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ARTICLE

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NMR determination of the hetero-association of phenanthridines with daunomycin and their competitive binding to a DNA oligomer

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Abstract 500 MHz ^{1}H NMR spectroscopy has been used to determine thermodynamic and structural information on the hetero-association of daunomycin (DAU) with the phenanthridine mutagenic dyes ethidium bromide (EB) and propidium iodide (PI). The NMR complexation data have been analysed by a statistical-thermodynamic model which takes into account indefinite association for both the self-association of the drugs and their hetero-association. The results have been used to estimate the effect of the side chains of the phenanthridines on the competitive binding between DAU and the mutagens with DNA. Knowledge of the equilibrium constants for self-association of the phenanthridines and DAU, their hetero-association and their complexation with a DNA fragment, the deoxytetranucleotide 5′-d(TpGpCpA), enabled the relative content of each of the EB-DAU, PI-DAU, EB-DAU-d(TGCA) and PI-DAU-d(TGCA) complexes to be calculated as a function of drug concentration in mixed solutions. The results provide some insight into the molecular basis of the action of combinations of biologically-active molecules. When intercalating drugs are used in combination, it is found that the decrease in binding of drug or mutagen with DNA is due both to formation of drug-mutagen hetero-association complexes in the mixed solution and to competition for the binding sites by the aromatic molecules; the relative importance of each process depends on the molecular properties of the drug or mutagen molecules being considered. Thus, the longer branched side chain of PI and the electrostatic contribution of the extra positive charge of the molecule compared with the ethyl group of EB results in lower affinity for self-association of PI molecules and their hetero-association with DAU, but increases the degree of binding of PI with DNA.

Keywords Competitive binding · DNA intercalators · Hetero-association · Phenanthridine · Daunomycin

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Introduction

Molecules which act as DNA intercalators comprise an important class of drugs that have been used for decades in the treatment of cancer (Feigon et al. 1984; Graves and Velea 2000). Such molecules have a planar polycyclic aromatic chromophore which is inserted between adjacent base pairs of the DNA duplex. In addition to involvement of the chromophore in binding, additional chemical substituents or side chains present in the molecule exhibit a marked influence on the binding energetics, complex formation and the sequence selectivity of the ligand-DNA interactions. These substituents may be simple groups such as methyl, methoxy, keto, hydroxyl or amino moieties or have more complex side chains which provide additional contributions to the binding energies through electrostatic and hydrophobic interactions (Graves and Velea 2000; Lane and Jenkins 2000). A good illustration is the fact that the 9-MeCO group in daunomycin (DAU) has been shown to be necessary for the biological activity of this intercalator, because anthracycline derivatives without this group are inactive (Graves and Velea 2000). An important aspect of the intercalation of DAU is that the 9-MeCO group is able to form hydrogen bonds with DNA (Graves and Velea 2000 and refs. therein). Such substituent groups are also known to be important in hetero-association of intercalators (Davies et al. 2000, 2001a, 2001b). The hydrogen bonding potential of the 9-MeCO group of DAU was also confirmed in the analysis of the hetero-association

between DAU and acridine dyes, where additional stabilization is found in the hetero-association complex of DAU + proflavine compared to hetero-association of DAU with acridine orange, in which such hydrogen bonds are unable to form (Davies et al. 2000).

It is known (Alberts et al. 1981; Birchall et al. 1991; Adel et al. 1993) that many DNA intercalating drugs are pharmacologically more active when used in combination, i.e. when more than one drug is given at the same time or in the same mixture. As "combination therapy" influences the activity of drugs, investigations of their hetero-association are necessary to quantify the effect of competitive binding of different aromatic molecules to receptors such as DNA or proteins, and to elucidate the molecular basis of the action of aromatic compounds as mediators of the pharmacological activity of drugs and as protectors of DNA from binding with mutagenic aromatic molecules (Traganos et al. 1991; Kapuscinsky and Kimmel 1993; Larsen et al. 1996; Davies et al. 2001b). Examination of the physical and chemical properties that drive aromatic drugs to bind to each other and to DNA is important for understanding the physical forces involved in interactions of aromatic molecules (e.g. hydrophobic effect, dispersive van der Waals interactions, hydrogen bonding) and for recognition of the molecular constituents responsible for drug-DNA (Graves and Velea 2000) and drug-drug binding specificity (Davies et al. 2000). The phenanthridine drugs such as ethidium bromide (EB) and propidium iodide (PI) are well-known mutagen agents which interact with the DNA double helix by intercalation (Ames et al. 1975; Gale et al. 1981; Graves and Velea 2000). As the phenanthridines have been used extensively as model intercalating drugs for drug-DNA binding studies (Neidle 1979; Feigon et al. 1984; Hopkins et al. 1990; Graves and Velea 2000 and refs. therein), they are also very attractive molecules for investigations of the hetero-association of aromatic molecules and their competitive binding with DNA. In order to estimate the relative importance of the hetero-association of aromatic drugs versus drug-DNA binding, it is necessary to know the relative magnitudes of the equilibrium constants of self-association of the intercalators, their hetero-association constants and also the equilibrium constants for their complexation with DNA under the same experimental conditions (Davies et al. 2001b). The self-association constants are known for EB (Davies et al. 1996a), PI (Davies et al. 1999) and DAU (Davies et al. 2000) in 0.1 M phosphate buffer, pD 7.1, as well as their complexation with the deoxytetranucleotide 5′-d(TpGpCpA) (Davies et al. 1996b, 2000; Veselkov et al. 2000). Thus, in this work, 1D and 2D 500 MHz ^{1}H NMR spectroscopy has been used to investigate the hetero-association of DAU with both EB and PI under the same solution conditions. The results have been interpreted in terms of the effect of the side chains of the phenanthridines on their hetero-association with DAU and on their competitive binding to a self-complementary DNA oligomer, the deoxytetranucleotide 5′-d(TpGpCpA).

