SLA} and F_{SLA} were measured, allowing the determination of the dissociation constant for the dimer. The average value was $K_{D_{\text{BLAD}}} = 10^{-5} \pm 10^{-6} \text{ M}$.

In order to compare the affinities of bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane and 9-amino-

Fig. 4. *Scatchard analysis of spin-labelled 9-aminoacridine binding to DNA*

A range of eight concentrations of spin-labelled aminoacridine from $0.5 \times 10^{-5} \text{ M}$ was used. The concentration of the bound spin-labelled 9-aminoacridine was taken as the difference between the total spin-labelled 9-aminoacridine present and the amount that remained free. All data points represent mean values from triplicate determination.

acridine, the dissociation constant of the intercalating monomer was determined under similar conditions of competition between 9-aminoacridine and spin-labelled 9-aminoacridine. In this way, the value obtained for $K_{D_{AA}}$ was $0.5 \times 10^{-5} \pm 10^{-6} \text{ M}$, which is in accordance with previous estimations by other physicochemical means (Drummond *et al.*, 1965; Le Pecq *et al.*, 1975; Yamaoka & Masujima, 1978).

Discussion

By using competitive effects between 9-aminoacridine and spin-labelled 9-aminoacridine on the one hand, and between bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane and spin-labelled 9-aminoacridine on the other, e.p.r. data provide accurate estimates of binding parameters for aminoacridine and for the bifunctional molecule. The dissociation constant of bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane was found to be similar to the corresponding K_D of echinomycin (Wakelin & Waring, 1976). The obtained value indicates that the affinity of bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane for DNA is very close to that for 9-aminoacridine.

The obtained affinity constant of bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane is smaller than the theoretical or observed values in the case of

Fig. 5. *Schematic representation of the different types of intercalation of the dimer bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane in DNA*

dimeric molecules where two aminoacridine molecules are separated by an aliphatic chain (Le Pecq *et al.*, 1975; Gaugain *et al.*, 1978). This result could be explained in terms of steric constraints. It can be considered that the initial interaction between bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane and DNA is the formation of an ionic complex between the side chain of the lysine molecules and the phosphate groups, which dictates the positioning of the intercalative moieties. Furthermore, the rigidity of the peptide bonds could limit the accessibility of the acridine chromophores to the intercalative sites. Under these conditions, several types of intercalation could be postulated, as shown in Fig. 5. In particular, a monointercalation could occur as observed for aliphatic bifunctional intercalators (Gaugain *et al.*, 1978) or bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane could cross-bridge between DNA molecules. Moreover, the participation of electrostatic interactions and steric constraints could

support the hypothesis that the dissociation rate of bis(lysyl- ϵ -acridinyl-lysyl)-1,8-diamino-octane is lower than that of aminoacridine, as previously reported for aliphatic dimers (Capelle *et al.*, 1979) or actinomycins (Müller & Crothers, 1968; Crothers, 1971).

Oligopeptides, which may bind to DNA via stacking of their aromatic side chains and by ionic linkages of their basic side chains, have been the subject of considerable interest for many investigators (Gabbay *et al.*, 1976; Toulmé & Hélène, 1977; Toulmé *et al.*, 1980). Introduction of a well-known intercalative moiety, such as aminoacridine, into a peptidic sequence in appropriate positions as described above, could exhibit interesting pharmacological properties or, at least, can be considered as a simple theoretical model providing information by which anti-tumour antibiotics, such as echinomycin, triostin or actinomycin, may 'recognize' specific sequences of nucleic acid.

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