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ARTICLE

Complexation of anthracycline drugs with DNA in the presence of caffeine

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Abstract The competitive binding of anthracycline antitumour drugs, [daunomycin (DAU), doxorubicin (DOX) or nogalamycin (NOG)], with caffeine (CAF) to a model DNA oligomer has been investigated by 500 MHz ^{1}H NMR spectroscopy under physiological solution conditions. The method depends on the stepwise analysis of one-component (self-association), two-component (hetero-association and DNA complexation) and three-component interactions, in order to de-convolute the overall binding of the anthracycline antibiotic and CAF to DNA into two competing processes, viz. hetero-association of the antibiotic-CAF ('interceptor' action of CAF) and CAF–DNA complexation ('protector' action of CAF). It is found that the complexation of DAU with DNA in the presence of CAF is mainly affected by the CAF–DNA complexation, whereas the binding of either DOX or NOG to DNA is affected approximately equally by both the CAF–DNA complexation and CAF-antibiotic hetero-association. Quantitative evaluation of the three-component mixture of drug–CAF–DNA has enabled the proportion of the antibiotic displaced from DNA on addition of CAF to be calculated over a large range of CAF concentration, which may provide a quantitative basis for the change in anthracycline-related toxicity on addition of CAF.

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Introduction

Anthracycline antibiotics are an important class of pharmaceutical drugs used to treat various human cancers in the clinic (Chu and DeVita 2003). The anthracyclines, such as daunomycin (DAU) and doxorubicin (DOX), act as topoisomerase II inhibitors, thereby affecting DNA replication and cell progression (Neidle and Waring 1983). It is now generally believed that the specific molecular mechanism of action involves intercalation of the planar aromatic chromophore of the anthracycline drug into the DNA duplex (Neidle and Waring 1983). It has also been shown that typical anthracycline antibiotics, such as DAU and DOX, associate in solution with caffeine (CAF) forming hetero-complexes (Piosik et al. 2002; Davies et al. 2001), which is thought to be the major mechanism responsible for the marked reduction in the toxicity of DOX in cultured cell lines treated with CAF (Piosik et al. 2002; Traganos et al. 1991a). The effect, which was termed the 'interceptor action' of CAF, has also been reported for other aromatic drugs (Traganos et al. 1991a; Piosik et al. 2003; Ulanowska et al. 2005; Larsen et al. 1996).

Recent NMR studies have shown that CAF complexes with a DNA oligomer in aqueous solution with an association constant of the same order of magnitude as the hetero-association constant between CAF and DAU (ca. 10^{2}–10^{3} M^{-1}) and much lower than the association constant of the antibiotic with the oligomer (ca. 10^{5}–10^{6} M^{-1}) (Davies et al. 2001). These results

suggest that there may be no competition between antibiotic-DNA and CAF–DNA complexation as assumed in a previous work with DOX and other anticancer drugs (Traganos et al. 1991a). On the other hand, the concentrations of CAF commonly required to observe of the effect of cell detoxification are in the millimolar range, which is at least two or three orders of magnitude higher than the concentrations of the anthracycline antibiotic normally used. Qualitatively, it means that the much larger concentration of CAF may compensate for its lower DNA affinity and lead to a commensurable amount of binding of CAF and the anthracycline antibiotics to DNA. Under these circumstances, CAF is able to block the potential DNA binding sites or remove the antibiotic already complexed with DNA; this mechanism has been described as the 'protector action' of CAF (Davies et al. 2001; Traganos et al. 1991b). Both interceptor and protector mechanism acting together are thought to be responsible for the observed scavenging effect of CAF on aromatic drug molecules.

In order to discriminate between the 'protector' and 'interceptor' mechanisms, it is necessary to quantify the three-component equilibrium of aromatic drug, CAF and DNA and develop a criterion for the discrimination between the two mechanisms. Quantititation of a three-component equilibrium of an aromatic drug, CAF and a DNA oligomer has been made (Davies et al. 2001), though no method for discrimination between the 'protector' and 'interceptor' mechanisms has been developed. In the present work, NMR measurements have been made on the complex multi-component equilibrium in solution of a typical anthracycline antibiotic (DAU, DOX or NOG, Fig. 1) and a model DNA oligomer d(TGCA), the latter being used to represent binding sites of nuclear DNA (Evstigneev et al. 2005). In order to complete the analysis of a three-component mixture of the anthracycline drug, CAF and DNA oligomer, d(TGCA), it is necessary to know the self-association constant of each component (DAU, DOX, NOG, CAF, DNA oligomer) and the complexation constant for each pair of molecules in two-component mixtures (DAU/DOX/NOG–CAF and DAU/DOX/NOG/CAF–DNA oligomer) measured under similar solution conditions and derived using a similar model and computational method. The self-association constants of DAU/NOG/CAF/TGCA and the two-component complexation constants between DAU/CAF/TGCA have previously been determined in aqueous solution (0.1 M Na-phosphate buffer, pH 7.1, T = 298 or 303 K) (Davies et al. 2001; Eaton et al. 2000). In order to provide all necessary data for complete analysis of the three-component