Materials and methods

The phenanthridine dyes EB and PI were purchased from Sigma and the anthracycline drug DAU from Fluka. The samples were lyophilized from D_2O and re-dissolved in 0.1 M phosphate buffer in 99.95% D_2O, pD = 7.1, containing 10^{-4} M EDTA. The concentrations of the stock solutions of the aromatic molecules were measured spectrophotometrically on appropriate dilution using the following molar extinction coefficients: $\epsilon = 5860$ L mol^{-1} cm^{-1} ($\lambda = 480$ nm) for EB (Bresloff and Crothers 1981); $\epsilon = 5900$ L mol^{-1} cm^{-1} ($\lambda = 493$ nm) for PI (Patel and Canuel 1977); $\epsilon = 11{,}500$ L mol^{-1} cm^{-1} ($\lambda = 477$ nm) for DAU (Huang and Phillips 1977; Chaires et al. 1982). The structures of the phenanthridine drugs and DAU are presented in Fig. 1.

500 MHz ^{1}H NMR spectra were recorded on a Bruker DRX FT NMR spectrometer with the residual water peak saturated during relaxation. Signal assignments of the non-exchangeable protons of the drugs were obtained previously using both two-dimensional homonuclear COSY/TOCSY and NOESY/ROESY experiments (Davies et al. 1996a, 1999, 2000). Chemical shift measurements of the non-exchangeable protons of the aromatic molecules were made as a function of concentration at two temperatures (303 and 313 K) and measurements as a function of temperature were made at constant concentration in the temperature range 278–348 K. All NMR measurements were made in the fast-exchange condition on the NMR time-scale with at least 32

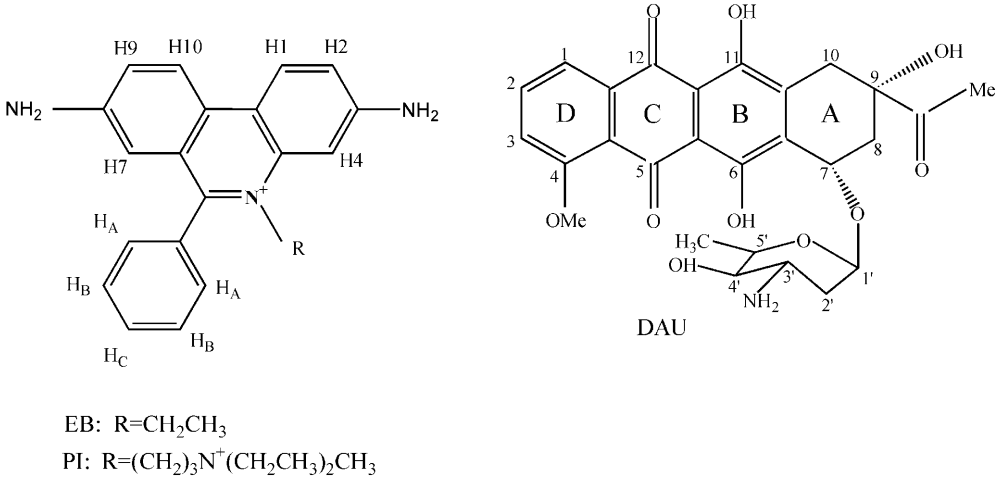

Fig. 1 Structures of the aromatic drugs: daunomycin (DAU) and the phenanthridines, ethidium bromide (EB) and propidium iodide (PI)

scans for the highest concentrations and up to 256 scans for the lowest concentrations. 2D ROESY experiments were measured at 303 K employing standard pulse sequences described previously (Bax and Davis 1985), using 128 to 256 transients depending on sample concentration, with a mixing time of $\tau_m = 240$ ms, recycle delays of ~2.0 s, 512 points in the F1 dimension (zero filled by a factor of 2 in processing) and 4096 points in the F2 dimension. Chemical shifts were measured relative to the internal reference TMA (tetramethylammonium bromide) and recalculated with respect to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate), i.e. $\delta_{DSS} = \delta_{TMA} + 3.178$ (ppm). The sample temperature was regulated using a Bruker BVT-3000 unit.

The calculated fits of the model of the equilibrium reactions to the experimental chemical shift data were assessed by the magnitude of the discrepancy function Δ:

$$\Delta = \sum_{i=1}^{n} (\delta_{ei} - \delta_i)^2 \quad (1)$$

where n is the number of experimental points; δ_{ei} and δ_i are the experimental drug proton chemical shifts for the i-th concentration (or temperature) and the calculated values using the theoretical models. Minimization of Δ was performed using initial approximations produced by the law of accidental numbers over a wide range of variations of the parameters. In order to determine the global and not a local minimum, a large statistical set of data enabled the whole field of the possible values of minimization parameters to be investigated over a wide range of their variations. The values of Δ obtained in the calculations are in the range 10^{-5}–10^{-6} for at least 14 experimental points in each data set, which corresponds to an average deviation between observed and modelled chemical shifts in the range 0.002–0.0003 ppm, assuming an error of 0.001 ppm in the measurement of the chemical shifts; these results indicate a very good fit to the experimental data. Magnitudes of the equilibrium constants were calculated for each proton independently and the average value and mean deviation determined. The spatial representation of the structures was obtained with the help of Mathematica 2.2 software (Wolfram Research).

Results

NMR measurements of hetero-association of phenanthridines and DAU

In order to optimize the conditions for observation of hetero-association intermolecular contacts, 2D ROESY measurements of a 1:1 mixed solution of DAU with EB were made at relatively high drug concentrations (~6 mM) in 0.1 M phosphate buffer. The 2D ROESY spectrum of the DAU + EB molecular system exhibits a number intermolecular ROE contacts of medium (m) and weak (w) intensities between protons of EB and DAU, i.e. (DAU) 4-OMe with (EB) H-7 (m) and *ortho*-phenyl protons (m) and (DAU) H-1/3 with (EB) *ortho*-phenyl protons (w) and CH_3 (w). The medium intensity ROE cross-peaks are consistent with an orientation of the EB chromophore in the hetero-complex with DAU, in which the phenyl ring and H-7 of EB is situated close to the 4-OMe group of DAU, i.e. on the same side of the antibiotic as the amino-sugar ring. At the same time, the ROE contacts of small intensities are consistent with intermolecular interactions of the DAU (H-1/H-3) protons closely situated in space to the CH_3 and *ortho*-phenyl protons of ethidium bromide, in which the EB molecule is rotated by ~180° with respect to the longitudinal axis of the dye chromophore in the 1:1 EB + DAU complex, so that the phenyl ring of EB is situated on the opposite side (to the amino-sugar ring of antibiotic) of the stacked chromophores of the aromatic molecules. No intermolecular NOE/ROE cross-peaks were observed for PI + DAU mixed solutions even at the highest concentration of the drugs used.

