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EFFECT OF SIDE GROUPS OF PHENANTHRIDINES ON THEIR HETERO-ASSOCIATION WITH ANTIBIOTIC DAUNOMYCIN AND THE COMPETITIVE BINDING WITH A DNA OLIGOMER

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500 MHz ^{1}H NMR spectroscopy has been used to study the hetero-association of phenanthridine dyes, containing different side groups in the chromophore, with anthracycline antibiotic daunomycin (DAU) and the competitive binding of aromatic dye/drug molecules with a DNA oligomer 5'-d(TpGpCpA). The magnitudes of the hetero-association parameters of Ethidium Bromide (EB) and its azido-derivatives, 8-azido-Ethidium Bromide (EMB) and 3,8-Diazido-Ethidium Chloride (EDC) with DAU show a successive decrease with mono and di-substitution of the 3,8-amino groups of EB due to less probability of the formation of an intermolecular hydrogen bond between the amino groups of EB and the 9 MeCO group of DAU. Analysis has shown that the equilibrium balance of self-association, hetero-association and dye/drug-DNA binding depends on solution conditions. In order to elucidate the molecular complexation processes in multi-component equilibria occurring in mixed solution (including mutagen, aromatic antibiotic and DNA), it is necessary to take into account not only the hetero-association of the aromatic molecules but also the competition between drug and mutagen for the oligonucleotide binding sites.

KEY WORDS: Phenanthridines, Azido-derivatives, Daunomycin, NMR spectroscopy, Hetero-association, Competitive Binding to DNA.

A number of aromatic drug molecules exhibit their biological activity by intercalation between the base pairs of DNA and RNA molecules [1]. The geometry of many intercalative complexes has been investigated in both the crystal and solution states [1,2], but the correlation between the biological activity and physico-chemical properties that drive association of aromatic drugs and their binding with DNA is still under discussion. Such studies are important for understanding the physical forces involved in interaction of aromatic molecules (*e.g.* hydrophobic effect, dispersive van der Waals interactions, hydrogen bonding, *etc.*) and for recognition of the molecular constituents, which are responsible for drug-DNA and drug-drug binding specificity [2,3]. Particular groups attached to an aromatic chromophore in the 'correct' positions may play a significant role in determining the conformation and binding of drugs with DNA. 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 [2]. An important aspect of the intercalation of DAU is that the 9-MeCO group is able to form hydrogen bonds with DNA [2]. Such substituent groups have been also shown to be important in hetero-association of intercalators [3,4]. The hydrogen bonding potential of the 9-MeCO group of DAU has been confirmed in the analysis of the hetero-association between DAU and phenanthridine dye Ethidium Bromide (EB), where additional stabilization is found in the hetero-association complex of DAU+EB due to the formation of intermolecular hydrogen bonds between 9-MeCO group of DAU and 3,8 amino-groups of phenanthridine chromophore of EB compared to hetero-association of DAU with acridine orange, where such hydrogen bonds are unable to form [3]. These studies enable to assume that replacement of amino-groups of EB by other chemical groups which are not capable to form of H-bonds with DNA and drug molecules, should result in reduction of the probability of both the dye-drug complex formation and dye/drug complexation affinity with DNA in solution.

Investigation of the hetero-association is also important from a pharmacological point of view since hetero-complexes and competitive binding may influence the activity of drugs [5]. Hence, in order 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 [4,6], it is necessary to quantify the effect of hetero-association and competitive binding of different aromatic molecules to receptors such as DNA.

In this work the hetero-association of the anthracycline aromatic drug DAU with different phenanthridines (EB, 8-Azido-Ethidium Bromide (EMB) and 3,8-Diazido-Ethidium Chloride (EDC)) and their competitive binding to a self-complementary DNA oligomer, the deoxytetranucleotide 5'-d(TpGpCpA), has been studied in

aqueous salt solution (0.1M phosphate buffer in D_2O) by one- and two dimensional 500 MHz NMR spectroscopy. Comparison of the structural and thermodynamical properties of the hetero-association of different phenanthridines with daunomycin and their binding parameters with DNA provides information on the role of amino side groups of EB in stabilization of hetero-association complexes with DAU and the efficacy of drugs binding with DNA in the mixed solution. Despite identical structures of the chromophores of EMB and EDC, these molecules show different binding affinities to DNA: the characteristics of EMB complexation with oligonucleotides are similar to those for EB and differ significantly from those for EDC [7-9]. Investigation of the self-association of EB [10], EMB and EDC [11] has also shown a substantial effect of azido-groups in the phenoxazone chromophore on the aggregation parameters of the dye molecules in aqueous solution.