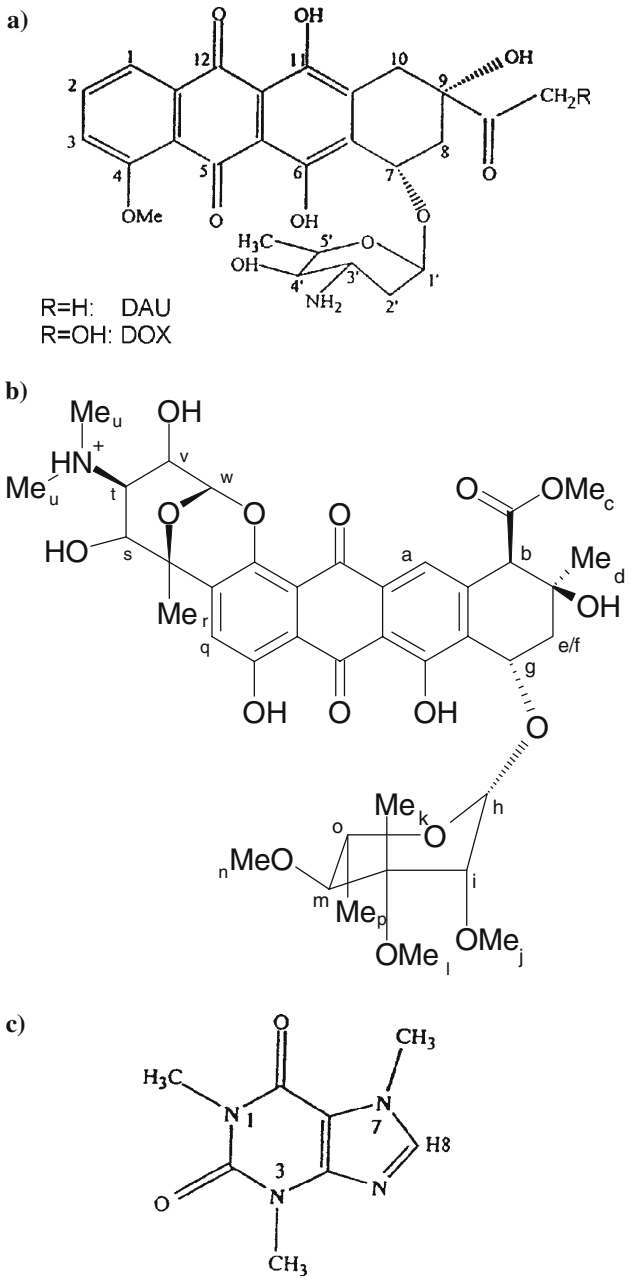

Fig. 1 Structures of **a** daunomycin and doxorubicin, **b** nogalamycin, and **c** caffeine

mixture, the self-association of DOX and the hetero-association DOX–CAF and NOG–CAF has been measured in this work as well as the three component system of DAU–CAF–DNA oligomer. On the basis of the NMR measurements, the analytical methodology has been validated, a criterion developed for discrimination between the 'protector' and 'interceptor' mechanisms and an estimate made of the average reduction in complexation of the anthracycline drug with DNA in the presence of CAF at different concentrations.

Experimental

Daunomycin from 'Fluka', DOX, NOG, CAF from 'Sigma' (Fig. 1) and deoxytetranucleotide 5′-d(TpGpCpA) from 'Metabion' were used without further purification. The samples were lyophilised from D_2O solutions and re-dissolved in 0.1 M phosphate buffer (NOG–CAF) or Tris (DOX, DOX–CAF) in 99.95% D_2O at pD 7.1 and 7.3, respectively, 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 TOCSY and ROESY experiments. Chemical shift measurements of the non-exchangeable protons of the aromatic molecules were made as a function of concentration of the antibiotic (CAF–DOX/NOG experiment at T = 303 K) or CAF [DAU–CAF–d(TGCA) experiment at T = 298 K] maintaining the concentration of CAF and DAU/dTGCA constant, respectively. In the hetero-association experiments, the variation of the concentration of the antibiotic was used because the self-association of anthracyclines is much greater than that for CAF, hence the chemical shift changes due to redistribution of different associated species in solution upon dilution are more pronounced. The variation of CAF in the DAU–CAF–d(TGCA) experiment was used in order to track the effect of removal of the drug from DNA.

The temperature dependence of the proton chemical shifts for all the mixtures was measured at constant concentration of drug molecules in the temperature range 278–353 K. 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.

Results and discussion

Analysis of one- (self-association) and two-component (hetero-association) systems

General method for analysis of experimental NMR data

The concentration dependences of proton chemical shifts of interacting molecules were used to provide information about the equilibrium self- or hetero-association constants and the induced proton chemical shifts within the aggregates and complexes in solution. Analysis of the concentration dataset was made as explained previously (Davies et al. 2001; Veselkov et al. 2001) using a model approach based on the law of mass action and mass conservation law, which enables an analytical expression for the observed chemical shift, $\delta(C, K, \delta_C)$, to be evaluated as a function of concentration C of the dissolved components, the equilibrium constants K for association reactions and the intrinsic chemical shift δ_C for each type of complex being formed in solution.

The temperature dependences of proton chemical shifts are used to provide thermodynamical information (enthalpy, ΔH, and entropy, ΔS) by incorporation of the van't Hoff's relation between the association constant and the thermodynamic quantities

$$K(T) = \exp(\Delta S/R - \Delta H/RT) \quad (1)$$

into the expression for $\delta(C, K, \delta_C)$.

Both the concentration and temperature datasets were fitted by computational adjustment of the K/δ_C or $\Delta H/\Delta S$ parameters to reach the minimum of the discrepancy function

$$\Delta = \sum_i (\delta_i - \delta_{ei})^2,$$

where δ_e is the experimentally observed proton chemical shift measured in the given concentration or temperature point i.

Self-association of doxorubicin

The experimental NMR data on the self-association of DOX (Fig. 2) have been analysed in terms of the indefinite non-cooperative model of association, which assumes that the aromatic molecules aggregate in solution with a self-association constant K_X. The dependence of the experimentally observed chemical shift, δ, on the concentration of antibiotic, x_0, is given by the following equation: (2)

$$\delta = \delta_m + (\delta_d - \delta_m)\frac{2K_X x_0 + 1 - \sqrt{4K_X x_0 + 1}}{K_X x_0} \quad (2)$$

where δ_m, δ_d are the chemical shifts in the monomer and dimer forms (or at the ends of an aggregate) of DOX in solution, respectively, and x_1 is the concentration of monomer (Davies et al. 1996). The equilibrium self-association parameters for DOX were determined from the experimental concentration