The structures and thermodynamics of complexation between DAU and the phenanthridine drugs have been investigated by analysis of the chemical shift changes of both molecules in mixed solutions as a function of concentration and temperature. As an example, the changes of chemical shifts with concentration for PI + DAU hetero-association at $T = 303$ K are shown in Fig. 2a; similar curves were observed for EB + DAU hetero-association. Measurements were made by keeping the concentration of PI constant and changing the content of DAU in solution; this experimental procedure was adopted because of the higher self-association constant of DAU (580 L mol^{-1}; Davies et al. 2000) compared with the phenanthridines (EB: 270 L mol^{-1}; PI: 46 L mol^{-1}; Davies et al. 1996a, 1999, respectively) and therefore changes of concentration of DAU affect the equilibrium distribution of the aggregates to a greater extent than keeping it constant and varying the concentration of the phenanthridine in solution.

Model and analysis

In the general model of molecular hetero-association of two components A and P it is assumed that there is a dynamic equilibrium that includes indefinite self-association of both A and P, as well as indefinite hetero-association reactions of different types, as shown in the following scheme (Davies et al. 1999, 2000):

$$\begin{aligned}
&A_1 + A_i \overset{K_A}{\rightleftharpoons} A_{i+1} && \text{(a)}\\
&P_1 + P_j \overset{K_P}{\rightleftharpoons} P_{j+1} && \text{(b)}\\
&P_j + A_i P_j \overset{K_{het}}{\rightleftharpoons} A_i && \text{(c)}\\
&P_j A_i + P_l \overset{K_{het}}{\rightleftharpoons} P_j A_i P_l && \text{(d)}\\
&A_m + P_j A_i \overset{K_{het}}{\rightleftharpoons} A_m P_j A_i && \text{(e)}
\end{aligned} \quad (2)$$

where A_1 and P_1 correspond to the monomers of DAU and phenanthridine dye, and A_i, A_m, P_j and P_l are the aggregates containing i, m monomers of DAU and j, l monomers of the dye, respectively. The equilibrium constants for the self-association reactions of DAU (K_A) and the dye (K_P) and for hetero-association of drug and dye molecules (K_{het}) are assumed to be independent of the number of molecules in the aggregates and complexes (Martin 1996).

The mass conservation law for the general case of indefinite association of aromatic molecules in the

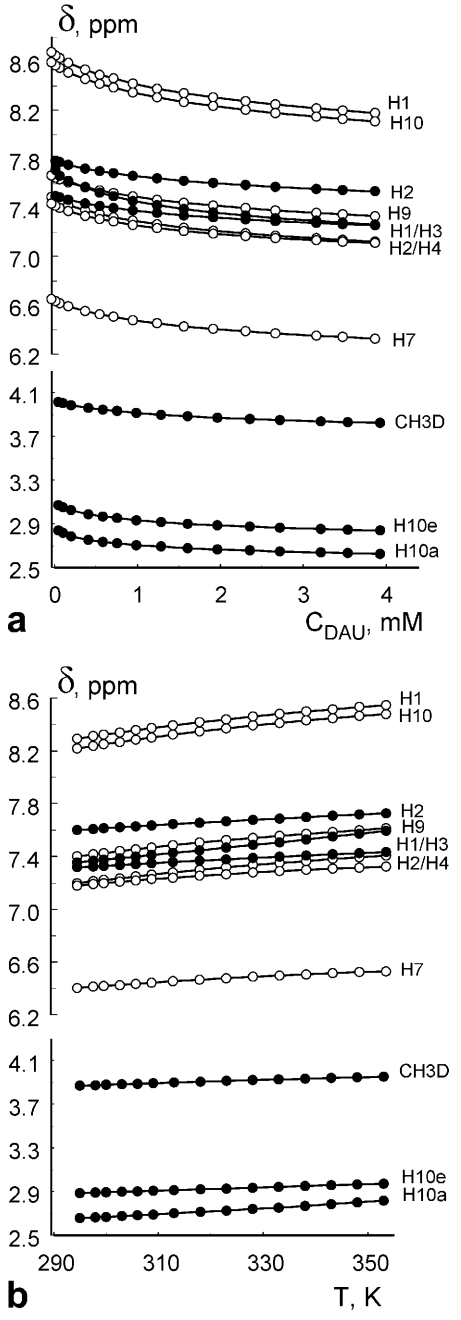

Fig. 2a, b 500 MHz ^{1}H NMR experiments on the hetero-association of DAU with PI in 0.1 M phosphate buffer, pD = 7.1. Changes of chemical shifts with: **a** concentration of DAU ([PI] = [P$_0$] = 0.79 mM = constant), T = 303 K; **b** temperature of DAU + PI ([A$_0$] = 1.61 mM, [P$_0$] = 0.79 mM)

reactions summarized in Eqs. (2) has the following form (Davies et al. 2000)[1]:

$$[a_0] = \frac{[a_1]}{(1-K_A[a_1])^2}\left[1 + K_{het}\frac{[p_1]}{1-K_P[p_1]} + \frac{K_{het}^2}{2}\frac{[p_1]^2}{(1-K_P[p_1])^2} + K_{het}^2\frac{[a_1][p_1]}{(1-K_A[a_1])(1-K_P[p_1])}\right] \quad (3)$$

where $[a_0]$ is the initial concentration of DAU and $[a_1]$ and $[p_1]$ are the monomer concentrations of DAU and the phenanthridines, respectively.