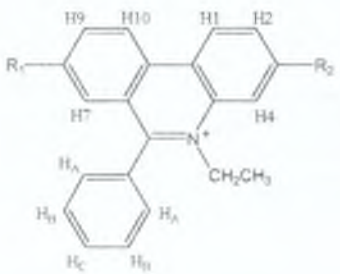
MATERIALS AND METHODS

The phenanthridine dyes, Ethidium Bromide (EB), Ethidium Monoazido Bromide (3-azido ethidium, EMB) and Diazido Ethidium Chloride (EDC) were purchased from "Sigma" (EB) and "Molecular Probes" (EMB and EDC) Chemical Companies. The anthracycline drug, daunomycin (DAU), was purchased from "Fluka" Chemical Company. The samples were used without further purification. 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: ε=5860 l mol^{-1} cm^{-1} (λ=480nm) for EB [12]; ε=5220 l mol^{-1} cm^{-1} (λ=458nm) for EMB [13]; ε=5850 l mol^{-1} cm^{-1} (λ=432nm) for EDC [13]; ε=11500 l mol^{-1} cm^{-1} (λ=477nm) for DAU [14,15]. The structures of the phenanthridine drugs and DAU are presented in Figure 1.

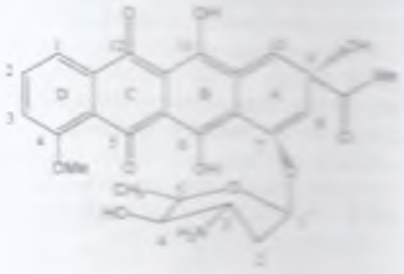
EB: $R_1 = NH_2$ $R_2 = NH_2$

EMB: $R_1 = N_3$ $R_2 = NH_2$

EDC: $R_1 = N_3$ $R_2 = N_3$



a)



b)

Fig. 1 Structures of ethidium derivatives (a) and daunomycin (b)

500 MHz ^{1}H-NMR spectra were recorded on a Bruker DRX FT NMR spectrometer. Signal assignments of the non-exchangeable protons of EMB and EDC were obtained using both two-dimensional homonuclear COSY/TOCSY and NOESY/ROESY experiments as done previously for EB and DAU [3,10]. Chemical shift measurements of the non-exchangeable protons of the aromatic molecules were made as a function of concentration at two temperatures (303 and 313K for DAU with EB; 298 and 308K for DAU with EMB and EDC) and measurements as a function of temperature were made at constant concentration in the temperature range 278-348K. All NMR measurements were made in the fast-exchange condition on the NMR time-scale. Chemical shifts were measured relative to an internal reference TMA (tetramethylammonium bromide) and recalculated with respect to DSS (sodium 2,2 dimethyl 2-silapentane-5-sulphonate).

RESULTS

Hetero-association of DAU with the phenanthridines

Quantitative analysis of the hetero-association of DAU with phenanthridine drugs requires determination of the structural and thermodynamical parameters of self-association of each of the drug molecules under the same experimental conditions [3,16]. The structures of the aggregates and thermodynamical parameters of self-association of DAU [17] and phenanthridine dyes (EB, EMB and EDC) [4,10,11] have been determined earlier. Comparison of the equilibrium constants of self-association of EB and its azido-analogues has shown [11] that the presence of azido-groups in the phenanthridine chromophore leads to lower probability of aggregation of dye molecules in aqueous solution. Thermodynamical parameters of dyes self-association confirm these conclusions [10,11]. It is likely that the interaction of electric dipole of the azido group (-N=N$^+$=N$^-$) with the electrons of the