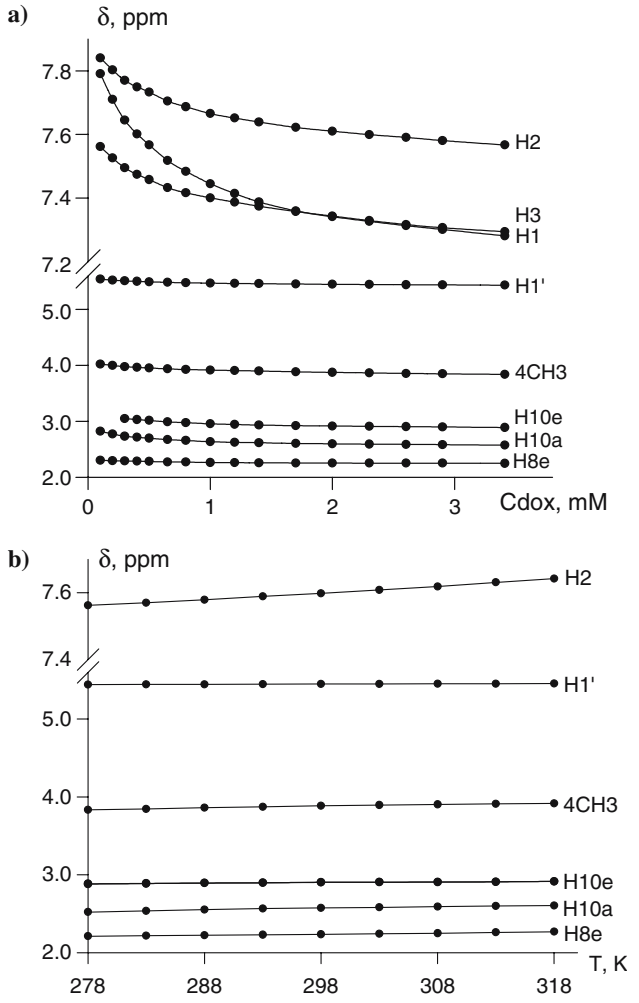

Fig. 2 Experimental dependence of ^{1}H NMR chemical shifts of doxorubicin in 0.1 M Tris buffer, p*D* 7.3. **a** On concentration, T = 303 K and **b** on temperature, C_{DOX} = 1.7 mM

(Fig. 2a) and temperature (Fig. 2b) dependences of the proton chemical shifts using Eqs. 1 and 2, and the results are summarised in Table 1.

The results on the self-association of DOX may be compared with those reported previously (Evstigneev et al. 2006a) for the self-association of DAU investigated under the same solution conditions (Table 1). Firstly, it is found that the intrinsic chemical shifts in the monomer (δ_m) and dimer (δ_d) are very similar for both DAU and DOX indicating that there is a negligible influence on the δ (within 0.06 ppm) of ring protons due to replacing the side chain CH_3 group in DAU by the CH_2OH group in DOX (Fig. 1a). It is also found that the magnitudes of the self-association constants of DOX and DAU are the same within experimental error. Both these observation suggest that there is a similar orientation of the aromatic

Table 1 Equilibrium parameters for self-association of doxorubicin (DOX) and daunomycin (DAU)

Proton		DAU[a]	DOX
H1	δ_m	7.91	7.95
	δ_d	7.47	7.47
H2	δ_m	7.89	7.90
	δ_d	7.62	7.66
H3	δ_m	7.61	7.61
	δ_d	7.37	7.35
H1′	δ_m	5.55	5.57
	δ_d	5.47	5.47
4Me	δ_m	4.06	4.05
	δ_d	3.89	3.87
H10e	δ_m	3.10	3.16
	δ_d	2.89	2.95
H10a	δ_m	2.88	2.86
	δ_d	2.66	2.64
H8e	δ_m	2.28	2.32
	δ_d	2.23	2.27
K (l/mol)		1,130 ± 170	1,500 ± 400
$-\Delta H$ (kJ/mol)		34.5 ± 2.7	26.0 ± 3.0
$-\Delta S$ (J/mol K)		55 ± 10	25.0 ± 8.0

[a] In 0.1 M Tris buffer, p*D* 7.3, T=303 K and taken from Evstigneev et al. (2006a)

moieties of these molecules in the aggregates, which results in their dispersive attraction being very similar. On the other hand, it is found that the thermodynamic parameters of the self-association of DOX and DAU are quite different, which is a consequence of the different properties of the CH_3 group in DAU compared to the CH_2OH group in DOX (Fig. 1a). The aggregation of aromatic molecules is known to be driven by stacking interactions, in which the main components are dispersive and hydrophobic attractions (Davies et al. 1996). The CH_2OH group results in an increase in the H-bonding ability of DOX with the aqueous environment and therefore greater ordering of water molecules around DOX as compared to DAU, which explains the observed difference in thermodynamical quantities (Table 1). These results are consistent with the differences in hydrophobicity of these molecules as judged from their different lipophilicity and membrane-binding properties (Gallois et al. 1998).

Hetero-association between caffeine and doxorubicin/nogalamycin

The dynamic equilibrium in solution containing two types of interacting aromatic molecules X (antibiotic) and Y (CAF) may be described using the following general scheme of reactions (Davies et al. 2001; Davies et al. 2002):

$$X_1 + X_i \overset{K_X}{\longleftrightarrow} X_{i+1} \quad \text{(a)}, Y_1 + Y_j \overset{K_Y}{\longleftrightarrow} Y_{j+1} \quad \text{(b)},$$
$$X_i + Y_j \overset{K_h}{\longleftrightarrow} X_i Y_j \quad \text{(c)}, Y_j X_i + Y_l \overset{K_h}{\longleftrightarrow} Y_j X_i Y_l \quad \text{(d)},$$
$$X_k + Y_j X_i \overset{K_h}{\longleftrightarrow} X_i Y_j X_k \quad \text{(e)}, \qquad (3)$$

where X_1 and Y_1 correspond to the monomers of the antibiotic and CAF, and X_i, X_k, Y_j, Y_l are the aggregates containing i, k monomers of antibiotic and j, l monomers of CAF, respectively; K_X, K_Y are the equilibrium self-association constants for X and Y, and K_h is the hetero-association constant. As shown in the previous work (Davies et al. 2001), reaction (3e) may be safely neglected for CAF–drug systems, which simplifies the analytical expressions for the observed dependence of proton chemical shifts of both X and Y components to the form (Davies et al. 2001) in Eq. 4

$$\begin{cases} \delta_X = \frac{x_1}{x_0}\left[\delta_{mX}\left(2(1+K_X x_1) - \frac{1}{(1-K_X x_1)^2}\right) + 2\delta_{dX}\left(\frac{1}{(1-K_X x_1)^2} - 1 - K_X x_1\right)\right. \\ \qquad \left. + \delta_{hX}\frac{K_h y_1}{(1-K_X x_1)^2(1-K_Y y_1)}\left(1 + \frac{K_h y_1}{2(1-K_Y y_1)}\right)\right] \\ \delta_Y = \frac{y_1}{y_0}\left[\delta_{mY}\left(2(1+K_Y y_1) - \frac{1}{(1-K_Y y_1)^2}\right) + 2\delta_{dY}\left(\frac{1}{(1-K_Y y_1)^2} - 1 - K_Y y_1\right)\right. \\ \qquad \left. + \delta_{hY}\frac{K_h x_1}{(1-K_Y y_1)^2(1-K_X x_1)}\left(1 + \frac{K_h y_1}{1-K_Y y_1}\right)\right] \end{cases}, \qquad (4)$$

where δ_h are the chemical shifts of X or Y protons in the hetero-complex. The values of δ_m, δ_d and the equilibrium self-association constants are known from self-association studies of DOX (this work), NOG (Eaton et al. 2000) and CAF (Davies et al. 2001). The monomer concentrations x_1 and y_1 may be derived from the solution of the mass conservation law for scheme (3). It follows that Eq. 4 is a function of two unknown quantities, δ_h and K_h (or ΔH, ΔS in the thermodynamical analysis), which may be determined from the concentration and temperature dependences of δ using the numerical procedure described above.