The dependence of observed proton chemical shifts of DAU (δ_A) in such a model is presented in symmetrical form with respect to the indexes "a" and "p". For DAU:

$$\delta_A = \frac{[a_1]}{[a_0]}\left[\begin{array}{l}\delta_{mA}\left(2(1+K_A[a_1]) - \frac{1}{(1-K_A[a_1])^2}\right) \\ +2\delta_{dA}\left(\frac{1}{(1-K_A[a_1])^2} - 1 - K_A[a_1]\right) \\ +\delta_{cA}\frac{K_{het}[p_1]}{(1-K_A[a_1])^2(1-K_P[p_1])}\left(1 + \frac{K_{het}[p_1]}{2(1-K_P[p_1])} + \frac{K_{het}[a_1]}{1-K_A[a_1]}\right)\end{array}\right] \quad (4)$$

As the values of chemical shifts in the monomer (δ_{mA}, δ_{mP}) and dimer forms (δ_{dA}, δ_{dP}) of both drugs and the equilibrium constants K_A and K_P for self-association of drug (A) and dye (P) have been independently determined (Davies et al. 1996a, 1999, 2000), it follows that the observed concentration dependences of proton chemical shifts of DAU and the phenanthridines in mixed solutions (e.g. Fig. 2a) are a function of two unknown quantities, δ_c and K_{het}. These have been determined using the computational procedure described previously (Davies et al. 2000), and the magnitudes of the calculated parameters K_{het} and δ_c are summarized in Table 1.

The thermodynamic parameters $\Delta H°_{het}$ and $\Delta S°_{het}$ of hetero-association of DAU with each of the phenanthridines were determined from measurements of the proton chemical shifts of the molecules in the mixed solution as a function of temperature (e.g. Fig. 2b), employing the same analytical method used for self-association of the phenanthridine dyes in aqueous solution, i.e. calculation of the thermodynamic parameters of hetero-association was made using Eq. (4) in which the dependence of the equilibrium constants on temperature is expressed by relation (5):

$$K(T) = \exp\left(\frac{\Delta S°}{R} - \frac{\Delta H°}{RT}\right) \quad (5)$$

The derived values of enthalpy and entropy of the hetero association reactions of DAU with the phenanthridine dyes in aqueous solution are summarized in Table 1.

Structures of the complexes

The most favourable structures of the 1:1 hetero-association complexes of DAU + phenanthridines were determined by analysis of the calculated values of the induced proton chemical shifts, $\Delta\delta = \delta_m - \delta_c$, for both DAU and the dyes (Table 1). The mutual orientation of the molecules in the complex was determined by

[1]As concentration equations and expressions for chemical shift changes with concentration are "symmetrical" with respect to the indexes a and p, those for DAU (A) are presented. Analytical equations for EB (or PI) may be obtained by simple substitution of indexes a for p (and vice versa) and K_A for K_P (and vice versa) (Davies et al. 2000)

Table 1 Thermodynamic parameters for the hetero-association of DAU with the phenanthridine dyes[a]

System	Protons of A	δ_{cA} (ppm)	δ_{mA} (ppm)	Protons of P	δ_{cP} (ppm)	δ_{mP} (ppm)	K_{het} (L mol^{-1})	$-\Delta H°_{het}$ (kJ mol^{-1})	$-\Delta S°_{het}$ (J K^{-1} mol^{-1})
EB + DAU	H-2	7.57	7.83	H-1	7.96	8.69	2700 ± 440	42.5 ± 3.3	74 ± 12
$K_P = 270 \pm 20$	H-1	7.31	7.78	H-10	7.88	8.63			
$K_A = 580 \pm 110$	H-3	7.25	7.55	H-9	7.19	7.66			
	OCH_3	3.87	4.02	H-4	7.09	7.55			
	H-10*e*	2.96	3.05	H-2	7.05	7.48			
	H-10*a*	2.72	2.81	H-7	6.23	6.67			
PI + DAU	H-2	7.61	7.83	H-1	7.81	8.71	560 ± 60	37.0 ± 7.0	70 ± 15
$K_P = 46 \pm 5$	H-1	7.31	7.78	H-10	7.74	8.64			
$K_A = 580 \pm 110$	H-3	7.32	7.55	H-9	7.08	7.70			
	OCH_3	3.93	4.02	H-4	6.92	7.50			
	H-10*e*	2.96	3.05	H-2	6.89	7.45			
	H-10*a*	2.75	2.81	H-7	6.13	6.67			

[a]In 0.1 M phosphate buffer solutions, pD 7.1; the self- and hetero-association constants correspond to $T = 303$ K; chemical shifts of the monomers (δ_{mA} and δ_{mP}) are taken from the self-association analysis of EB, PI and DAU (Davies et al. 1996a, 1999, 2000)

comparison of $\Delta\delta$ and their theoretical values derived from quantum-mechanical calculations of iso-shielding curves for aromatic molecules (Giessner-Prettre and Pullman 1987), as described in previous work (Davies et al. 2000). A detailed description of the calculations of iso-shielding curves for aromatic molecules is given by Giessner-Prettre and Pullman (1987). Taking into account the two-fold symmetry of the iso-shielding curves of the aromatic rings (rings B, C, D) of the DAU chromophore, there are two conformations of the EB chromophore with respect to DAU in the hetero-complex. In each conformation the planes of the dye chromophore and DAU molecule are parallel to each other and situated 0.34 nm apart, as shown for one conformation of the 1:1 hetero-complex in different spatial projections in Fig. 3. This conformation is consistent with one set of ROE intermolecular contacts. The second conformation has the EB chromophore rotated ca. 180° with respect to its longitudinal axis in the 1:1 EB-DAU hetero-complex, which is consistent with the second set of ROE contacts.

It should be noted that for both orientations of the EB chromophore (i.e. the EB phenyl ring situated on the same or opposite side to the amino-sugar ring of DAU) in the 1:1 EB-DAU hetero-complexes, the 3/8-diamino groups of EB and the 9-MeCO group of DAU are oriented so that intermolecular hydrogen-bond formation is possible. A similar structure was obtained from analysis of limiting chemical shifts for the DAU + PI hetero-complex in aqueous solution, though their structures could not be confirmed by ROE/NOE measurements.