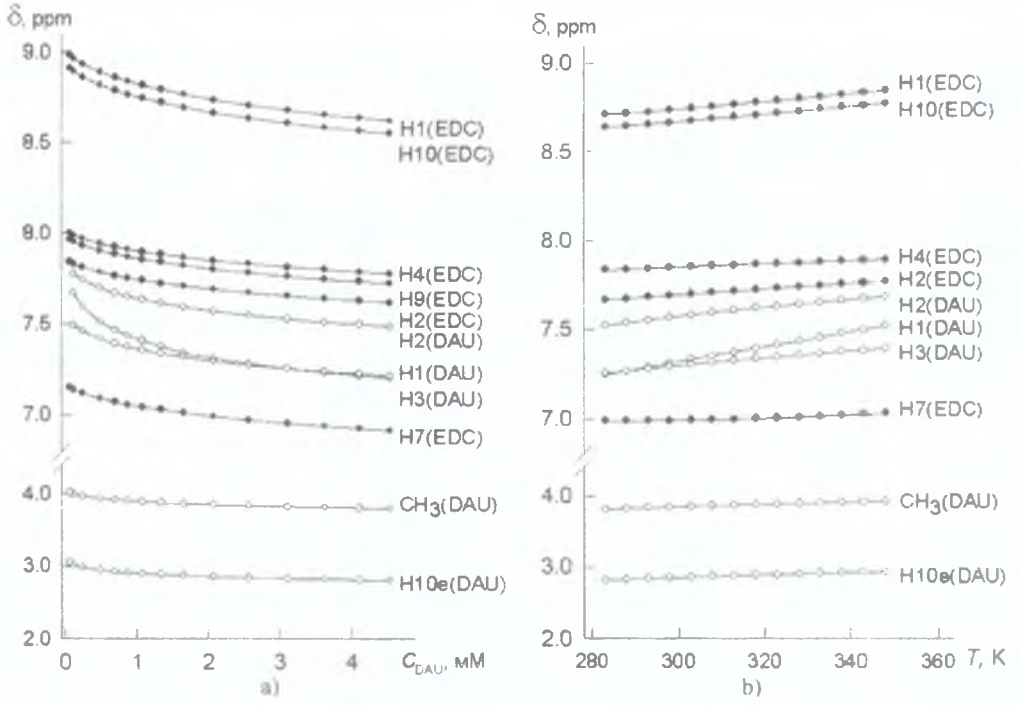


Fig. 2 Examples of 500 MHz ^{1}H NMR experiments of the hetero-association of DAU with EDC in 0.1M phosphate buffer, pD=7.1. Changes of chemical shifts on: (*a*) concentration of antibiotic ([EDC]=p_0=1.08mM=const), T=298 and (*b*) temperature of EDC+DAU (a_0=2.073 mM, p_0=1.08 mM)

aromatic rings of the chromophore leads to weakening of dispersive interactions in the formation of stack of the azido derivatives of EB.

The structures and thermodynamics of hetero-association between DAU and the phenanthridine drugs have been investigated by analysis of the chemical shift changes of both molecules in mixed solution as a function of concentration and temperature (Fig.2). Experimental data were analyzed in terms of the general model of molecular hetero-association of two components A and P [3,16]. It is assumed in this model 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 scheme (1):

$$A_1 + A_i \xleftrightarrow{K_A} A_{i+1} \qquad P_1 + P_j \xleftrightarrow{K_P} P_{j+1} \qquad P_j + A_i \xleftrightarrow{K_C} P_j A_i$$
$$P_j A_i + P_l \xleftrightarrow{K_C} P_j A_i P_l \qquad A_k + P_j A_i \xleftrightarrow{K_C} A_k P_j A_i \tag{1}$$

where indexes i,j,k,l denote the number of molecules in the aggregates of the dye (P) and DAU (A). Analysis of the scheme of reactions (1) leads to the following expression for the observed proton chemical shift of the component A [3]:

$$\delta_A = \frac{a_1}{a_0}\left[\delta_{mA}\left(2(1+K_A a_1) - \frac{1}{(1-K_A a_1)^2}\right) + 2\delta_{dA}\left(\frac{1}{(1-K_A a_1)^2} - 1 - K_A a_1\right) + \right.$$
$$\left. + \delta_{CA}\frac{K_C p_1}{(1-K_A a_1)^2(1-K_P p_1)}\left(1 + \frac{K_C p_1}{2(1-K_P p_1)} + \frac{K_C a_1}{1-K_A a_1}\right)\right] \tag{2}$$

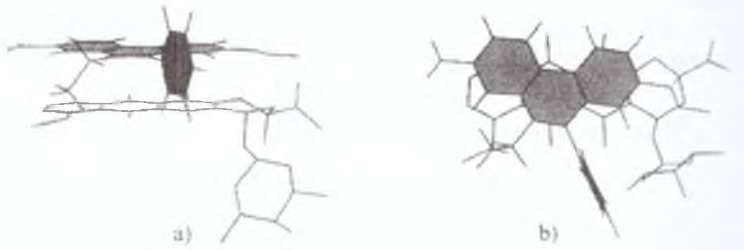
The values of chemical shifts for the protons of the P component can be obtained from eqn.(2) by means of substitution of indexes a for p and *vice versa* [3].