^{1}H NMR chemical shift measurements of the non-exchangeable protons of the aromatic molecules for hetero-association of CAF with either DOX or NOG were made as a function of concentration of the antibiotic and the experimental results for the CAF–DOX system are shown in Fig. 3a as an example. In the mixed solution of CAF and the anthracyclines, the experimental ^{1}H NMR curves show an increase in average shielding of CAF protons on successive additions of the antibiotic in solution measured at constant concentration of CAF (Fig. 3a). Similar observations were made previously for hetero-association mixtures of CAF with a number of molecules having three fused aromatic rings and were interpreted in terms of stacking of their aromatic chromophores (Davies et al. 2001). It is likely that the shielding of CAF protons results from complexation between CAF and DOX/NOG, though the small deshielding (δ_m–δ_h<0, Tables 1, 2) of some protons of the antibiotics in the hetero-complex with CAF probably results from the significant difference in the size of the chromophores of anthracycline and CAF (Fig. 1). In such case, these protons can be outside the shielding cone of the CAF molecule and therefore experience deshielding from stacking of their aromatic chromophores.

The calculated magnitudes of the hetero-association constant for CAF–DOX/NOG complexation are equal to each other, which is just a coincidence given the error limits, and have values in between those for the self-association constants of CAF and DOX/NOG (Tables 2, 3). The thermodynamical parameters are also intermediate in value between those for self-association of CAF and DOX/NOG (Tables 1, 2). The results are in accord with previous studies of CAF–drug systems (Piosik et al. 2002; Davies et al. 2001; Larsen et al. 1996) and indicate that stacking interactions are a major driving force in the formation of CAF–DOX/NOG hetero-complexes. It is also worth noting that the equilibrium constant for CAF–DOX hetero-association obtained in this work by NMR spectroscopy (Table 2) correlates well with that calculated from spectrophotometric titration data obtained for similar solution conditions (K_h = 128±10 l/mol, T=298 K (Piosik et al. 2002)).

The hetero-association of CAF with two of the anthracycline drugs, DOX and NOG, appears to be more pronounced than with DAU (Table 3). It is known that the self-association constant of NOG (Eaton et al. 2000) is much greater than that of DAU (Evstigneev et al. 2006a), which reflects a greater hydrophobic contribution for self-association of NOG compared to DAU. It is likely that this effect is also responsible for the greater magnitude of the hetero-association constant of CAF–NOG compared to the CAF–DAU complexation.

Analysis of three-component systems

Model for the analysis of the three-component mixture of drug–CAF–DNA

Following the previous work on the competitive binding of a drug and vitamin with DNA (Evstigneev et al. 2005), the tetrameric oligonucleotide, d(TpGpCpA), is used as a model DNA receptor in the analysis of the competitive binding of drug and CAF in this work. It needs to be taken into consideration that the three-component mixture of Anthracycline drug, CAF and the tetramer, might comprise all possible interactions between the dissolved molecules, i.e. self-association of the drug (X), CAF (Y) and d(TGCA), hetero-association of drug–CAF and complexation of both the drug and CAF with d(TGCA). The scheme of drug–DNA interactions should include complexation of the drug with both single-stranded (N) and double-stranded (N_2) forms of the tetramer in solution (Davies et al. 2000). Hence, the basic scheme of reactions describing the complex equilibrium of drug, CAF and the DNA tetramer in solution is a summation of self-association, hetero-association and complexation reactions in Eq. (5):

Self-association

$$X_1 + X_i \overset{K_X}{\longleftrightarrow} X_{i+1} \quad \text{(a)}, Y_1 + Y_j \overset{K_Y}{\longleftrightarrow} Y_{j+1} \quad \text{(b)},$$

$$N + N \overset{K_N}{\longleftrightarrow} N_2 \quad \text{(c)}.$$

Hetero-association

$$X_i + Y_j \overset{K_h}{\longleftrightarrow} X_iY_j \quad \text{(d)}, X_iY_j + X_k \overset{K_h}{\longleftrightarrow} X_iY_jX_k \quad \text{(e)},$$

$$Y_l + X_iY_j \overset{K_h}{\longleftrightarrow} Y_lX_iY_j \quad \text{(f)}.$$

DNA complexation

$$X + N \overset{K_{1X}}{\longleftrightarrow} XN \quad \text{(g)}, X + N_2 \overset{K_{2X}}{\longleftrightarrow} XN_2 \quad \text{(h)},$$

$$Y + N \overset{K_{1Y}}{\longleftrightarrow} YN \quad \text{(i)}, Y + N_2 \overset{K_{2Y}}{\longleftrightarrow} YN_2 \quad \text{(j)}, \qquad (5)$$

where K_{1X}, K_{1Y} and K_{2X}, K_{2Y} are complexation constants of Antibiotic and CAF with single- and double-stranded form of d(TGCA), respectively.

Each reaction in scheme (5) contributes its own term to the mass conservation law (6) and to the observed NMR proton chemical shift (7) acquired in the fast exchange regimen for all components in the mixed solution:

$$\begin{cases} \frac{x_1}{(1-K_Xx_1)^2}\left(1+\frac{K_hy_1}{1-K_Yy_1}+\frac{K_h^2y_1^2}{2(1-K_Yy_1)^2}\right)+x_1N(K_{1X}+K_{2X}K_NN)=x_0 \\ \frac{y_1}{(1-K_Yy_1)^2}\left(1+\frac{K_hx_1}{1-K_Xx_1}+\frac{K_h^2x_1y_1}{(1-K_Yy_1)(1-K_Xx_1)}\right)+y_1N(K_{1Y}+K_{2Y}K_NN)=y_0 \\ 2K_NN^2(1+K_{2X}x_1+K_{2Y}y_1)+N(1+K_{1X}x_1+K_{1Y}y_1)=N_0 \end{cases}, \qquad (6)$$

