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Biophysical Chemistry 121 (2006) 84–95

Biophysical Chemistry

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Complexation of norfloxacin with DNA in the presence of caffeine

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Received 4 November 2005; received in revised form 5 December 2005; accepted 7 December 2005
Available online 7 February 2006

Abstract

^{1}H NMR spectroscopy (500 MHz) has been used to quantify the complexation of the antibacterial antibiotic Norfloxacin (NOR) with DNA in the presence of Caffeine (CAF). Separate studies have been made for the self-association of NOR, its hetero-association with CAF and complexation with a model self-complementary DNA tetramer, 5′-d(TpGpCpA), in order to determine the equilibrium parameters (induced chemical shifts, association constants, enthalpy and entropy) of the two-component mixtures to aid the analysis of the three-component systems. Investigations of the self-association of NOR and its hetero-association with CAF show that the aggregation of NOR molecules and association with CAF in solution are driven by the stacking of aromatic chromophores. The complexation of NOR with d(TGCA) has been analysed in terms of intercalation with the double-stranded form and non-intercalative binding with the single-stranded form of DNA. Investigations of the competitive binding of NOR and CAF with DNA show that at physiological concentrations of NOR (μM) and CAF (mM) the dominant mechanism influencing the affinity of NOR with DNA is the displacement of bound NOR molecules from DNA due to CAF–DNA complexation (i.e. the protector action of Caffeine).

Keywords: Norfloxacin; Caffeine; Self-association; Hetero-association; DNA complexation; Competitive binding

1. Introduction

Quinolones are antibacterial agents that effectively inhibit DNA replication and are commonly used as treatment for many infections. Norfloxacin (NOR, Fig. 1a), one of the first clinically effective quinolones to be discovered, is active against most Gram-positive bacteria, numerous Gram-positive cocci as well as a few dangerous organisms like staphylococci, streptococci and *Pseudomonas aeruginosa* [1,2]. Like other piperazine-substituted fluoroquinolones (e.g. ciprofloxacin and ofloxacin), norfloxacin is a specific inhibitor of DNA gyrase, a bacterial type II topoisomerase, which unwinds the supercoiled DNA helix prior to replication and transcription [3]. It is known that the drug does not inhibit the gyrase but complexes to DNA in a highly cooperative mode of binding [4–6]. It has been suggested that the action of DNA gyrase forms a unique binding pocket on DNA, which can accommodate a dimer of NOR molecules with a sandwich-type arrangement of the chromophores and stabilised by π–π stacking [6,7]. The self-association ability of quinolones has been proposed from crystal structure analysis and molecular modeling studies [7], though nothing is known to date about the thermodynamics of the self-association of NOR in solution. The assumption about the aggregation of NOR resulting from stacking interactions of aromatic chromophores [7] indicates that complexation of the drug could occur with other aromatic molecules in solution and evidence of such interactions has recently been reported for NOR–mononucleotide studies [8]. On the other hand, the opposite conclusion has been made as no complexation between the NOR and ATP has been detected [9].

It is known that a mixture of NOR and Caffeine (CAF, Fig. 1b) given simultaneously exhibits biological synergism in vivo [2,10]. It has been recently shown that CAF, a purine derivative, effectively forms hetero-complexes with a number of aromatic DNA intercalators, which may account for the observed changes of biological activity of these drugs in the presence of Caffeine, i.e. the '*interceptor*' action of Caffeine [11,12]. Another mechanism of action has been proposed, in which there is competition between CAF and aromatic drug for the binding

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doi:10.1016/j.bpc.2005.12.003

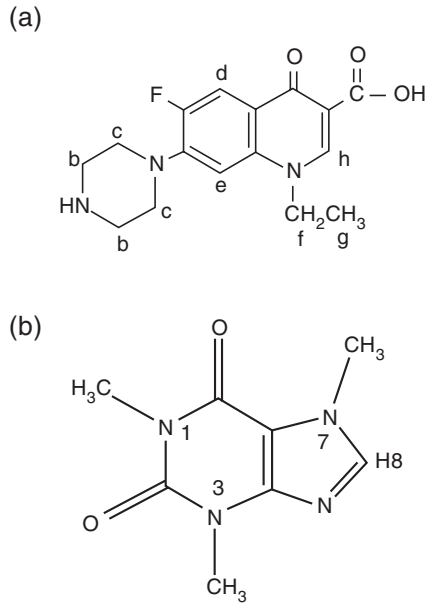

Fig. 1. Structures and atom numbering of (a) Norfloxacin and (b) Caffeine.

sites on DNA, i.e. the '*protector*' action of Caffeine [12,13]. It is likely that both mechanisms can act simultaneously and therefore must be taken into consideration when analysing a three-component equilibrium of the Drug, CAF and DNA [12,13].