The values of δ_m, δ_d and the equilibrium constants K_A and K_P have been determined from the studies of the self-association of A and P molecules under similar experimental conditions [10,11]. It follows that the observed concentration dependences of proton chemical shifts of dye and DAU in mixed solutions (*e.g.* Fig.2a) are a function of two unknown quantities δ_C and K_C, which have been determined using the computational procedure described previously [3]. The magnitudes of the calculated parameters K_C, δ_{CA} and δ_{CP} are summarized in Table 1. The thermodynamical parameters ΔH^0_{het} and ΔS^0_{het} of hetero-association of aromatic molecules have been

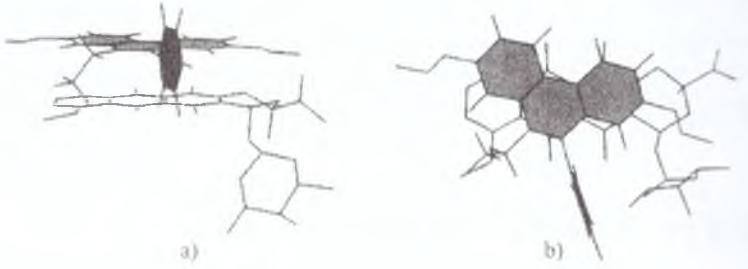
Table 1. Hetero-association parameters of DAU with the phenanthridine dyes in 0.1 mol l^{-1} phosphate buffer solutions, pD 7.1, T=298K

System	Protons of A	δ_{cA}, ppm	δ_{mA}, ppm	Protons of P	δ_{cP}, ppm	δ_{mP}, ppm	K_{het}, $l \cdot mol^{-1}$	$-\Delta H^{o}_{het}$, $kJ \cdot mol^{-1}$	$-\Delta S^{o}_{het}$, $J \cdot K^{-1} \cdot mol^{-1}$
EB+DAU*)	H2	7.57	7.83	H1	7.96	8.69			
K_P=305±14	H1	7.31	7.78	H10	7.88	8.63			
K_A=720±130	H3	7.25	7.55	H9	7.19	7.66	3850±630	42.5±3.3	74±12
	OCH_3	3.87	4.02	H4	7.09	7.55			
	H10*e*	2.96	3.05	H2	7.05	7.48			
	H10*a*	2.72	2.81	H7	6.23	6.67			
EMB+DAU	H2	7.60	7.83	H1	7.59	8.76			
K_P=276±17	H1	7.21	7.78	H10	7.62	8.80			
K_A=720±130	H3	7.29	7.55	H9	7.00	7.86	660±100	23.5±2.0	25±5
	OCH_3	3.87	4.02	H4	6.81	7.57			
	H10*e*	2.92	3.05	H2	6.79	7.52			
	H10*a*	2.70	2.81	H7	6.26	7.05			
EDC+DAU	H2	7.71	7.83	H1	7.96	8.68			
K_P=19±3	H1	7.34	7.78	H10	7.88	8.62			
K_A=720±130	H3	7.41	7.55	H9	7.19	7.66	320±65	10.1±2.0	13±4
	OCH_3	3.95	4.02	H4	7.09	7.55			
	H10*e*	2.95	3.05	H2	7.05	7.48			
	H10*a*	2.73	2.81	H7	6.23	6.67			

* Data taken from ref. [18].



(1) Structure of DAU+EMB complex



(2) Structure of DAU+EDC complex

Fig. 3. The calculated NMR structure of the 1:1 hetero-association complexes of phenanthridines (EMB and EDC) with DAU: a) side view of the hetero-complexes b) view looking perpendicular to the planes of the chromophores of aromatic molecules.

determined from measurements of the proton chemical shifts of the molecules in the mixed solutions as a function of temperature (Fig.2,b) using the analytical method described previously [5,16]. The derived values of enthalpy and entropy of the hetero-association reactions of DAU with phenanthridine dyes are also presented in Table 1.The most favourable structures of the 1:1 hetero-association complexes of aromatic molecules have been determined by analysis of the calculated values of induced proton chemical shifts, $\Delta\delta=\delta_c-\delta_m$, for both *A* and *P* components (Table 1). The mutual orientation of the molecules in the complex is determined by comparison of $\Delta\delta$ and their theoretical values derived from quantum-mechanical calculations of ring-current shielding maps for aromatic molecules [19]. A detailed description of the calculations of ring-current shielding maps for aromatic

molecules is given by Giessner-Prettre and Pullman [19]. The most favourable calculated structures of the 1:1 hetero-association complexes of DAU+phenanthridines are presented in different spatial projections in Fig.3.