$$\begin{cases} \delta_X=\frac{x_1}{x_0}\left[\delta_{mX}\left(2(1+K_Xx_1)-\frac{1}{(1-K_Xx_1)^2}\right)+2\delta_{dX}\left(\frac{1}{(1-K_Xx_1)^2}-1-K_Xx_1\right)\right. \\ \quad \left.+\delta_{hX}\frac{K_hy_1}{(1-K_Xx_1)^2(1-K_Yy_1)}\left(1+\frac{K_hy_1}{2(1-K_Yy_1)}\right)+N(\delta_{1X}K_{1X}+\delta_{2X}K_{2X}K_NN)\right] \\ \delta_Y=\frac{y_1}{y_0}\left[\delta_{mY}\left(2(1+K_Yy_1)-\frac{1}{(1-K_Yy_1)^2}\right)+2\delta_{dY}\left(\frac{1}{(1-K_Yy_1)^2}-1-K_Yy_1\right)\right. \\ \quad \left.+\delta_{hY}\frac{K_hx_1}{(1-K_Yy_1)^2(1-K_Xx_1)}\left(1+\frac{K_hy_1}{1-K_Yy_1}\right)+N(\delta_{1Y}K_{1Y}+\delta_{2Y}K_{2Y}K_NN)\right] \end{cases}, \qquad (7)$$

where δ_1, δ_2 are proton chemical shifts of the drug and CAF in a complex with single- and double-stranded tetramer, respectively.

Each type of complexation in the model (6, 7) (self-association, hetero-association, DNA binding) is investigated in separate NMR studies of systems measured under similar solution conditions and analysed by single methodological approach. It is expected that all types of complexations in the three-component mixture should remain unchanged from those observed in the separate one- or two-component studies, i.e. the association/complexation parameters derived from one/two-component studies can be utilised in evaluations of the three-component mixture. This hypothesis needs to be tested.

In order to verify the model developed in Eqs. 6 and 7, the dependence of proton chemical shifts of DAU and CAF on the concentration of CAF has been measured in the three-component mixture consisting of the antibiotic, CAF and d(TGCA) and the results are summarised in Fig. 4. The experimental data on the three component system can be completely described using Eqs. 6 and 7 and the equilibrium parameters (association/complexation constants and limiting/induced chemical shifts) of all the one- and two-component systems in the DAU–CAF–TGCA mixture as summarised in Table 3 and references therein. The calculated discrepancy function for all the aromatic protons of both DAU and CAF shows that the average

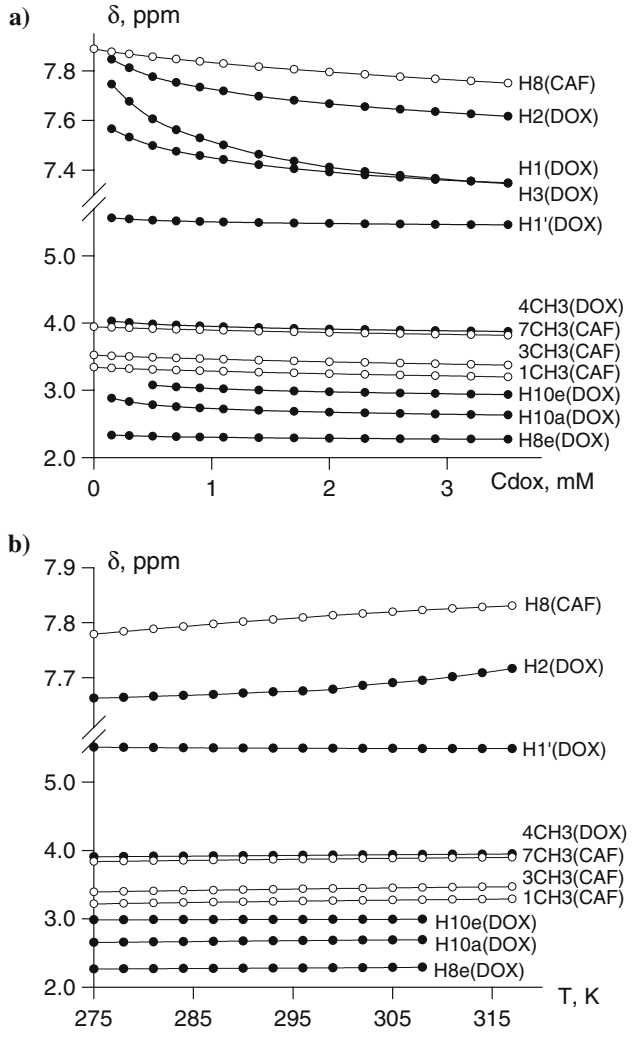

Fig. 3 Experimental dependence of ^{1}H NMR chemical shifts of doxorubicin and caffeine in 0.1 M Tris buffer, p*D* 7.3. **a** On DOX concentration, T = 303 K, C_{CAF} = 2.53 mM and **b** on temperature, C_{DOX} = 1.4 mM, C_{CAF} = 2.53 mM

discrepancy per experimental point is less than 0.01 ppm, which indicates a very good fit of the experimental data to the model. This means that utilisation of equilibrium parameters for one- and two-component systems is valid for quantitative descriptions of the dynamic equilibrium of three-component mixtures of aromatic drugs and DNA model based on Eqs. 6 and 7.

Method to analyse the simultaneous binding of CAF and an anthracycline drug with DNA

All the anthracycline drugs studied in this work are known to exert their major effects by binding to double stranded DNA (Neidle and Waring 1983), and so it is reasonable to exclude the single-stranded form of d(TGCA) from the analysis (Evstigneev et al. 2005; Evstigneev et al. 2006b). Hence reactions (5g) and (5i) can be excluded from reaction scheme (5), which reduces to the form of Eq. 8:

$$\begin{cases} \frac{x_1}{(1-K_X x_1)^2}\left(1+\frac{K_h y_1}{1-K_Y y_1}+\frac{K_h^2 y_1^2}{2(1-K_Y y_1)^2}\right)+K_{2X}x_1N_2=x_0 \\ \frac{y_1}{(1-K_Y y_1)^2}\left(1+\frac{K_h x_1}{1-K_X x_1}+\frac{K_h^2 x_1 y_1}{(1-K_Y y_1)(1-K_X x_1)}\right)+K_{2Y}y_1N_2=y_0, \\ N_2(1+K_{2X}x_1+K_{2Y}y_1)=N_0 \end{cases} \quad (8)$$

where the quantity N_2 now stands for the unbound double-stranded species of d(TGCA)$_2$ in solution.