As the NOR molecule contains two fused aromatic rings just like CAF, it is assumed that it is able to form hetero-association complexes with CAF and that it can bind with DNA just like CAF. Hence, one might expect that the observed biological synergism of NOR and CAF [2,10] may be due to the hetero-association of NOR with CAF and/or competition of NOR and CAF for DNA binding sites. In order to rationalize biological consequences of the action of antibiotic Norfloxacin, when combined with other aromatic drugs such as Caffeine, the solution behaviour of NOR in the presence of CAF and DNA needs to be investigated. This will also entail separate studies of NOR self-association, NOR–CAF hetero-association and NOR–DNA complexation. In the present work we have used ^{1}H NMR spectroscopy (500 MHz) to quantify the complexation of NOR with Caffeine and DNA. As in previous work [12,14] we took the self-complementary DNA oligomer, 5′-d(TpGpCpA), as a model DNA sequence to analyse competitive binding in a three-component system. The choice of the tetramer is dictated by the fact that short self-complementary DNA sequences are thought to be appropriate model systems for competitive binding studies of molecules in equilibrium [14] and that the 5′-d(TGCA) sequence contains the central the GC step, which was proposed to be the binding site for NOR [8].

2. Materials and methods

Norfloxacin (Fig. 1a) and Caffeine (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 (self-association experiment), concentration of CAF (NOR–CAF hetero-association experiment) and tetramer (NOR–TGCA experiment) at two temperatures (298 and 308 K) maintaining the concentration of NOR constant. The temperature dependences of proton chemical shifts for the NOR, NOR–CAF and NOR–TGCA solutions were measured at constant concentration of interacting molecules in the temperature range 278–348 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.

2.1. Molecular mechanics calculations

Calculations of the spatial structures of the 1:1 NOR–NOR and NOR–CAF complexes in an aqueous environment have been made as previously [15,16] by the molecular mechanics methods using X-PLOR software with the Charmm22 force field. Modeling of aqueous environment was performed by water molecules in the form of TIP3P [17], placed in rectangular box (1100 molecules). Topology of Norfloxacin and Caffeine molecules and parametrization of their valent interactions have been obtained with the help of XPLO2D-software [18] using crystal structures from PDB databank [19]. Parameters of non-valent interactions between atoms corresponded to force field MM3 [20]. Prior to energy minimisations the initial structures of the NOR dimer and 1:1 hetero-complex with Caffeine were built up from analysis of the induced chemical shifts of the protons of interacting molecules.

3. Results and discussion

3.1. Self-association of norfloxacin

Assignment of the non-exchangeable protons of NOR (Fig. 1a) has been made using two-dimensional TOCSY and ROESY experiments and are in good agreement with the results published previously [21]. The structural and thermodynamical parameters of the self-association of NOR have been determined from the experimental concentration and temperature dependences of proton chemical shifts (Fig. 2a,b). It can be seen from Fig. 2a that increasing the concentration of the drug results in upfield shifts (low frequency shifts) of aromatic protons, which is indicative of self-association of NOR by formation of stacked aggregates [22–24].

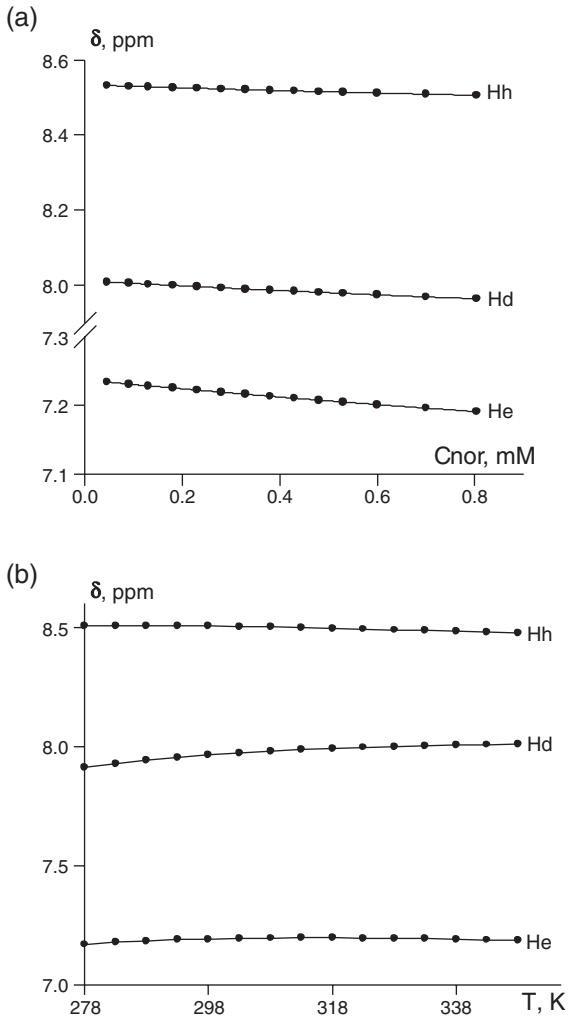

Fig. 2. Experimental dependence of ^{1}H NMR chemical shifts of Norfloxacin in 0.1 M Na–phosphate buffer, pD 7.1 on: (a) concentration, T=298 K; (b) temperature, C_{NOR}=0.8 mM.