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), was made using the additive model of drug-DNA complexation in solution, in which the self-association reactions of the drug and oligonucleotide ($N + N \xleftrightarrow{K_N} N_2$) 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 [17,20,21]:

$$\begin{array}{llll} A + N \xleftrightarrow{K_1} AN & (a) & A + N_2 \xleftrightarrow{K_2} AN_2 & (b) \\ A + AN \xleftrightarrow{K_3} A_2N & (c) & A + AN_2 \xleftrightarrow{K_4} A_2N_2 & (d) \\ AN + N \xleftrightarrow{K_5} AN_2 & (e) & & \end{array} \qquad (3)$$

where A and N are the monomer forms of the ligand and tetranucleotide, respectively.

The equilibrium constants K_1 -K_5 for complexation of DAU and EB with both the monomer and duplex form of the deoxytetranucleotide d(TpGpCpA) were calculated from observed concentration dependences of drug proton chemical shifts [17,20] and the results are presented in Table 2.

NMR analysis of the complexation of EMB with the tetramer shows that the binding constants, within error limits, coincide with those for formation of the EB+d(TGCA) complex, in agreement with the results obtained for EB and EMB binding with macromolecular DNA [7,9]. As for EDC, it has been shown [7-9] that the equilibrium constant of complexation of this drug with deoxyoligonucleotides and with DNA molecules is 2-3 times smaller when compared to those for EB and EMB.

Table 2. Equilibrium constants of complex formation between EB, DAU and the deoxytetranucleotide 5'-d(TpGpCpA)[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
DAU[c]	31 ± 8	430 ± 100	5.4 ± 0.4	12.5 ± 0.5	-[d]

[a] Determined from NMR measurements in 0.1 M phosphate buffer solutions, pD 7.1.
[b] Data taken from ref. [20]
[c] Data taken from ref. [17]
[d] The value of this association constant turned out to be negligible and was not included in the calculations

DISCUSSION

Hetero-association of DAU with the phenanthridines

The value of hetero-association constant of EB+DAU (3850±630 l mol^{-1}) [18] is substantially higher than the self-association constants of these molecules (Table 2) indicating that formation of hetero-complexes between EB and DAU is energetically more favourable than self-aggregation of these aromatic dye/drug molecules. It may be assumed that such an extra-stabilization in the 1:1 EB-DAU hetero-complex is due to formation of a hydrogen bond between the 3,8-amino group of EB and the 9MeCO group of DAU analogous to that observed between the 3,6-amino groups of the acridine chromophore of Proflavine (PF) and the 9-MeCO group of DAU in the 1:1 PF-DAU hetero-complex [3]. The regular decrease in K_{het} values of EMB+DAU (660±100 l mol^{-1}) and EDC+DAU (320±65 l mol^{-1}) compared to EB+DAU is consistent with hydrogen bond formation between the 9MeCO group of DAU and the (3,8) amino groups of EB, because of the successive substitution of amino groups in EMB (one group) and EDC (no amino groups) by the azido groups, which cannot form such a hydrogen bond.

The values of the thermodynamical parameters of hetero-association of DAU with the phenanthridine drugs (Table 1) confirm qualitatively an extra-stabilization of DAU-dye complexes by intermolecular H-bond. Hydrogen bond formation in aqueous solution contributes to negative values of enthalpy and entropy [22] and the magnitude of the enthalpy of hydrogen bond formation in aqueous solution is estimated to be from -8 to -13 kJmol^{-1} [22]. A regular decrease in absolute values of changes in enthalpy and entropy is observed for hetero-association of DAU with EB, EMB and EDC (Table 1), which is consistent with a decrease in probability of hydrogen bond formation due to substitution of amino with azido groups in the phenanthridine chromophore.

The most favourable structures of the 1:1 hetero-association complexes of DAU and the phenanthridine dyes could be reliably determined using the known isoshielding curves of daunomycin and limiting proton chemical shifts of the phenanthridine in the 1:1 hetero-complex (Table 1). Analysis of the induced proton chemical shifts of EB and its azido-analogues in 1:1 hetero-complexes with DAU (Table 1) shows similar behaviour of $\Delta\delta$ ratios for the non-exchangeable protons of all the phenanthridines. It follows that the mutual orientation of the DAU and phenanthridine chromophore in the 1:1 hetero-complexes is similar for the molecular systems studied (Fig.3). The planes of the chromophores of the dye and the DAU molecule are parallel to each other (situated 0.34 nm apart for EB-DAU hetero-complex and *ca.* 0.31nm for EMB-DAU and EDC-DAU hetero-complexes). The difference in distances between the chromophores may be caused by the dipole moment of azido groups, which affects the electron distribution in the aromatic rings of phenanthridine chromophore.