In order to calculate the relative fraction of each type of complex in the three-component mixture, it is necessary to solve Eq. 8 with respect to x_1, y_1 and N_2, which can be achieved using the set of known equilibrium constants for contributing one and two-component systems summarised in Table 3. Unfortunately, the exact values of K_2 constant for complexation of either NOG or DOX with d(TGCA)$_2$ cannot be determined by NMR equilibrium studies because their complexes with the tetramer are in intermediate exchange on the NMR timescale. As in the previous work (Davies et al. 2002), the magnitudes of the complexation constants of NOG and DOX with d(TGCA) have been estimated by relating them to the known complexation constants of DAU/DOX/NOG with native DNA (Ibrahim 2001) and then scaling them with respect to the known constant for DAU–TGCA (Davies et al. 2000) observed under similar solution conditions (Table 3). Having values of all necessary contributing equilibrium constants enables the dynamic equilibrium in Eq. 8 to be quantified for the three component system of antibiotic, CAF and DNA.

In order to quantify the 'protector' and 'interceptor' mechanisms of CAF on simultaneous binding with DNA in the presence of the anthracyclines, one may use the criterion R_D, the relative decrease in complexes of antibiotic-d(TGCA)$_2$. A similar criterion was developed previously (Evstigneev et al. 2005) to analyse the competitive binding to DNA of DAU with vitamin B_2, if the vitamin is considered as an interceptor of DAU. R_D may be calculated for two circumstances: (1) under the 'switched off' hetero-association of drug–CAF and 'switched on' complexation of CAF with d(TGCA)$_2$ ($K_h = 0$, $K_{2Y} \neq 0$), i.e. $f^X{}_{C2(C)}$, and (2) under the 'switched on' hetero-association of drug–CAF and 'switched off' complexation of CAF with d(TGCA)$_2$ ($K_h \neq 0$, $K_{2Y} = 0$), i.e. $f^X{}_{C2(H)}$:

$$R_D = \frac{f^X_{C2(0)} - f^X_{C2(C)}}{f^X_{C2(0)} - f^X_{C2(H)}}, \quad (9)$$

Table 2 Equilibrium parameters for the hetero-association of caffeine (CAF) with DOX and nogalamycin (NOG)

DOX–CAF hetero-association[a]											
DOX protons								CAF protons			
H2	H1	H3	H1′	4Me	H10e	H10a	H8e	H8	7Me	3Me	1Me
7.86	7.68	7.57	5.59	4.05	3.17	2.93	2.37	7.23	3.33	2.80	2.65
K_h = (180 ± 30) l/mol, ΔH_h = –(22,000 ± 1,000) J/mol, ΔS_h = –(30 ± 5) J/(mol K)											
NOG–CAF hetero-association[b]											
H_a	H_q	H_w	H_h	H_g	H_b	H8	7Me	3Me	1Me		
7.59	7.35	5.92	5.52	5.19	4.20	7.40	3.66	3.02	3.03		
K_h = (180 ± 40) l/mol, ΔH_h = –(23,000 ± 3,000) J/mol, ΔS_h = –(33 ± 8) J/(mol K)											

Thermodynamical parameters of the hetero-association of CAF: ΔH_h = –(21,000 ± 400) J/mol, ΔS_h = –(50 ± 1) J/(mol K) (Davies et al. 2001); NOG: ΔH_h = –(30,100 ± 4,000) J/mol, ΔS_h = –(27 ± 4) J/(mol K) (Eaton et al. 2000)

[a] In 0.1 M Tris buffer, pD 7.3, at T = 303 K

[b] In 0.1 M Na-phosphate buffer, pD 7.1, at T = 303 K

where ${f^X}_{C2(0)}$ is the mole fraction of drug–d(TGCA)$_2$ complexes with 'switched off' hetero-association and CAF–DNA complexation. The range of $R_D > 1$ corresponds to the predominance of CAF–DNA complexation over drug–CAF hetero-association (i.e. the 'protector action' of CAF) and $R_D < 1$ corresponds to hetero-association being the major contribution of the displacement of drug molecules from DNA (i.e. the 'interceptor action' of CAF).

An estimate of the amount of drug displaced from DNA due to the presence of CAF can be made using the quantity A_D (Evstigneev et al. 2006b), which corresponds to the relative amount of drug molecules removed from DNA on addition of CAF,

$$A_D = \left(f^X_{C2(0)} - f^X_{C2}\right) \Big/ f^X_{C2(0)}, \tag{10}$$

Table 3 Equilibrium constants (l/mol, T = 298 K) for self-association (K), hetero-association with CAF (K_h) and complexation with single-stranded (K_1) and double-stranded (K_2) forms of d(TGCA) in aqueous solution for anthracycline drugs

	DAU	DOX	NOG	CAF
K	1,300 ± 300[a]	1,800 ± 500[b]	7,400 ± 1,500[c]	11.8 ± 0.3[d]
K_h	72 ± 4[d]	210 ± 40[b]	210 ± 50[b]	–
K_1	53,000 ± 13,000[e]	–	–	34 ± 4[d]
K_2	560,000 ± 13,000[e]	~ 800,000[f]	~ 1,200,000[f]	246 ± 18[d]

[a] Taken from Evstigneev et al. (2006a)

[b] This work

[c] Taken from Eaton et al. (2000)

[d] Taken from Davies et al. (2001)

[e] Taken from Davies et al. (2000)

[f] Estimated using Davies et al. (2000) and Ibrahim (2001)

where ${f^X}_{C2}$ is the relative amount of drug bound to d(TGCA)$_2$ in the presence of CAF. As the biological action of the anthracycline drug probably originates from the fraction of the drug molecules bound to DNA (${f^X}_{C2}$), it is likely that the relative displacement of drug from DNA, A_D, is related to the change in cytotoxicity of drug on addition of CAF.

Analysis of the simultaneous binding of anthracycline drugs with DNA in the presence of CAF

It is known that hetero-association of CAF with an aromatic drug decreases the fraction of the monomer form of the drug available for binding with DNA in solution (Piosik et al. 2002 2003; Davies et al. 2001; Traganos et al. 1991a, b; Ulanowska et al. 2005; Larsen et al. 1996), and that competition between CAF and the drug for DNA binding sites results in displacement of the bound drug with DNA (Davies et al. 1996; Traganos et al. 1991b). Both processes may act simultaneously and, in both cases, a decrease in binding of drug with DNA should affect the biological activity in the given system (Piosik et al. 2002, 2003; Ulanowska et al. 2005; Traganos et al. 1991a, b).

