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Complexation of daunomycin with a DNA oligomer in the presence of an aromatic vitamin (B_2) determined by NMR spectroscopy

M.P. Evstigneev [a,*], Yu.V. Mykhina [a], D.B. Davies [b]

[a] *Department of Physics, Sevastopol National Technical University, Studgorodok, Sevastopol, 99053, Crimea, Ukraine*
[b] *School of Biological and Chemical Sciences, Birkbeck College, University of London, Malet Street, London WC1E 7HX, UK*

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Abstract

The effect of simultaneous binding of the anthracycline antibiotic Daunomycin (DAU) and the Vitamin B_2 derivative, Flavin-mononucleotide (FMN), with the DNA oligomer, $d(TGCA)_2$, in solution has been investigated quantitatively by ^{1}Í-NMR spectroscopy (500 MHz). The equilibrium reaction constants and the thermodynamical parameters (ΔH, ΔS) of the hetero-association FMN-DAU and complexation of FMN with $d(TGCA)_2$ have been determined by analysis of the concentration and temperature dependences of chemical shifts of the aromatic protons in terms of a competitive binding model. A criterion for discrimination between hetero-association and DNA complexation has been developed and applied to the analysis of the simultaneous binding of the antibiotic and the vitamin with DNA. Under the conditions of the experiment, it is found that both the hetero-association of FMN with DAU and the complexation of FMN with DNA contribute approximately equally to the decrease of DAU binding with DNA oligomer. Such competitive complexation of aromatic vitamin and drug with DNA could affect the biological activity of such drugs.

Keywords: Antitumour antibiotic; Flavin-mononucleotide; Daunomycin; Hetero-association; Competitive binding; NMR spectroscopy

1. Introduction

Doxorubicin (DOX) and its close analogue daunomycin (DAU) (Fig. 1a) belong to the family of anthracycline antibiotics used extensively for the treatment of a wide variety of human malignant leukemias, bone sarcomas, and carcinomas of the urinary and reproductive tracts [1]. The effectiveness of DOX/DAU as an antineoplastic agents is well established, but the most serious side effect of its use is acute cardiotoxicity, which in later stages culminates in irreversible congestive heart failure [1–3]. Due to the great importance of the anthracyclines in chemotherapy much effort has been expended in order to prevent or attenuate the side effects of anthracycline administration. It is now widely accepted that oxidative stress and the production of free radicals are involved in DOX/DAU action, both in terms of antitumour effects and cardiotoxicity [2,3]. An important strategy of trying to reduce the toxic effects of the anthracyclines without interfering with their antitumour properties is the delivery of the drug combined with an antioxidant in order to reduce oxidative stress [3]. Among these agents the antioxidants derived from diet, such as aromatic vitamins, are used widely in clinical practice [3,4].

It has been previously shown that Vitamin B_2 (Riboflavin, RBF) and its physiological derivative, Flavin-mononucleotide (FMN) (Fig. 1b), are able to modulate the toxicity of DOX in vivo [5,6]. A number of mechanisms have been suggested, including the formation of hetero-complexes between DOX and RBF leading to chemical degradation of the antibiotic [6,7], and the ability of DOX and several of its metabolites to mimic flavines and compete successfully for their binding sites on a number of flavin-containing enzymes [6,8]. Bearing in mind that the anthracyclines DOX/DAU function primarily at the DNA level by blocking replication and transcription processes due to binding with nuclear DNA [9], it also may be suggested that Vitamin B_2 and its derivatives can interfere with the DNA binding of aromatic anthracycline antibiotics. Recently the protective effect of RBF on the toxicity of different aromatic carcinogens has been reported [10,11],

* Corresponding author. Fax: +38 692 243 590.
E-mail address: max_evstigneev@mail.ru (M.P. Evstigneev).

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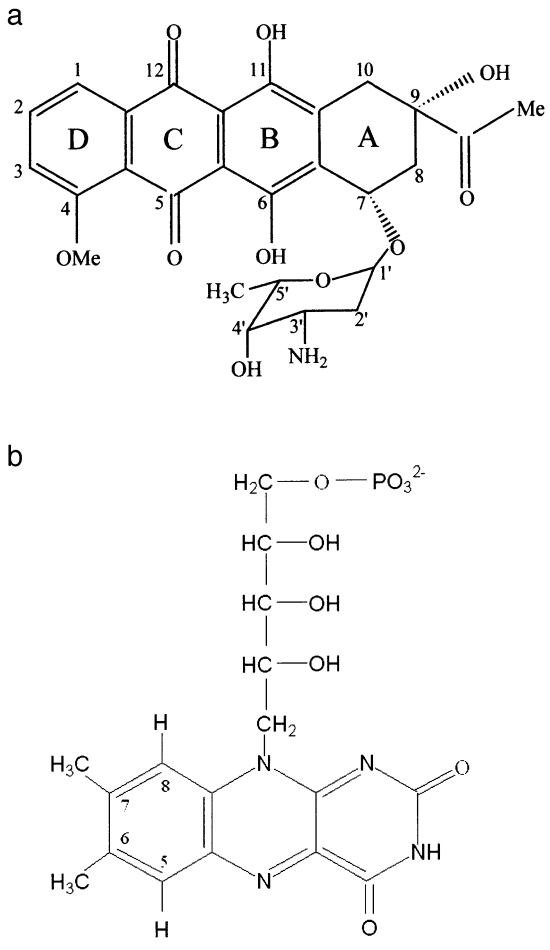

Fig. 1. Structures of (a) daunomycin and (b) flavin-mononucleotide.

which was monitored by a significant change in carcinogen-DNA binding in the presence of the vitamin. The ability of other aromatic drugs such as caffeine to intercept anthracyclines via hetero-association and to compete with them for the binding sites on DNA has also been thoroughly investigated [12–14]. It is likely that complexation of the anthracycline antibiotic with both Vitamin and DNA may contribute to the observed change in anthracycline toxicity in the presence of Riboflavin. Hence a method to discriminate between two processes is needed to understand how Vitamin B_2 and its derivatives modify the effect of anthracycline antibiotics.