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

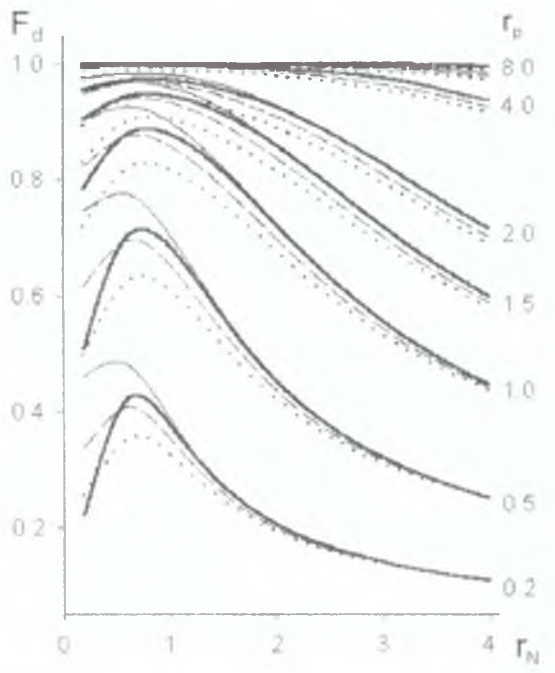


Fig. 4 Relative decrease in content of the complexes of DAU with the 5'-d(TpGpCpA) duplex calculated (from results in Tables 1 and 2) as a function of $r_N=[N_0]/[A_0]$ at different ratios (r_p) of the dye to DAU concentrations in solution, when EB (*thick solid lines*), EMB (*thin solid lines*), EDC(2) (*dashed lines*) and EDC(3) (*dotted 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 computational procedure described in previous work [4,21] 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 of self-, and hetero-association of drug molecules, and their complexation with 5'-d(TpGpCpA), and the mass conservation law, and, hence, determination of the relative content of different complexes in solution. The relative decrease in content of the complexes of aromatic ligand-deoxytetranucleotide duplex has been calculated as a function of tetramer concentration for different amounts of phenanthridine/DAU in the mixed solution, using equilibrium constants summarized in Table 1. The results for all the molecular systems studied in the mixture with d(TpGpCpA) are summarised in Fig.4 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 different for the different phenanthridines at relatively small r_N values ($r_N< 1$), when the dye acts as an "interceptor" of DAU in solution, which affects the complexation of DAU with DNA due to the differences in the equilibrium constants of self-association of the dyes and their hetero-association with DAU and in the dye-DNA complexation constants (Tables 1 and 2). In the calculations for the molecular system EDC+DAU+d(TGCA), the effective complexation constant of EDC with the DNA fragment was taken two (EDC(2)) and three (EDC(3)) times smaller compared with EB and EMB [7-9]. The calculated dependences $F_d(r_N)$ have pronounced maxima at $r_N\leq 1$ for EB+DAU (Fig. 4, thick solid lines) owing to competition between EB-DAU hetero-association and dye-DNA binding. Such a situation is hardly observed for EMB+DAU (Fig.4, thin solid lines), because hetero-association of EMB and DAU plays a relatively small role in the complex equilibrium in solution compared to competitive binding of EMB and DAU with DNA at low r_N values.

Qualitatively the same situation is observed for propidium iodide (PI) in the mixture with DAU and d(TpGpCpA), because the binding affinity of PI with the DNA duplex is substantially higher compared with that for EB-DNA complexation, but, on the other hand, the PI-DAU hetero-association constant is much smaller than K_{het} of EB+DAU system [18]. It is interesting that for the EDC+DAU system (Fig.4, dashed-dotted lines) the $F_d(r_N)$ dependences also show maxima, though less pronounced than for EB+DAU, at relatively small DNA concentrations. This is obviously due to a smaller hetero-association affinity of EDC with both DAU and DNA compared with EMB and EB. It follows that the dynamic equilibrium in the mixed solutions depends

substantially on the interplay of the magnitudes of equilibrium constants for formation of different molecular complexes. At $r_N > 1$ the greater "intercepter" action of EB and EMB than that of EDC obviously results from the smaller complexation affinity of EDC compared to EB and EMB. An increase in content of the DNA oligomer in solution at $r_p \leq 2$ leads to a substantial decrease of F_d as a function of r_N, where the phenanthridines act as "interceptors" of DAU molecules (Fig.4). 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 content in solution is further increased ($r_p > 2$), this effect becomes less pronounced (Fig.4). In principle, such an analysis enables the optimum concentration of drug/mutagen to be determined for any defined reduction in ligand binding to DNA [4,21].