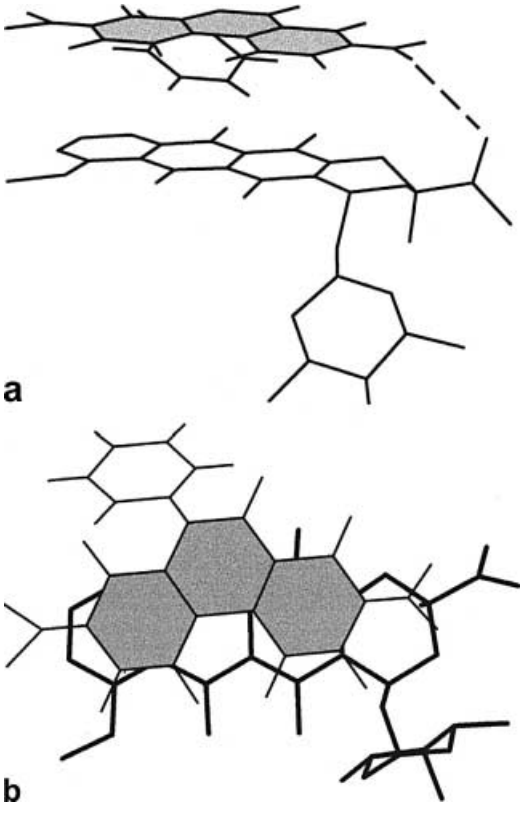

Fig. 3a, b The calculated NMR structure of the 1:1 hetero-association complex of EB (*shaded chromophore*) with DAU. **a** Side view of the hetero-complex (the possible hydrogen bond between the 3-amino group of the dye chromophore and the 9-MeCO group of DAU is indicated by a *dashed line*); **b** view looking perpendicular to the planes of the chromophores of the aromatic rings

Discussion

Hetero-association complexes are stabilized by intermolecular hydrogen bonding

It is seen from Table 1 that the hetero-association constant of EB + DAU (2700 ± 440 L mol^{-1}) is substantially higher than the self-association constants of these molecules, which indicates that formation of hetero-complexes between EB and DAU is energetically more favourable than their self-association. The most probable explanation of such an extra stabilization in the 1:1 EB-DAU hetero-complex is formation of a hydrogen bond between the 3,8-amino groups of EB and the 9-MeCO group of

DAU, analogous to that observed previously between the 3,6-amino groups of the acridine chromophore of proflavine and the 9-MeCO group of DAU in the 1:1 PF-DAU hetero-complex (Davies et al. 2000). Other results obtained in this work for the EB-DAU system are also consistent with hydrogen-bond formation in the hetero-complex, i.e. the calculated structures of the 1:1 hetero-complexes between EB and DAU have the 3,8-diamino groups of EB and the 9-MeCO group of DAU situated close to each other in space for formation of hydrogen bonds in both possible conformations (denoted by the dashed line in Fig. 3 for one of the orientations of the EB chromophore) and also by observation of intermolecular ROE contacts, which are consistent with the EB-DAU structure. However, as in the previous work on PF + DAU (Davies et al. 2000), H-bonding in the EB-DAU hetero-complex in 90% H_2O/D_2O solution could not be directly detected, because a separate NH_2 resonance signal was not observed by NMR, probably due to rapid intermolecular exchange of the protons with water. It should be noted also that the hetero-association constants, K_{het}, for EB + DAU and PF + DAU (Davies et al. 2000) systems are substantially higher than the self-association constants of the interacting molecules, whereas for many other hetero-association systems studied previously in our laboratory (Davies et al. 1999, 2001a, 2001b) the values of K_{het} are intermediate between the self-association constants of the two aromatic molecules, for molecular systems in which there was little likelihood of intermolecular H-bonding on hetero-complex formation. Hence, the relative magnitude of the hetero-association constant of the aromatic molecules is a direct indication of the contribution of additional stabilization (such as hydrogen bonding) to the hetero-association of drug-mutagen molecules compared to their self-association. The relatively high magnitude of K_{het} for the PI-DAU system (560 ± 60 L mol^{-1}) compared to the relatively low value of the self-association constant of PI (46 ± 5 L mol^{-1}) may also reflect additional stabilization of the hetero-complex consistent with H-bond formation.

The similarity in thermodynamic parameters of PI + DAU and EB + DAU (Table 1) is consistent with the formation of hydrogen bonds between the 9-MeCO groups of DAU and the 3,8-amino groups of both phenanthridines. Compared to the magnitudes of the thermodynamic parameters for self-association of EB and PI (Davies et al. 1996a, 1999), the relatively large negative ΔH°_{het} and ΔS°_{het} values for complexation of the phenanthridines with DAU in aqueous solution indicate that dispersive van der Waals forces and H-bond formation in the 1:1 hetero-complex provide the main contributions to stabilization of the complex. Dispersive van der Waals interactions are characterized both by negative enthalpy and negative entropy (Ross and Subramanian 1981). H-bond formation in aqueous solution also contributes to negative values of enthalpy and entropy (Record et al. 1978; Ross and Subramanian 1981) and the magnitude of the enthalpy of H-bond formation in aqueous solution is estimated to be –8 to –13 kJ mol^{-1} (Ross and Subramanian 1981). Differences in ΔH for hetero-association of EB + DAU compared to self-association of EB and DAU is –19 kJ mol^{-1} and –9 kJ mol^{-1}, respectively, and is –11 kJ mol^{-1} for hetero-association of PI + DAU compared to self-association of PI; these differences in ΔH correspond to that expected for H-bond formation.

The lower value of the hetero-association constant (Table 1) of PI-DAU compared to EB-DAU probably reflects increased steric barriers to hetero-association resulting from the much longer side chain of PI compared to the ethyl group in EB, also mirrored in the differences in self-association constants of EB and PI (Davies et al. 1996a, 1999). The absence of intermolecular NOE cross-peaks in PI + DAU hetero-association may be due to the relatively small value of K_{het} compared to that for the EB + DAU system, which results in a relatively small content of PI-DAU hetero-association complexes in solution. Calculation of the relative content of each DAU-containing molecular complex as a function of drug concentration in the mixed solution confirms the hypothesis mentioned above. Using the values of equilibrium constants (Table 1), the results of the calculations are shown in Fig. 4 for hetero-association in solution for EB + DAU (Fig. 4a) and PI + DAU (Fig. 4b). It is seen from Fig. 4a that for the EB + DAU system the fraction of hetero-association complexes in solution is greater than self-association over the whole range of drug concentrations studied, whereas it is seen from Fig. 4b that for the PI + DAU system the total contribution of hetero-complexes (A_iP_j and $A_iP_jA_l$) to the dynamic equilibrium in solution is relatively small (the contribution of $A_iP_jA_l$ hetero-complexes is negligible and is not shown in Fig. 4b). It is also found that the relative amount of monomers of PI (P_1) in the mixed solution with DAU (Fig. 4b) is substantially higher than that of EB (Fig. 4a).