The effect of CAF on removal of an aromatic drug from native DNA has been investigated by other than NMR experimental techniques for a number of typical aromatic intercalators, viz. acridine orange (Lyles and Cameron, 2002a, b), propidium iodide (Bedner et al. 2001) and ethidium bromide (Bedner et al. 2001). This effect is manifested in the present study by the changes in chemical shift of the DAU protons on varying the concentration of CAF in the mixture with d(TGCA). It is seen in Fig. 4 that the aromatic protons of DAU

move downfield on increasing the concentration of CAF. The complexation constant of DAU with $d(TGCA)_2$ (K_{2X} = 560,000 l/mol (Davies et al. 2000)) is two or three orders of magnitude greater than that for its self-association (Table 1) or hetero-association with CAF (Table 3), and so in solution with the oligonucleotide DAU mainly exists in the complexed form with the tetramer duplexes, which has an averaged shielding per proton of $\overline{\Delta\delta} = 0.32$ ppm (Davies et al. 2000). On increasing the concentration of CAF in the DAU–nucleotide mixture, the proportion of CAF–DAU hetero-complexes increases, in which the averaged shielding per DAU proton ca.$\overline{\Delta\delta} = 0.04$ ppm (Davies et al. 2001) is much lower than that in the complex with the oligonucleotide. The significant differences in shielding effects explains the downfield changes of DAU protons on addition of CAF (Fig. 4) and provides confirmatory evidence of the decrease in proportion of DAU molecules complexed with the tetramer.

The competition between the 'protector' and 'interceptor' mechanisms of added CAF is manifested by the variation of the parameter R_D over a large range of concentrations (0–10 mM) of CAF as shown in Fig. 5a and b by the relative displacement (A_D) of the anthracycline drug from DNA over the same range of CAF concentrations. For these calculations, it was assumed that the concentration of drug is in physiological range (x_0 = 0.01 mM) and the concentration of the free

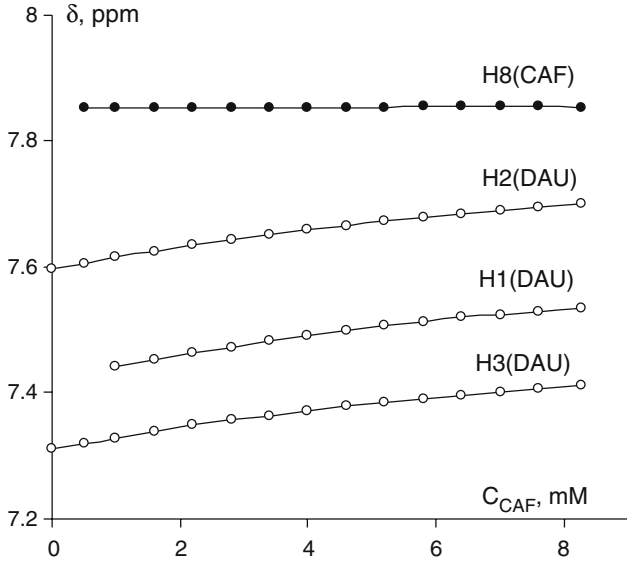

Fig. 4 Experimental dependence of (selected) ^{1}H NMR chemical shifts of daunomycin and caffeine in the mixture with d(TGCA) on the concentration of CAF (C_{DAU} = 0.57 mM, C_{TGCA} = 0.82 mM, 0.1 M Na-phosphate buffer, pD 7.1, T = 298 K)

tetrameric DNA regions to be N_0 = 0.01 mM. From previous studies of DOX-induced apoptosis in cultured leukaemia cells it is known that, on addition of CAF in millimolar concentrations, cell survival rises to a few dozen percent compared to decaffeinated cells (Traganos et al. 1991a) (i.e. the same order of magnitude as in Fig. 5b). So we think that a good estimate of the concentration of DNA sites free from histone regions in nuclear DNA is N_0 = 0.01 mM.

It is seen from Fig. 5a that up to $C_{CAF} \sim 1$ mM, the main mechanism affecting the DNA binding properties of DAU is the 'protector' action of CAF ($R_D > 1$) due to CAF–DNA complexation. On the other hand, CAF acts approximately equally as an 'interceptor' and 'protector' for the DOX and NOG systems over the same concentration range. The difference in behaviour of DAU vs DOX/NOG can be understood in terms of the differences in their hetero-association constants with CAF (Table 3). The hetero-association constants K_h(DOX-CAF) and K_h(NOG-CAF) are equal to each other, whereas the value for K_h(DAU-CAF) is much lower, and so CAF possesses weaker intercepting ability in CAF–DAU system compared to DOX and NOG. The result is that the R_D curve for CAF–DAU has much higher values than those for DOX and NOG (Fig. 5a). It is interesting to note that, because the R_D(DOX-CAF) and R_D(NOG-CAF) curves are essentially the same, the R_D factor appears to depend mainly on the hetero-association constant. These results and the R_D profile in Fig. 5a remain unchanged for variations of the DNA concentration (N_0) over a large range as may be judged from Fig. 6.

The effect of CAF on the binding of aromatic drugs with DNA is commonly detectable in vitro for CAF in the concentration range 1 mM < C_{CAF} < 10 mM (Piosik et al. 2002; Traganos et al. 1991a). At these concentrations, there are comparable contributions of both the interceptor and protector mechanisms in all three systems as shown quantitatively in Fig. 5a. Hence, the qualitatively deduced view (Larsen et al. 1996) that the interceptor action is an important mechanism in attenuation of the toxicity of anthracycline antibiotics is now confirmed quantitatively in this work. Even so, previous work (Larsen et al. 1996) did not take into account that the protector mechanism could be dominant, as shown for DAU in this work, and which may lead to erroneous results in other cases.