In order to quantify the process of simultaneous binding of the anthracycline antibiotic and Vitamin B_2 with DNA, it should just be necessary to know the equilibrium parameters of DAU-RBF hetero-association, and complexation of DNA with DAU and RBF. Separate studies of two-component systems can be performed under similar solution conditions and the derived equilibrium constants used to calculate the relative importance of hetero-association and DNA complexation. Three questions immediately arise: (i) Is it relevant to use the equilibrium parameters derived from two-component studies for analysis of three-component mixtures? (ii) What criterion can be used to discriminate between hetero-association and the competition of two aromatic molecules for DNA binding sites? (iii) and Which DNA receptor should be chosen for such studies? The first two questions will be investigated in this work and the latter one discussed now in relation to the situation for nuclear DNA and the need to study systems in fast exchange in equilibrium in order to determine thermodynamic parameters of complexation by NMR spectroscopy.

Although DNA is associated with histones and other nuclear proteins in chromatin [15], some studies have reported that Daunomycin complexes predominantly with protein-free regions of DNA available in linker regions of a nucleosome [16,17]. This suggests that a non-polymeric DNA sequence would be more suitable to probe competitive binding and that an oligomeric sequence should contain no more than one binding site. An exclusion parameter for DAU/DOX is known to be greater than 3 base pairs [18], so a tetrameric DNA sequence is the best choice for competitive analysis. For a number of self-complementary deoxytetranucleotides it has been shown that an equilibrium exists between single- and double-stranded forms and that drug complexation with such oligonucleotides is in fast exchange and suitable for determining thermodynamic parameters by NMR spectroscopy [13]. And finally what nucleotide sequence should be selected? In order to attenuate a contribution of the difference in sequence-specificity of DNA binding of competing drugs on the results of analysis, the tetrameric sequence 5′-d(TpGpCpA) containing all four naturally occurring nucleotides is suitable.

In the present work we have studied by NMR spectroscopy the hetero-association of the anthracycline antibiotic Daunomycin with Vitamin B_2 derivative, Flavin-mononucleotide, and the complexation of DAU with deoxyoligonucleotide 5′-$d(TpGpCpA)_2$ in the presence of FMN in aqueous solution. Since no information is currently available for the binding of FMN with double-stranded DNA, NMR experiments have also been conducted in order to obtain the equilibrium complexation parameters between FMN and d(TGCA). A model for the competitive binding assay has been developed; a criterion for the discrimination between the hetero-association and DNA complexation has been formulated and applied for analysis of the simultaneous binding of the antibiotic and the vitamin with DNA.

2. Experimental

Daunomycin (Fig. 1a) from Fluka, flavin-mononucleotide (Fig. 1b) from Sigma and 5′-d(TpGpCpA) from Metabion were used without further purification. The samples were lyophilized from D_2O solutions and re-dissolved in 0.1 M phosphate buffer in 99.95% D_2O (pD 7.1) containing 10^{-4} M EDTA.

500 MHz ^{1}H NMR spectra were recorded on a Bruker DRX spectrometer with the residual water peak saturated during relaxation. Signal assignments of the non-exchangeable protons of the drugs were obtained using both two-dimensional homonuclear COSY (TOCSY) and NOESY (ROESY) experiments. Chemical shift measurements of the non-exchangeable protons of the aromatic molecules were made as a function of concentration of the antibiotic (DAU-FMN experiment) or tetramer (FMN-TGCA experiment) at two temperatures (298 and 308 K) maintaining the concentration of FMN constant