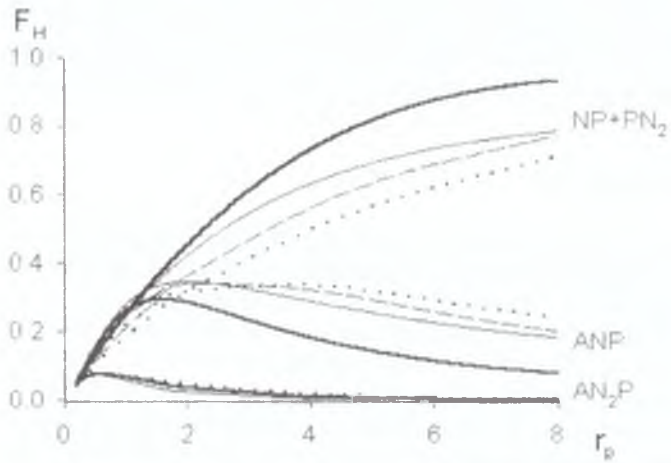


Fig. 5 Relative content, F_c, of the complexes of DAU (*A*) and dye (*P*) with the tetranucleotide (*N*) 5'-d(TpGpCpA), calculated (from results in Tables 1 and 2) as a function of r_p, the ratio of dye to DAU concentrations in the mixed solution at r_N= $[N_0]/[A_0]$=1; *PN+PN₂* - are complexes of the dye with deoxytetranucleotide in the monomer and duplex forms; *APN* and *APN₂* are hetero-association complexes: (EB, *thick solid lines*; EMB, *thin solid lines*; EDC(2), *dashed lines*; EDC(3), *dotted lines*).

Using the 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.5).

Qualitatively similar curves were obtained for the PI+DAU system in the mixture with d(TpGpCpA) under the same experimental conditions [18]. 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 dye-DAU-DNA hetero-complexes (APN and APN_2) depends on the relation between the equilibrium constants for complexation with the deoxytetranucleotide of the drug and dye, as well as for drug-dye hetero-association in solution.

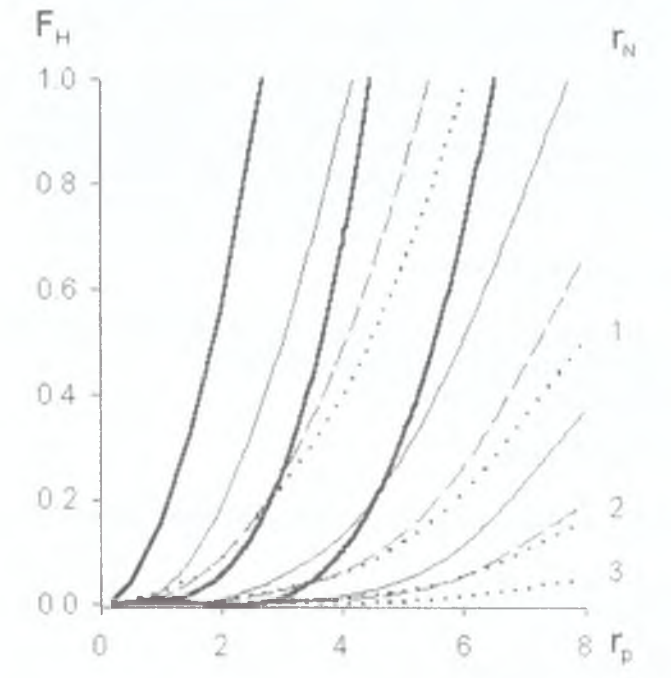


Fig.6 Ratio (F_H) of the content of "dye-DAU" hetero-association complexes with respect to the total amount of complexes of dye with deoxytetranucleotide 5'-d(TpGpCpA), calculated (from results in Tables 1and 2) as a function of the ratio (r_p) of dye and DAU concentrations in the mixed solution of dye+DAU+d(TGCA) at different r_N=$[N_0]/[A_0]$ values: thick solid, thin solid, dashed and dottes lines correspond to EB, EMB, EDC(2) and EDC(3), respectively, acting as interceptors of DAU.

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. The results of such calculations in Fig.6 show that for different phenanthridine dyes 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 values for the molecular systems studied may be presented in the following order:

EB-DAU-DNA > EMB-DAU-DNA > EDC(2) -DAU-DNA > EDC(3) -DAU-DNA

in accordance with dye-drug hetero-association constants and their complexation affinity with DNA (Tables 1, 2). At $r_N = 1$ the value of F_H becomes predominant ($F_H > 1$) when $r_p > 2$ for EB-DAU-DNA, at $r_p > 3$ for EMB-DAU-DNA, at $r_p > 4$ for EDC(2)-DAU-DNA and at $r_p > 5$ for EDC(3)-DAU-DNA system. At $r_N = 2$ and $r_N = 3$ this situation ($F_H > 1$) will be observed at much higher r_p values (Fig. 6).