The quantitative displacement by CAF of drugs bound to DNA increases with CAF concentration as shown in Fig. 5b, where A_D% reflects the cumulative effect of the 'protector' and 'interceptor' mechanisms. The sequence of curves is in the order of DOX > NOG > DAU, which is found to be independent of

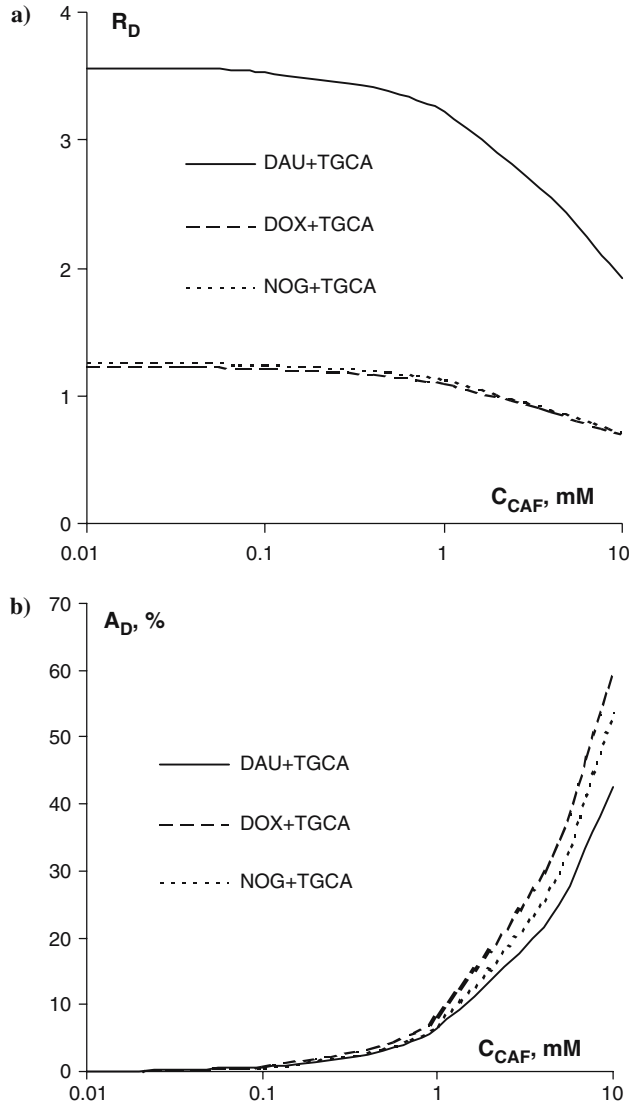

Fig. 5 Dependence on the concentration of caffeine. **a** Factor R_D, a measure of the effectiveness of the interceptor and protector action of caffeine and **b** A_D, fraction of bound drug removed from the DNA oligomer on addition of caffeine

variations in DNA concentration (N_0) over a large range (not shown). As the same concentration of CAF exerts a greater displacement effect for DOX and NOG compared to DAU, it suggests that the reduction in toxicity of these anthracycline drugs in vitro due to added CAF will follow the same sequence, assuming that the two dominating mechanisms (protector and interceptor) are valid and the magnitudes of equilibrium constants correspond to those in cell media. For the anthracycline drugs, it is found that the A_D curves depend on both the hetero-association and drug–DNA complexation constants, in contrast to the R_D factor which mainly depends on the hetero-association constant.

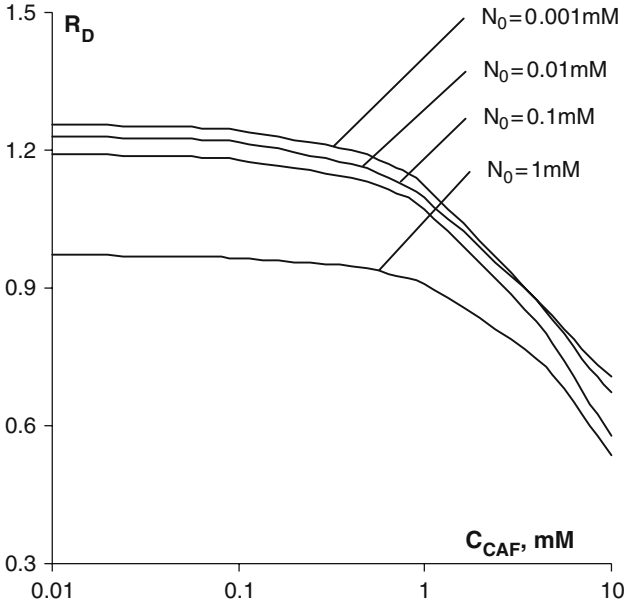

Fig. 6 Factor R_D as a function of caffeine concentration in the presence of a constant concentration of a DNA oligomer, N_0, for three orders of magnitude of DNA concentrations

Conclusions

Investigations have been made by NMR spectroscopy on the complexation of anthracycline antitumour drugs (DAU, DOX or NOG) to a model DNA tetrameric sequence and the competitive binding equilibrium on addition of CAF. The method depends on the stepwise analysis of one-component (self-association), two-component (hetero-association and DNA complexation) and three-component interactions under the physiological conditions; each stage of the analysis utilises as input data the parameters derived in the previous step. Such an approach was verified experimentally using ^{1}H NMR titration data on the three-component mixture of DAU, CAF and the DNA tetramer, d(TGCA). In order to complete the set of data necessary for three-component analysis for each of the anthracycline drugs, the self-association of DOX and the hetero-association of CAF with DOX/NOG were measured under the same solution conditions.

The overall binding equilibrium of anthracycline drug, CAF and DNA was quantitatively analysed in terms of the competition between two processes, which are known to affect drug–DNA binding, viz. hetero-association of CAF–drug ('interceptor' action of CAF) and CAF–DNA complexation ('protector' action of CAF). The results may be reflected in different effects of CAF on the toxicity of the different anthracycline drugs: the complexation of DAU with DNA is mainly affected by CAF–DNA complexation, whereas both

CAF–DNA complexation and CAF–drug hetero-association contribute approximately equally to the binding of DOX/NOG to DNA. The result is in agreement with previous conclusions on the binding of aromatic drugs with DNA in the presence of CAF (Larsen et al. 1996), though that work did not take into account the protector action of CAF as a possible mechanism, which in other cases could lead to erroneous results.

Quantitiative evaluation of the three-component mixture of drug–CAF–DNA has enabled the proportion of the antibiotic displaced from DNA upon addition of CAF to be calculated over a large range of concentrations of CAF. Assuming that the protector and interceptor mechanisms are valid and the magnitudes of equilibrium constants correspond to those in cell media, these results provide a quantitative basis for the change in anthracycline-related toxicity on addition of CAF. Potentially, it can be used as a strategy of regulation of the medico-biological activity of aromatic drugs in clinical practice, say, e.g. in reduction of the consequences of drugs' overdosing during chemotherapy or in production of antimutagenic effects in vivo.

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