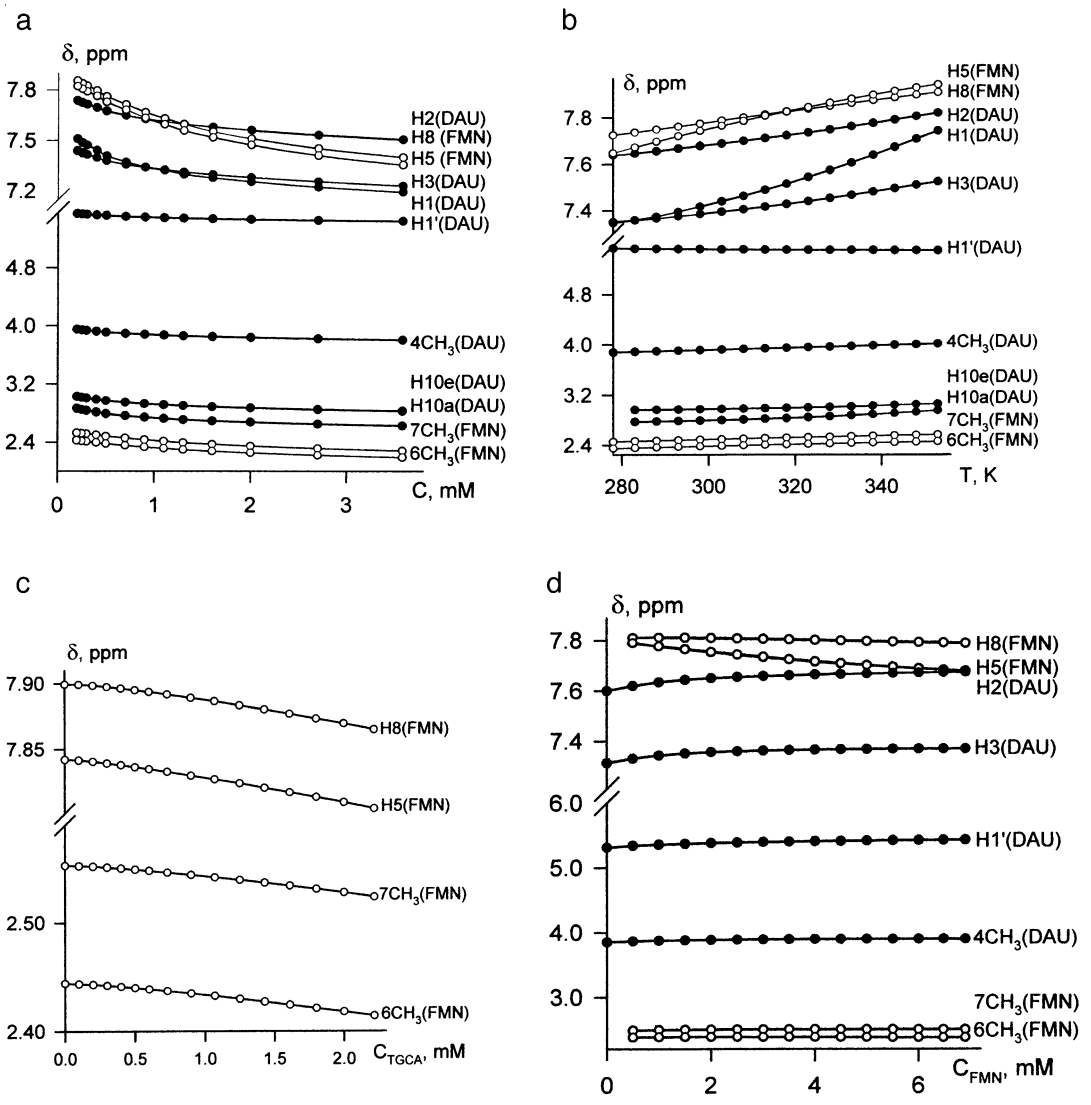

Fig. 2. Dependence of proton chemical shifts of FMN (–○–) and DAU (–●–): (a) on concentration of DAU (T=298 K, C_{FMN}=1 mM) in the DAU-FMN experiment; (b) on temperature (C_{FMN}= 1 mM, C_{DAU}=0.5 mM) in the DAU-FMN experiment; (c) on concentration of tetramer (T=298 K, C_{FMN}=1.89 mM) in the FMN-TGCA experiment; and (d) on concentration of FMN (T=298 K, C_{DAU}=0.55 mM, $C_{d(TGCA)}$=0.824 mM) in the FMN-DAU-TGCA experiment.

(Fig. 2a,c). The temperature dependences of proton chemical shifts for the DAU–FMN mixture were measured at constant concentration of drug molecules in the temperature range 278–353 K (Fig. 2b). A competitive experiment with the mixture of DAU, FMN and d(TGCA) was performed by varying the concentration of FMN and keeping the concentrations of DAU and d(TGCA) constant (Fig. 2d). All sets of NMR measurements were made in the fast-exchange condition on the NMR timescale. Chemical shifts were measured relative to TMA (tetramethylammonium bromide) as an internal reference and recalculated with respect to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate), i.e. DSS=TMA+3.178 (ppm). The sample temperature was regulated using the Bruker BVT-3000 unit.

3. Results and discussion

3.1. Hetero-association of DAU with FMN

Analysis of the hetero-association of the antibiotic Daunomycin with Flavin-mononucleotide has been made as in previous work [19–21] using a general model of indefinite molecular hetero-association in solution based on reaction scheme (1):

$$D_i + F_j \overset{K_h}{\leftrightarrow} D_iF_j, \quad D_iF_j + D_k \overset{K_h}{\leftrightarrow} D_iF_jD_k, \quad F_l + D_iF_j \overset{K_h}{\leftrightarrow} F_lD_iF_j, \tag{1}$$

Table 1
Hetero-association parameters of DAU (D) with FMN (F) in 0.1 mol l^{-1} phosphate buffer solutions, pD 7.1 at 298 K

T (K)	Protons of (DAU)	δ_{hD} (ppm)	δ_{mD} (ppm)	Protons of (FMN)	δ_{hF} (ppm)	δ_{mF} (ppm)	K_{het} (M^{-1})
T= 298 K							
K_D=720±140 M^{-1}; K_F=265±38 M^{-1}	H2	7.62	7.83	H8	7.03	7.99	453±28
	H1	7.03	7.78	H5	6.59	8.00	
	H3	7.28	7.55	$7CH_3$	1.89	2.59	
	H1′	5.58	5.52	$6CH_3$	1.72	2.49	
	$40CH_3$	3.85	4.02				
	H10*e*	2.95	3.05				
	H10*a*	2.88	2.81				
T= 308 K							
K_D=466±90 M^{-1}; K_F=176±25 M^{-1}	H2	7.58	7.83	H8	7.03	7.99	283±13
	H1	7.03	7.78	H5	6.59	8.00	
	H3	7.23	7.55	$7CH_3$	1.89	2.59	
	H1′	5.58	5.52	$6CH_3$	1.72	2.49	
	$40CH_3$	3.83	4.02				
	H10*e*	2.95	3.05				
	H10*a*	2.88	2.81				