Dependences of $F_d(r_N)$, $F_c(r_p)$ and $F_H(r_p)$ have been also calculated using an effective complexation constant of EDC with DNA ten times smaller compared with that for EB and EMB. The calculations have shown that the observed dependences are qualitatively similar to those for EDC(2) and EDC(3), but the "interceptor action" of the phenanthridine dye with substantially smaller binding affinity to DNA, is obviously much less pronounced.

CONCLUSIONS

NMR investigations of the hetero-association of the aromatic dyes, containing different side groups in the phenanthridine chromophore, with DAU and the competitive binding of aromatic dye-drug molecules with a DNA oligomer, 5'-d(TpGpCpA), have shown that:

1. The presence of groups which may act as H-bond donor and acceptor groups in aromatic molecules can result in higher association and binding affinity of intercalators: extra- stabilization in the 1:1 EB-DAU hetero-complex by formation of a hydrogen bond between the 3,8-amino groups of EB and the 9 MeCO group of DAU is confirmed by the regular decrease in thermodynamic parameters (K_{as}, ΔH) for complexation of DAU with EB azido- derivatives, having successive substitution of amino groups in EMB (one group) and EDC (no amino groups) by the azido groups which cannot form such a hydrogen bond;
2. Substitution of amino- with azido-groups in the phenanthridine dye molecules leads to a decrease in the binding affinity of intercalators with DNA;
3. The equilibrium balance of self-association, hetero-association and dye-drug-DNA binding depends on solution conditions. When phenanthridines are added in combination with DAU at relatively small DNA concentration ($r_N < 1$, $r_p < 2$), the major role is played by the relation between the equilibrium constants of dye-drug hetero-association and their complexation with DNA. Under these conditions, the dominant effect for EMB is competition for DNA binding sites, whereas for EB and EDC it is hetero-association with DAU. At higher DNA concentrations ($r_N > 2$) the complexation of phenanthridines with DNA becomes more important;
4. In order to elucidate the molecular complexation processes in multi-component equilibria occurring in mixed solution (including mutagen, aromatic antibiotic and DNA), it is necessary to take into account not only the hetero-association of the aromatic molecules but also the competition between drug and mutagen for the oligonucleotide binding sites: the model and analytical method outlined in this work enables the relative importance of each complexation reaction to be determined quantitatively.

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ВПЛИВ БОКОВИХ ГРУП ФЕНАНТРИДІНОВИХ БАРВНИКІВ НА ГЕТЕРОАСОЦІАЦІЮ З АНТИБІОТИКОМ ДАУНОМІЦИНОМ ТА КОНКУРЕНТНЕ ЗВ'ЯЗУВАННЯ ЛІГАНДІВ З ОЛІГОМЕРОМ ДНК

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Методом ^{1}H ЯМР спектроскопії досліджено гетероасоціацію фенантридинових барвників, вмщуючих різні групи у бокових ланцюгах хромофору, з антрациклиновим антибіотиком дауноміцином (DAU) та їх конкурентне зв'язування з олігомером ДНК 5'-d(TpGpCpA). При послідовному заміщенні 3,8 аміно-груп на азидо-групи спостерігається зменшення значень параметрів гетероасоціації азидо-аналогів бромистого етідію (EB) – моноазиду (EMB) и диазиду (EDC) етідія з DAU, що свідчить про зниження імовірності утворення міжмолекулярного водневого зв'язку між аміно-групами фенантридинового хромофора та 9MeCO групою DAU. Аналіз показав, что рівноважні процеси самоасоціації, гетероасоціації та комплексоутворення лігандів з ДНК залежать від співвідношення концентрацій компонентів у змішаному розчині. Для встановлення молекулярного механізму комплексоутворення молекул в багатокомпонентному розчині, вмщуючому мутагени, ароматичні антибіотики і фрагменти ДНК, необхідно враховувати не тільки гетероасоціацію ароматичних молекул, але і конкуренцію між антибіотиком і мутагеном за місця посадки на олігонуклеотидну послідовність.

КЛЮЧОВІ СЛОВА: Фенантридіни, Азидо-аналоги, Дауноміцин, ЯМР спектроскопія, Гетероасоціація, Конкурентне зв'язування з ДНК.