Comparison of the complexation of DAU and the phenanthridines with 5′-d(TpGpCpA)

Quantitative analysis of the complexation of each of the aromatic drugs (A) with a DNA fragment, 5′-d(TpGpCpA), used the additive model of drug-DNA complexation in solution, in which the self-association reactions of the drug and oligonucleotide ($N + N \overset{K_N}{\rightleftharpoons} N_2$) were considered in addition to the complexation reactions of the ligand with both the single-stranded (N) and double-stranded (N_2) form of the oligonucleotide:

$$\begin{aligned} &A + N \overset{K_1}{\rightleftharpoons} AN && \text{(a)}\\ &A + N_2 \overset{K_2}{\rightleftharpoons} AN_2 && \text{(b)}\\ &A + AN \overset{K_3}{\rightleftharpoons} A_2N && \text{(c)}\\ &A + AN_2 \overset{K_4}{\rightleftharpoons} A_2N_2 && \text{(d)}\\ &AN + N \overset{K_5}{\rightleftharpoons} AN_2 && \text{(e)} \end{aligned} \qquad (6)$$

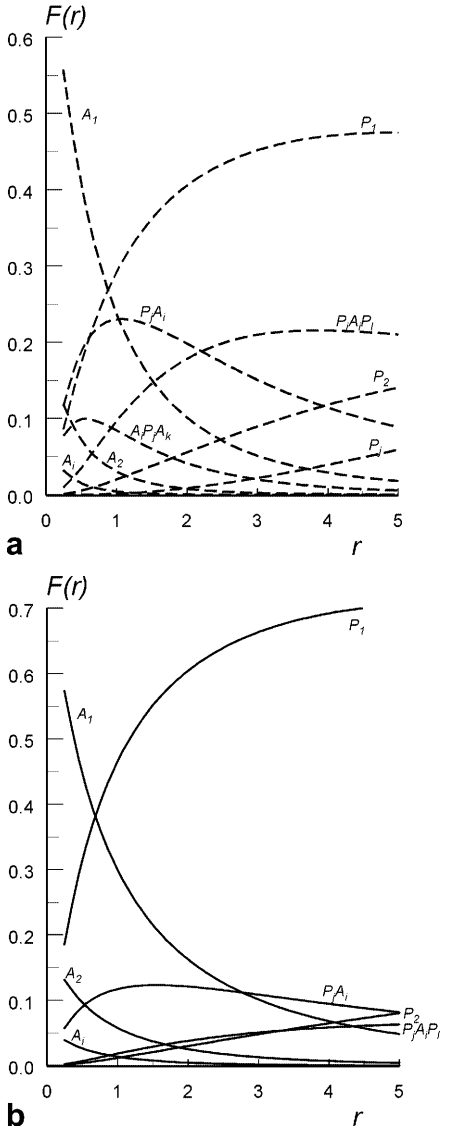

Fig. 4a, b Calculated relative content $F(r) = C_i/C_0$ of **a** EB + DAU and **b** PI + DAU molecular complexes in mixed solutions as a function of r ($r = [A_0]/[P_0]$) at $T = 303$ K

where A and N are the monomer forms of the ligand and tetranucleotide, respectively (Davies et al. 1996b).

The equilibrium constants K_1–K_5 for complexation of DAU and EB/PI with both the monomer and duplex form of the deoxytetranucleotide d(TpGpCpA) were calculated from observed concentration dependences of drug proton chemical shifts (Davies et al. 1996b, 2000; Veselkov 2000) and the results are summarized in Table 2. Although the patterns of equilibrium constants of 1:1, 2:1, 1:2 and 2:2 complex formation are qualitatively similar for both EB + d(TpGpCpA) (Davies et al. 1996b) and PI + d(TpGpCpA) complexation (Veselkov et al. 2000), the magnitude of K_2 for 1:2 complexation of PI with d(TpGpCpA) is approximately three times greater than that for EB at the same temperature. It is likely that the longer and positively charged side chain in the PI molecule creates energetically more favourable contacts with the deoxyribose-phosphate backbone of the oligonucleotide sequence than the ethyl group of EB, i.e. $K_2(\mathrm{PI}) > K_2(\mathrm{EB})$, whereas the side chain in PI is likely to result in greater steric barriers for the binding of the second drug molecule with the tetranucleotide duplex, i.e. $K_2 > K_4$ for PI and $K_4 > K_2$ for EB. The relation between the calculated constants K_1–K_4 confirms that PI, as well as EB, preferentially intercalates into *pyr-pur* sequences, d(T-G) and d(C-A) sites, of the tetramer duplex and that the binding corresponds to the "excluded neighbour" model (McGhee and von Hippel 1974) in which intercalation of the drug molecule between adjacent base pairs is impossible.

Competition between the hetero-association of phenanthridines with DAU and their complexation with 5′-d(TpGpCpA)

In addition to self-association (reactions 2a and 2b), hetero-association (reactions 2c–e) and reactions (6) of drug complexation with d(TGCA), the following reactions of hetero-complex formation between the drugs and the monomer and duplex form of the tetranucleotide need to be taken into account (Davies et al. 2001b):

$$\begin{aligned} &\mathrm{AN} + \mathrm{P} \overset{K_3^{\mathrm{P}}}{\rightleftharpoons} \mathrm{APN} && \text{(a)} \\ &\mathrm{AN_2} + \mathrm{P} \overset{K_4^{\mathrm{P}}}{\rightleftharpoons} \mathrm{APN_2} && \text{(b)} \\ &\mathrm{PN} + \mathrm{A} \overset{K_3^{\mathrm{A}}}{\rightleftharpoons} \mathrm{APN} && \text{(c)} \\ &\mathrm{PN_2} + \mathrm{A} \overset{K_4^{\mathrm{A}}}{\rightleftharpoons} \mathrm{APN_2} && \text{(d)} \end{aligned} \qquad (7)$$

where A, P and N are the monomer forms of DAU, phenanthridine dye and tetranucleotide, respectively;

Table 2 Equilibrium constants of complex formation between different ligands and the deoxytetranucleotide 5′-d(TGCA)[a]