Thermodynamic parameters of hetero-association of FMN and DAU: $\Delta H^{\circ}_{het}=-35.8$ (±5.4) kJ/mol; $\Delta S^{\circ}_{het}=-69.5$ (±9.1) J/(mol·K); $\Delta G^{\circ}_{het}=-15.1$ (±0.2) kJ/mol.

where K_h is the hetero-association constant of DAU (D) with FMN (F); i, j, k, l are the numbers of molecules in D or F aggregates within the hetero-complexes. The NMR dependence of the aromatic protons for both DAU and FMN as a function of concentration and temperature are summarised in Fig. 2a,b. The algorithm of analysis [19–21] includes a fitting of the experimental data using model equations derived from (1) with three search parameters for the concentration runs [equilibrium constant K_h and chemical shifts of DAU (δ_{hD}) and FMN (δ_{hF}) within 1:1 hetero-complex] and two search parameters for the temperature runs [enthalpy (ΔH_h) and entropy (ΔS_h)]. The results are presented in Table 1. The calculated values of δ_h were used to build the initial structure of the 1:1 DAU-FMN hetero-complex, whose calculated energy in an aqueous environment was minimized using the XPLOR molecular modeling software according to the methodology published previously [20,21]. The most probable calculated spatial structure of 1:1 DAU-FMN hetero-complex is presented in Fig. 3.

It is seen from the Table 1 that the equilibrium hetero-association constant K_h and the enthalpy/entropy falls between the self-association constants of DAU (K_D) and FMN (K_F), analogous to results for complexation of other aromatic molecules (caffeine, acridine orange) to Daunomycin reported previously [13,19]. It has been shown that stacking interactions, which include both dispersive and hydrophobic interactions, play a major role in stabilization of the sandwich-type hetero-complexes of aromatic chromophores in solution [13,19,21]. The structure of the DAU-FMN hetero-complex presented in Fig. 3 clearly demonstrates the co-planarity of aromatic chromophores of DAU and FMN, which supports the conclusion about the stacking as a major stabilizing force in the given system.

3.2. The complexation of FMN with d(TGCA) in aqueous solution

Calculations of equilibrium complexation parameters of FMN with d(TGCA) have been made over the concentration dataset of proton chemical shifts of FMN in the presence of the oligonucleotide in solution (Fig. 2c) using equilibrium Scheme (2), as in previous work [22,23]:

$$F + F_i \overset{K_F}{\leftrightarrow} F_{i+1}\ (a) \quad N + N \overset{K_N}{\leftrightarrow} N_2\ (b) \quad F + N \overset{K_{11}}{\leftrightarrow} FN\ (c) \quad F + N_2 \overset{K_{12}}{\leftrightarrow} FN_2\ (d) \quad F + FN \overset{K_{21}}{\leftrightarrow} F_2N\ (e) \quad F + FN_2 \overset{K_{22}}{\leftrightarrow} F_2N_2\ (f), \tag{2}$$

where K_N is the self-association constant of the tetramer [22,23]; K_{11}, K_{12}, K_{21}, K_{22} are equilibrium constants of the formation of 1:1, 1:2, 2:1 and 2:2 complexes of FMN with d(TGCA) in solution; N and N_2 are monomer and duplex form of the tetramer in solution. Analytical expressions for the observed proton chemical shift, derived from (2), are taken from Refs. [22,23] and the algorithm of fitting of experimental data using 8 varying quantities (four complexation constants and four chemical shifts for each type of complex in (2)) is similar to that used in the hetero-association study (see above). The results of the calculations are presented in Table 2.

Analysis of the equilibrium complexation parameters in Table 2 clearly demonstrates a predominant role of FMN binding with the duplex form of d(TGCA) in solution featuring a strongly anticooperative character of the complexation process, which may be

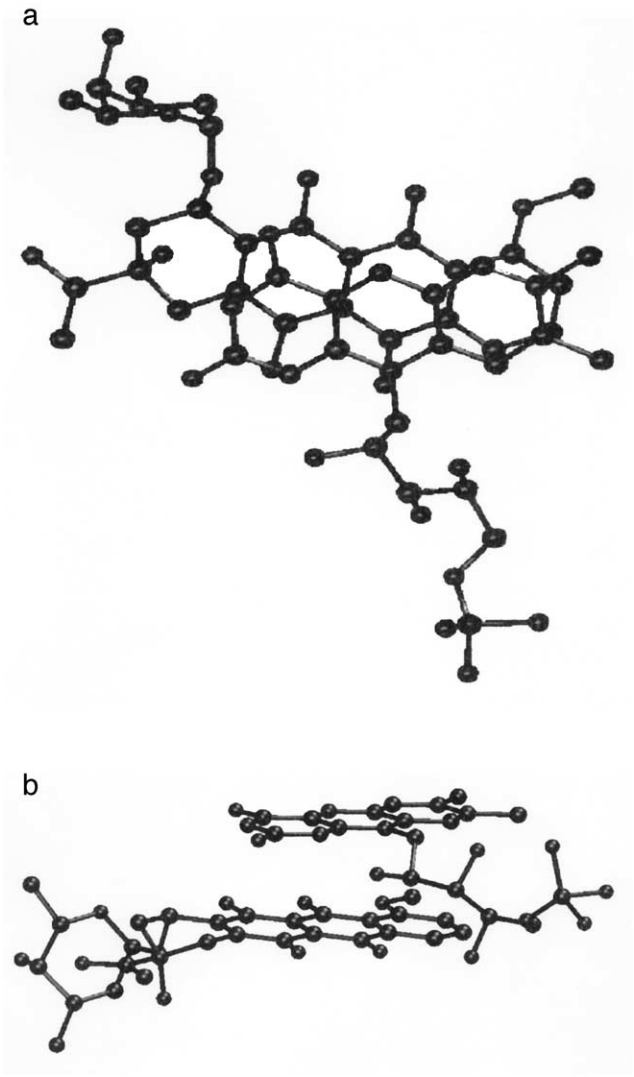

Fig. 3. The calculated spatial structure of the 1:1 hetero-association complex of FMN with DAU: a) view looking perpendicular to the planes of the chromophores of aromatic molecules, and b) side view of the hetero-complex.

deduced from the relatively small magnitudes of equilibrium constants K_{21} and K_{22} with respect to K_{11} and K_{12}. The anticooperative effect is likely to result from steric and electrostatic hindrance to the binding of the second molecule to the tetramer, originating from the charged side chain of the FMN molecule. Unfortunately, no intermolecular contacts were observed in 2D NOESY spectra and the present data makes it impossible to conclude unambiguously about the mode of FMN binding with DNA. However, the induced chemical shift δ_{12} of the FMN molecule in the 1:2 complex with the duplex form of the tetramer (Table 2) is lower by 0.3 ppm on average than for the monomeric form of FMN, δ_m. Shielding of aromatic protons is known to be a distinctive feature of the intercalation process [22–24] and therefore it is likely that FMN intercalates or partially intercalates with the DNA duplex.

3.3. General scheme for analysis of the simultaneous binding of DAU and FMN with the deoxytetranucleotide

In order to rule out the influence of a concentration range used for analysis, the model of the three-component equilibrium in solution should comprise all types of interactions, i.e. self-association of DAU (3a), FMN (3b) and d(TGCA) (3c); the hetero-

Table 2
Parameters of the complexation of FMN with d(TGCA) in 0.1 mol l^{-1} phosphate buffer solutions, pD 7.1, T=298 K

Complex	K_C, l/mol	H8	H5	7Me	6Me
1:1	100±40	7.93	7.70	2.53	2.45
1:2	8000±2000	7.71	7.65	2.45	2.34

association of DAU with FMN (3d–f) and the complexation of DAU/FMN with both single-(N) and double-stranded (N_2) forms of the tetramer in solution (3g–j):

$$\begin{array}{l}\text{Self}-\text{association}\\ D+D_i \overset{K_D}{\leftrightarrow} D_{i+1}\ (\text{a}) \quad F+F_j \overset{K_F}{\leftrightarrow} D_{j+1}\ (\text{b}) \quad N+N \overset{K_N}{\leftrightarrow} N_2\ (\text{c})\\ \text{Hetero}-\text{association}\\ D_i+F_j \overset{K_h}{\leftrightarrow} D_iF_j\ (\text{d}) \quad D_iF_j+D_k \overset{K_h}{\leftrightarrow} D_iF_jD_k\ (\text{e}) \quad F_l+D_iF_j \overset{K_h}{\leftrightarrow} F_lD_iF_j\ (\text{f})\\ \text{DNA complexation}\\ D+N \overset{K_{1D}}{\leftrightarrow} DN\ (\text{g}) \quad D+N_2 \overset{K_{2D}}{\leftrightarrow} DN_2\ (\text{h})\\ F+N \overset{K_{1F}}{\leftrightarrow} FN\ (\text{i}) \quad F+N_2 \overset{K_{2F}}{\leftrightarrow} FN_2\ (\text{j})\end{array} \tag{3}$$

where K_D and K_F are the self-association constants of DAU and FMN, respectively; K_{1D}, K_{1F} and K_{2D}, K_{2F} are complexation constants of DAU and FMN with the single- and double-stranded form of d(TGCA) respectively. For the case of DAU and FMN, which bind to the tetramer in a highly anticooperative manner, the DNA complexation scheme (3g–j) does not include contributions from 2:1 and 2:2 complexes.

Each reaction in scheme (3) contributes its own term to the mass conservation law (4) and to the observed NMR proton chemical shift (5) acquired in the fast exchange regimen for all components in the mixed solution:

$$\begin{cases} \frac{D}{(1-K_DD)^2}\left(1+\frac{K_hF}{1-K_FF}+\frac{K_h^2F^2}{2(1-K_FF)^2}+\frac{K_h^2FD}{(1-K_FF)(1-K_DD)}\right)+DN(K_{1D}+K_{2D}K_NN)=D_0\\ \frac{F}{(1-K_FF)^2}\left(1+\frac{K_hD}{1-K_DD}+\frac{K_h^2D^2}{2(1-K_DD)^2}+\frac{K_h^2FD}{(1-K_FF)(1-K_DD)}\right)+FN(K_{1F}+K_{2F}K_NN)=F_0\\ 2K_NN^2(1+K_{2D}D+K_{2F}F)+N(1+K_{1D}D+K_{1F}F)=N_0 \end{cases} \tag{4}$$