Ligand	K_1 (10^{-3} L mol^{-1})	K_2 (10^{-3} L mol^{-1})	K_3 (10^{-3} L mol^{-1})	K_4 (10^{-3} L mol^{-1})	K_5 (10^{-3} L mol^{-1})
EB[b]	12 ± 3	42 ± 5	4.8 ± 1.6	68 ± 12	0.2 ± 0.1
PI[c]	19 ± 3	127 ± 16	10.3 ± 2.1	62 ± 17	0.2 ± 0.1
DAU[d]	31 ± 8	430 ± 100	5.4 ± 0.4	12.5 ± 0.5	–[e]

[a]Determined from NMR measurements in 0.1 M phosphate buffer solutions, pD 7.1, recalculated for $T = 303$ K
[b]Davies et al. (1996b)
[c]Veselkov et al. (2000)
[d]Davies et al. (2000)
[e]The value of this association constant turned out to be negligible and was not included in the calculations

K_3^P, K_4^P and K_3^A, K_4^A are the equilibrium constants of EB/PI-nucleotide and DAU-nucleotide complexation. Reactions (7) are analogous to the complexation reactions summarized in Eqs. (6c) and (6d) and so it is assumed that the equilibrium constants K_3^P, K_4^P or K_3^A, K_4^A for phenanthridine/DAU binding with the 1:1 (AN or PN) complex and with the 1:2 (AN_2 or PN_2) complex are equal to the corresponding K_3 and K_4 values in Eqs. (6). Such an assumption is reasonable, because the binding of aromatic ligands to DNA corresponds to the "excluded neighbour" model (McGhee and von Hippel 1974), and so the mutual influence of each of the ligands, not containing large side chains or groups, is negligible when intercalated into the oligonucleotide sequence. The computational procedure described in previous work (Davies et al. 2001b) has been used to calculate the equilibrium concentrations of P, A and N by solving a system of non-linear equations, based on the mass law equations for reactions (2), (6) and (7) and the mass conservation law, and, hence, determination of the relative content of different complexes in solution.

The relative decrease in content of the aromatic ligand-deoxytetranucleotide duplex complexes has been calculated as a function of tetramer concentration for different amounts of EB/DAU and PI/DAU in the mixed solution, using equilibrium constants summarized in Tables 1 and 2. The results for both EB/DAU (dashed line) and PI/DAU (solid line) in the mixture with d(TpGpCpA) are summarized in Fig. 5 in terms of the relative decrease in content of the complexes of DAU with the 5′-d(TpGpCpA) duplex (F_d) calculated as a function of $r_N = [N_0]/[A_0]$ (the ratio of oligonucleotide and drug concentrations) at different concentrations of the dyes ($r_P = [P_0]/[A_0]$, the ratio of the dye and DAU concentrations in solution). The results show that F_d is substantially higher at relatively small r_N values ($r_N < 1$), when PI acts as an "interceptor" of DAU molecules in solution (solid lines), compared with that of EB (dashed lines) affecting DAU binding with DNA due to the differences in the dye-DNA complexation constants (Table 2).

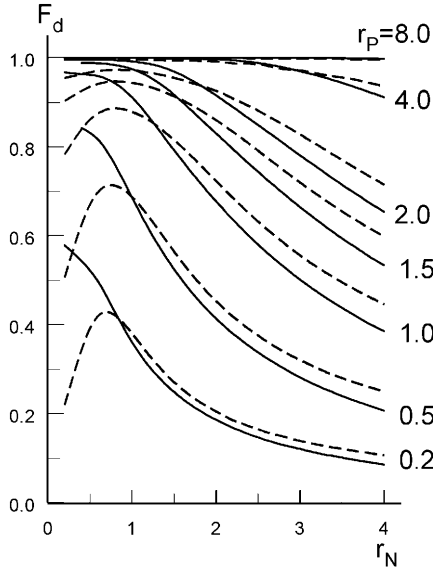

Fig. 5 Relative decrease in content of the complexes of DAU with the 5′-d(TpGpCpA) duplex calculated (from results in Tables 1 and 2) as a function of $r_N = [N_0]/[A_0]$ at different ratios (r_p) of the dye to DAU concentration in solution, when EB (*dashed lines*) and PI (*solid lines*) act as interceptors; $F_d = (f_0 - f_p)/f_0$, where f_p, f_0 are the fractions of the complexes between DAU and duplex of the deoxytetranucleotide in the presence or absence of the dye in solution, respectively

The calculated dependences $F_d(r_N)$ have pronounced maxima at $r_N \leq 1$ for EB + DAU (Fig. 5, dashed lines) owing to competition between EB-DAU hetero-association and drug-DNA binding. Such a situation is not observed for PI + DAU, because the PI-DAU hetero-association constant is considerably smaller than K_{het} for complex formation between EB and DAU (Table 1), whereas the binding affinity of PI with the DNA duplex is substantially higher compared with that for EB-DNA complexation (Table 2), i.e. hetero-association of PI and DAU plays a relatively small role in the complex equilibrium in solution compared to competitive binding of PI and DAU with the DNA at low r_N values. At $r_N > 1$ the greater "interceptor" action of EB than that of PI (Fig. 5) obviously results from the higher hetero-association affinity of EB compared to PI with DAU. The observed differences may be explained by the differences in dye-drug and dye-DNA interactions due to the electrostatic contribution of the extra positive charge and the longer branched side chain of the PI molecule compared with the ethyl group of EB. An increase in content of the DNA oligomer in solution at $r_p \leq 1$ leads to a substantial decrease of F_d as a function of r_N, when both EB and PI act as "interceptors" of DAU molecules (Fig. 5). At $r_p > 4$ the value of F_d practically equals 1, indicating that there is little binding of DAU with double-stranded DNA, i.e. the dye entirely "blocks" the binding of DAU with DNA. The largest changes in the complexation of dye/DAU with the oligonucleotide duplex are observed in the range of $r_p = 0.2$–2; when the dye/DAU content in solution is further increased ($r_p > 2$), this effect becomes less pronounced (Fig. 5). In principle, such an analysis enables the optimum concentration of drug/mutagen to be determined for any defined reduction in ligand binding to DNA.