$$\begin{cases} \delta_D=\frac{D}{D_0}\left[\delta_{mD}\left(2(1+K_DD)-\frac{1}{(1-K_DD)^2}\right)+2\delta_{dD}\left(\frac{1}{(1-K_DD)^2}-1-K_DD\right)+\delta_{hD}\frac{K_hF}{(1-K_DD)^2(1-K_FF)}\times\left(1+\frac{K_hF}{2(1-K_FF)}+\frac{K_hD}{1-K_DD}\right)+N(\delta_{1D}K_{1D}+\delta_{2D}K_{2D}K_NN)\right]\\ \delta_F=\frac{F}{F_0}\left[\delta_{mF}\left(2(1+K_FF)-\frac{1}{(1-K_FF)^2}\right)+2\delta_{dF}\left(\frac{1}{(1-K_FF)^2}-1-K_FF\right)+\delta_{hF}\frac{K_hD}{(1-K_FF)^2(1-K_DD)}\times\left(1+\frac{K_hD}{2(1-K_DD)}+\frac{K_hF}{1-K_FF}\right)+N(\delta_{1F}K_{1F}+\delta_{2F}K_{2F}K_NN)\right] \end{cases} \tag{5}$$

where D_0, F_0, N_0 and D, F, N are the total and monomer concentrations of DAU, FMN and d(TGCA), respectively; δ_m, δ_d, δ_h, δ_1, δ_2 are proton chemical shifts of DAU (D) and FMN (F) in the monomer, dimer form, 1:1 hetero-complex DAU-FMN and in a complex with single- and double-stranded tetramer, respectively. Parameters K_N, K_D, K_{1D}, K_{2D}, δ_{1D}, δ_{2D}, δ_{mD}, δ_{dD} have been determined previously [22] and so have parameters K_F, δ_{mF}, δ_{dF} [25]; other parameters result from the hetero-association of DAU-FMN and complexation of FMN-d(TGCA) determined above.

It is seen that the equations for DAU and FMN in Eqs. (4) and (5) can be simply derived for each other by substituting indexes D for F and vice versa, which follows from the symmetry of reaction scheme (3) with respect to D and F. The self-association terms in (4) and (5) correspond to the indefinite non-cooperative self-association model for both DAU and FMN and to dimeric self-association for the oligonucleotide [26], the hetero-association term is taken from the general model for molecular hetero-association [19–21] and the DNA complexation term is taken from Refs. [13,22,23].

3.4. Verification of the general scheme of simultaneous binding of DAU/FMN with the tetramer

The appropriateness of reaction scheme (3) for analysis of the three-component mixture has been investigated by using Eqs. (4) and (5) to recalculate the magnitudes of proton chemical shifts for DAU and FMN in the concentration range of FMN and total concentrations of DAU and d(TGCA) adopted in DAU-FMN-d(TGCA) experiment and comparing the results with the experimental concentration curves (Fig. 2d). As an accumulation of discrepancies takes place while going towards the three-component mixture through the self-association, hetero-association and complexation stages, a small variation of the induced proton chemical shifts to within 0.1 ppm and a variation of equilibrium constants to within 20% error in all types of complexes has been adopted. The discrepancy between the experimental and calculated data set was better than 0.02 ppm, which indicates the adequacy of reaction scheme (3) and hence the possibility of using equilibrium parameters derived from one- (self-association) and two-component studies (hetero-association and complexation with DNA), in order to analyse the complexation of the antibiotic DAU with the deoxytetranucleotide d(TGCA) in solution in the presence of the Vitamin B_2 derivative, Flavin-mononucleotide.

Using the competitive binding model (3)–(5) the relative contribution of different types of complexes in solution has been calculated as a function of FMN concentration in the millimolar range of experimental concentrations of interacting components in solution (Fig. 4)). It is easily seen that both the hetero-association (f_h) and the DNA complexation (f_{C2}) give a comparable contribution to the dynamic equilibrium and so all types of interactions in solution are important and must be taken into consideration in the model and analysis.

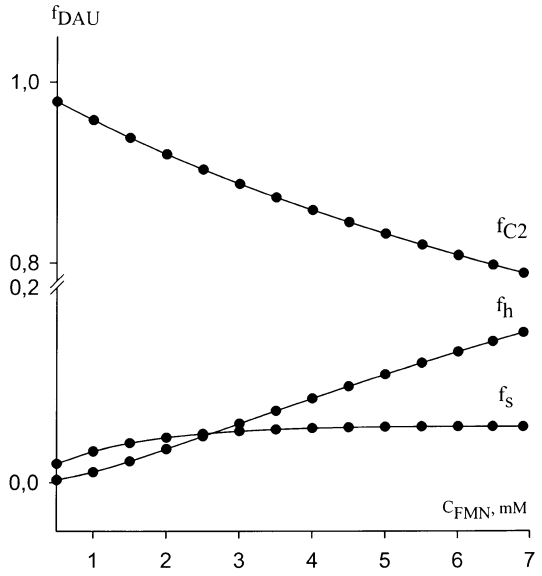

Fig. 4. Relative contents of different types of molecular complexes of DAU in solution as a function of FMN concentration: self-association (f_s), hetero-association (f_h) and DNA complexation (f_{C2}).

3.5. Formulation of the criterion for the discrimination between the hetero-association and complexation with DNA

There are two primary molecular processes which may influence the lowering of binding of a Drug1 with DNA in the presence of another aromatic Drug2: hetero-association of Drug1–Drug2 and the complexation of Drug2 with DNA. Hetero-association lowers the number of monomer species of Drug1 available for binding with DNA in solution and so the process may be called the 'interceptor' action of Drug2 on Drug1 [12–14]; alternatively, the complexation of Drug2 with DNA partially displaces the fraction of Drug1 bound to DNA thus lowering the fraction of Drug1–DNA complexes and so has been called the 'protector' action of Drug2 on DNA [11,13]. In either case, the fraction of the Drug1–DNA complex decreases in the presence of Drug2 and hence the biological activity of Drug1 changes.

The simplest way to distinguish between the two processes (interceptor and protector) would be to calculate the fraction of DAU (Drug1) in the hetero-complexes (f_h) and the complexes of DAU with DNA (f_{C2}), and then to compare them as in previous work [13]. Both f_h and f_{C2} come from the solution of the system of Eq. (4) and therefore they are both dependent on the effectiveness of the complexation, K_{2F}, and the hetero-association, K_h, respectively. It means that the quantities f_h and f_{C2} are interdependent, so a real discrimination between the two processes cannot be achieved and such an approach may lead to erroneous results. The correct criterion should therefore compare f_h and f_{C2} in their 'pure' forms, which contain no contribution from the competition process, or just 'switch off' the FMN-DNA complexation or DAU-FMN hetero-association, respectively.

An important factor should be taken into consideration prior to quantitative analysis. The modeling of the competitive binding of two drugs with the tetramer in the present work is targeted on DNA. Both DAU and FMN exert their predominant affinity to DNA in the duplex form and so the analysis would ideally be performed under the conditions of pure double-stranded form of d(TGCA) in the mixed solution, i.e. when reactions (3g and i) are excluded from the scheme (3) and no interference of complexation with a single-stranded form distorts the results.

In order to estimate the influence of hetero-association (interceptor action of FMN) and DNA complexation (protector action of FMN) on the complexation affinity of DAU with DNA in the presence of the vitamin, it is suggested that the relative decrease of DAU-d(TGCA)$_2$ complexes (R_D) is calculated for two circumstances: (i) under the 'switched off' hetero-association DAU-FMN and 'switched on' complexation of FMN with d(TGCA)$_2$ ($K_h=0$, $K_{2F}\neq 0$), $f^D_{C2(C)}$, and (ii) under the 'switched on' hetero-association DAU-FMN and 'switched off' complexation of FMN with d(TGCA)$_2$ ($K_h\neq 0$, $K_{2F}=0$), $f^D_{C2(H)}$:

$$R_D = \frac{f^D_{C2(0)} - f^D_{C2(C)}}{f^D_{C2(0)} - f^D_{C2(H)}}, \tag{6}$$

where $f^D_{C2(0)}$ is the mole fraction of DAU-d(TGCA)$_2$ complexes with a 'switched off' hetero-association and FMN-DNA complexation. The range of $R_D>1$ corresponds to the predominance of the FMN-DNA complexation over the DAU-FMN hetero-association (protector action of FMN) and $R_D<1$ means a major contribution of the hetero-association to the displacement of DAU from DNA (interceptor action of FMN).

3.6. Analysis of the competitive binding

The relative decrease of binding of DAU with $d(TGCA)_2$ calculated over a large range of FMN concentrations (F_0), whilst keeping the concentrations of DAU ($D_0 = 0.01$ mM) and tetramer ($N_0 = 1$ mM) constant, is presented in Fig. 5. In the 1–50 mM concentration range of F_0, it is seen that complexation of FMN with the oligomer dominates over hetero-association with DAU, whereas at very small (<1 mM) and very high (>50 mM) concentrations of FMN there are approximately equal contributions of complexation and hetero-association to the decrease of DAU binding with the tetramer. These conclusions should be noted because the affinity of FMN to $d(TGCA)_2$ (Table 2) is much higher than that to DAU (Table 1), thus implying a predominance of DNA complexation over hetero-association. If the concentration of vitamin is small compared to the oligonucleotide, any addition of FMN will not be able to displace a DAU bound to DNA due to the predominant complexation of FMN with free oligomer, which is in excess in solution. In any case, hetero-association apparently takes place in solution, lowering the fraction of monomeric DAU available for binding with the tetramer, which explains the comparable roles of hetero-association and complexation in the range of $F_0 < 1$ mM. On increasing F_0, the amount of the oligomer complexed with FMN gradually increases, shifting the R_D value towards complexation. Saturation of the tetramer by FMN occurs when the condition $N_0 \approx F_0$ occurs (see C_{2F} curve in Fig. 5). Further addition of the vitamin just increases the concentration of the hetero-complexes, whilst the concentration of FMN-TGCA complexes remains constant. Hence R_D is shifted towards hetero-association resulting in a maximum, which can be clearly seen and corresponds to the condition $N_0 \approx F_0$ (Fig. 5).

Interestingly, R_D value remains practically unchanged with respect to any variation of DAU over a large range of concentrations, because addition of DAU gives comparable contributions to the numerator and denominator of (6) thus hiding the effect of DAU in the competitive binding system expressed in terms of R_D. In contrast the variation of the tetramer concentration results in quite remarkable perturbation of the R_D profile (Fig. 6). The decrease of N_0 shifts the maximum towards the lower concentrations of FMN due to earlier saturation of tetramer by both FMN and DAU leading to a predominance of the complexation process in micromolar range of FMN concentrations.

The equilibrium complexation constants determined in the current study have been obtained under standard physiological conditions (pH 7.1, 0.1 M ionic strength, $T = 298$ K) and whether the results are physiologically relevant will depend on the concentrations of each species in solution. Normally the concentrations of DAU and FMN used in in vitro studies fall in micromolar range [6,11], which may be varied over an order of magnitude depending of the type of experiment. Hence the concentrations of DAU and FMN considered in the current study (0...100 mM) cover both the physiological and experimental range. The concentration of oligonucleotide should correspond to the total concentration of a tetramer sequence in oligomeric regions of nuclear DNA that are free of proteins in the cell. Although there is no estimate of the latter, it is assumed that the local concentration of the nuclear DNA region when drugs compete for binding sites is likely to be greater than the concentrations of DAU and FMN. Assuming millimolar concentrations of N_0, it is expected that the region of $F_0 < 0.1$ mM in Fig. 5 is the most physiologically relevant situation. Under these conditions, both hetero-association of FMN with DAU and complexation of FMN with DNA contribute approximately equally to the decrease of DAU binding with DNA, and presumably both would contribute to any observed alteration of biological activity of the antibiotic.