Using equilibrium constants summarized in Tables 1 and 2, the relative content of the dye-tetranucleotide (PN + PN_2) and the dye-DAU-nucleotide hetero-complexes (APN and APN_2) was calculated as a function of r_p, the ratio of dye and drug concentrations in solution, i.e. when the dye acts as "interceptor" (Fig. 6). The results show that the relative proportions of the DAU-tetranucleotide (PN + PN_2) complexes increase with increasing dye concentration, i.e. the dye "blocks" the binding sites of DAU on the deoxytetranucleotide. The relative amount of EB-DAU-DNA and PI-DAU-DNA hetero-complexes (APN and APN_2) depends on the relation between the equilibrium constants for complexation with the deoxytetranucleotide of the drug

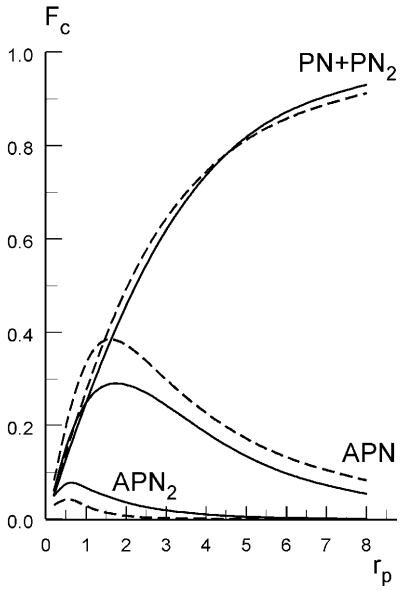

Fig. 6 Relative content, F_c, of the complexes of DAU (A) and dye (P) with the tetranucleotide (N) 5′-d(TpGpCpA), calculated (from results in Tables 1 and 2) as a function of r_P, the ratio of DAU to dye concentration in the mixed solution at $r_N = [N_0]/[A_0] = 1$; $PN + PN_2$, complexes of the dye (PI, *solid lines*; EB, *dashed lines*) with deoxytetranucleotide in the monomer and duplex forms; APN and APN_2 are hetero-association complexes

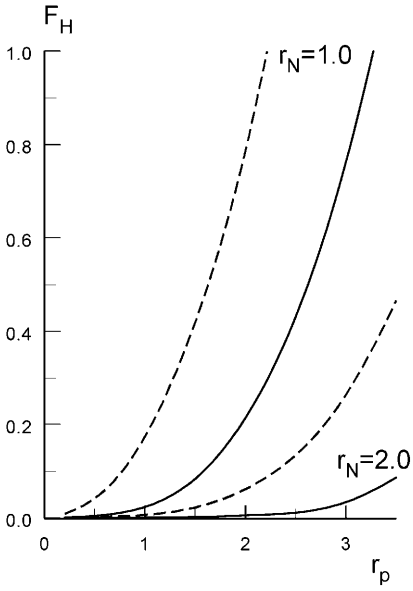

Fig. 7 Ratio (F_H) of the content of "dye-DAU" hetero-association complexes with respect to the total amount of complexes of dye with the deoxytetranucleotide 5′-d(TpGpCpA), calculated (from results in Tables 1 and 2) as a function of the ratio (r_p) of dye and DAU concentrations in the mixed solution of EB + DAU + d(TGCA) at different $r_N = [N_0]/[A_0]$ values; *solid* and *dashed lines* correspond to PI and EB molecules, respectively, acting as interceptors of DAU

and dye, as well as for drug-dye hetero-association in solution.

In order to determine which process prevails in the effect of dye/DAU on the degree of intercalative binding of aromatic ligands with DNA (i.e. competition by dye and drug for the binding sites of the oligonucleotide, or formation of "dye-DAU" hetero-complexes in solution), the proportion of the "dye-DAU" hetero-complex was calculated relative to the complexes of dye/DAU with the deoxytetranucleotide at different dye/DAU concentrations in solution, using the equilibrium constants summarized in Tables 1 and 2. It is seen from the results of such calculations in Fig. 7 for EB (dashed lines) and PI (solid lines) that the contribution of the "dye-DAU" hetero-complex to the decrease in DAU binding with the tetranucleotide (F_H) depends substantially on r_N, the ratio of the oligonucleotide and drug concentrations. It is found that F_H for the EB-DAU-DNA system (where EB acts as "interceptor") is much higher than that in the PI-DAU-DNA mixture. At $r_N = 1$ the value of F_H becomes predominant ($F_H > 1$) when $r_p > 2$ for EB-DAU-DNA and at $r_p > 3$ for the PI-DAU-DNA system; at $r_N = 2$ this situation ($F_H > 1$) will be observed at much higher r_p values (Fig. 7).

Conclusions

As a result of investigations of the hetero-association of the phenanthridine drugs EB and PI with DAU and the competitive binding of aromatic dye/drug molecules with a DNA oligomer, it may be concluded that:

1. The presence of groups which may act as hydrogen-bond donors and/or acceptors in aromatic drug molecules (i.e. the 3,8-amino groups in EB/PI and the 9-MeCO of DAU) results in higher hetero-association constants and affects the binding affinity of intercalators to DNA oligomer.
2. The longer size and extra positive charge of the side chain of PI compared to that of EB results in lower values of the self-association constant of PI and its hetero-association with DAU, but increases the binding affinity of PI with DNA.
3. The equilibrium balance of self-association, hetero-association and dye/drug-DNA binding (Fig. 5) depends on the solution conditions. When phenanthridines are added in combination with DAU at relatively small DNA concentrations ($r_N < 1$, $r_p < 2$), the dominant effect for PI is competition for DNA binding sites, whereas for EB it is hetero-association with DAU. Under these conditions, PI is more "competitive" than EB for DNA binding sites, but at higher DNA concentrations ($r_N > 2$) the complexation of EB with DAU becomes more important.

In order to elucidate the molecular basis for the competitive binding of mutagens and aromatic antibiotics with DNA, it has been shown, in this work, that it is necessary to take into account not only the hetero-association of the molecules but also the competition

between drug and mutagen for the oligonucleotide binding sites. The model and analytical method outlined in this work has general application to understanding molecular complexation processes in multi-component equilibria, as it enables the relative importance of each complexation reaction to be determined quantitatively.

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