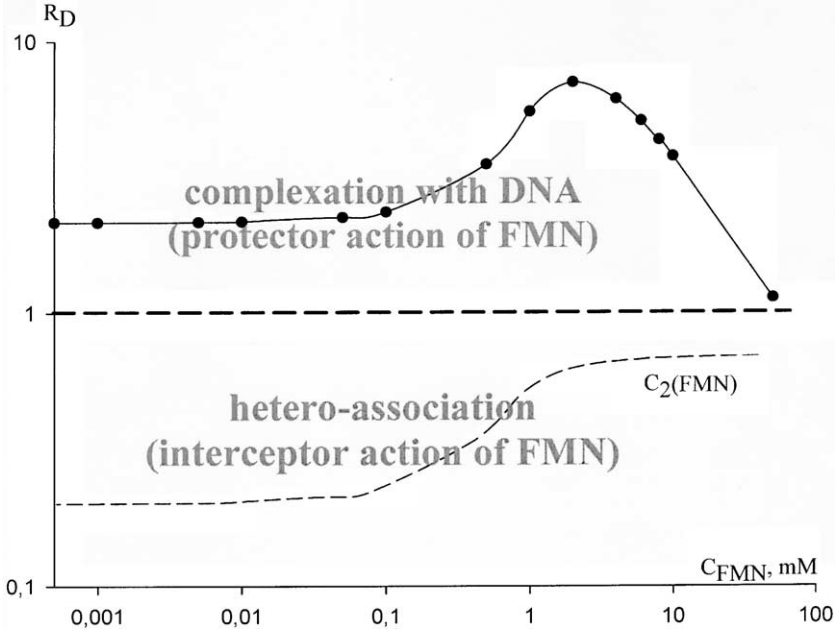

Fig. 5. Relative decrease of DAU binding with $d(TGCA)_2$ (R_D) as a function of the concentration of FMN ($D_0 = 0.01$ mM, $N_0 = 1$ mM); the absolute content of the FMN-$d(TGCA)_2$ complex (C_{2F}) as a function of FMN concentration is depicted schematically.

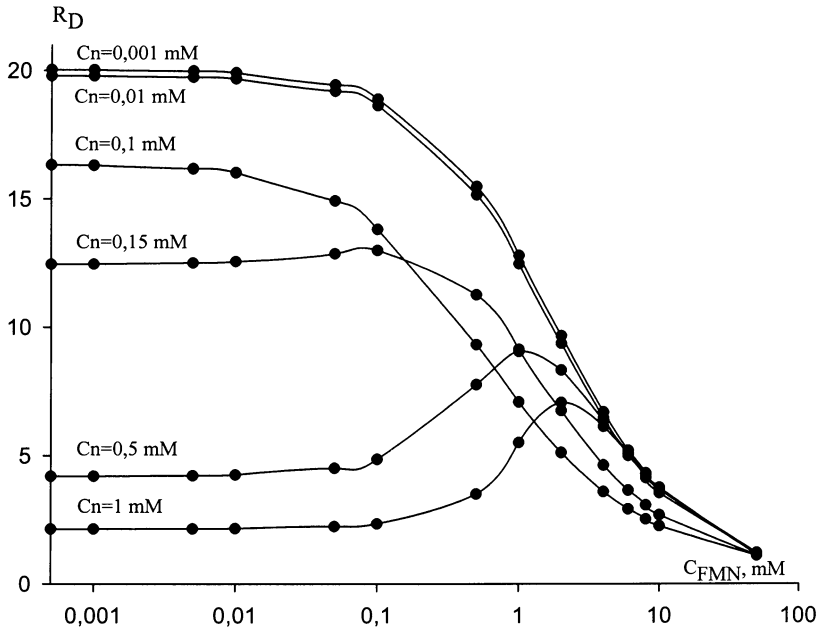

Fig. 6. Relative decrease of DAU binding with d(TGCA)$_2$ (R_D) as a function of FMN concentration (D_0=0.01 mM) at different concentrations of tetramer.

3.7. Conclusions

In the present work NMR spectroscopy has been used to investigate the complexation of the anthracycline antibiotic daunomycin in the presence of a Vitamin B_2 derivative, flavine-mononucleotide, with a DNA oligonucleotide as a model for the competitive binding of the drug and vitamin with nuclear DNA in the cell. It has been shown that a model and results for a complicated dynamic equilibrium of three species in solution are consistent with results from separate measurements on self-association, hetero-association and complexation of the drugs with DNA under the same solution conditions. Hence the quantitation of the competitive binding of two drugs with DNA is now possible. For example, a quantitative criterion has been formulated to discriminate between the interception of DAU by FMN via hetero-association and the displacement of the bound DAU by FMN via the complexation of FMN with DNA. It is found that both molecular process (hetero-association or complexation) contribute to the decrease in binding of DAU with DNA and, as expected, their relative importance depends on the concentration of DNA and FMN. Assuming a predominance of the concentration of vacant sites for DNA binding over the local concentration of DAU and FMN in the cell, it is found that both interceptor and protector mechanisms are of comparable importance, which should be taken into consideration when quantifying or interpreting the results of synergistic biological action of an antibiotic and a vitamin in cell systems.

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