The experimental NMR data have been analysed in terms of indefinite cooperative and non-cooperative models of association as described previously [23,24]. The key relations are given by the dependence of the experimentally observed chemical shift, δ, on the concentration of antibiotic, x_0, for the non-cooperative (1a) and cooperative (1b) models [23,24]:

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

$$\delta = \delta_m + 2(\delta_d - \delta_m)\left[1 - \frac{x_1}{x_0} - \frac{\sigma\cdot K_X x_1^2}{x_0(1 - K_X x_1)}\right] \quad (1b)$$

where δ_m, δ_d are the chemical shifts in monomer and dimer forms (or at the ends of an aggregate) of NOR in solution, respectively; K_X is the equilibrium constant for self-association of NOR; x_1 is the monomer concentration; σ is a cooperativity parameter reflecting the formation of aggregates of higher order than a dimer (for $\sigma<1$ the self-association is cooperative, $\sigma=1$ it is non-cooperative, and for $\sigma>1$ it is anti-cooperative) [23,24].

The experimental concentration dependences (Fig. 2a) can be described completely by Eqs. (1a) and (1b), in which there are three unknown parameters (K_X, δ_m, δ_d) in the non-cooperative model and four unknown parameters (K_X, δ_m, δ_d, σ) in the cooperative model. Using the computational method described previously [23], the results of the calculations are summarised in Table 1. The thermodynamical parameters, enthalpy (ΔH_X) and entropy (ΔS_X), for the self-association of NOR have been calculated (results in Table 1) as in previous work [18,19] from the temperature dependences of proton chemical shifts (Fig. 2b) replacing the K_X value in Eqs. (1a) and (1b) by the corresponding relation $K_X(T)$ according to the van't Hoff's formalism:

$$K_X(T) = \exp(\Delta S_X/R - \Delta H_X/RT). \quad (2)$$

It is found (Table 1) that the magnitude of the self-association constant of NOR, K_X, is of the same order of magnitude ($\sim 10^2$–10^3 M^{-1}) as those found previously [23] for molecules with three fused aromatic rings, in contrast to the self-association of Caffeine, which also contains two fused aromatic two rings but has a K_X which is 10 times lower than

Table 1
Equilibrium parameters of the self-association of Norfloxacin in 0.1 M Na–phosphate buffer, pD 7.1

Proton	δ_m, ppm	δ_d, ppm	K_X, l/mol	σ
T=298 K				
H_h	8.53	8.39	130±14	0.97±0.06
H_d	8.01	7.76		
H_e	7.24	6.96		
H_f	4.45	4.28		
H_c	3.58	3.50		
H_b	3.49	3.45		
H_g	1.50	1.42		
T=308 K				
H_h	8.53	8.40	100±16	0.98±0.06
H_d	8.02	7.80		
H_e	7.23	7.00		
H_f	4.44	4.27		
H_c	3.58	3.50		
H_b	3.49	3.44		
H_g	1.50	1.43		

Thermodynamic parameters of self-association

$-\Delta G^0$, kJ/mol	$-\Delta H^0$, kJ/mol	$-\Delta S^0$, J/(mol K)
12.0±1.6	18.0±3.6	20±5

that of NOR. The thermodynamical parameters of self-association, ΔH and ΔS (Table 1), are both negative, which is indicative of dispersive interactions as the main contributor to the energy of the self-association [23,25]. Assuming that dispersive interactions account for the main stabilisation of aggregates of aromatic molecules in solution with high ionic strength (0.1 M Na^+) [6,23,24], the observed difference in equilibrium self-association constants of NOR and CAF could originate from some additional stabilisation of NOR aggregates. The NOR molecule contains a bulky piperazine ring, which is absent in the structure of CAF (see Fig. 1), and also has groups which can form either hydrogen bonds or ionic interactions depending on pH, viz. a carboxylate group and both a secondary and tertiary amino group in the piperazine ring. Previously we have shown that 'bulky hydrophobic' substituents attached to an aromatic chromophore may result in a significant contribution to the energy of self-association, which may lead to an increase of the self-association constant up to 10 times or more, e.g. compare the self-association constants of the structurally similar acridine dyes Proflavine (K_{PF}=700 M^{-1}), which contain two amino groups, and Acridine Orange (K_{AO}=4600 M^{-1}) [23]), which contains dimethylamino groups in analogous positions. So, in addition to dispersive forces, a hydrophobic contribution may account for the relatively high self-association constant, K_X, for NOR compared to CAF. In addition, both the enthalpy and entropy of self-association of NOR are smaller in absolute value than the same parameters for CAF (Table 2), which is in agreement with the assumption that there is a significant hydrophobic contribution to the self-association of NOR, because hydrophobic interactions add positive terms to ΔH and ΔS of the association process.

Table 2
Equilibrium parameters of the hetero-association of Norfloxacin (NOR) with Caffeine (CAF)[a] in 0.1 M Na–phosphate buffer, pD 7.1

Protons NOR (X)	δ_{cX}, ppm	δ_{mX}, ppm	Protons CAF (Y)	δ_{cY}, ppm	δ_{mY}, ppm	K_h, l/mol
T=298 K						
H_h	8.49	8.53	H8	7.74	7.89	30±10
H_d	7.47	8.01	$7CH_3$	3.70	3.95	
H_e	6.86	7.24	$3CH_3$	3.09	3.54	
H_f	4.26	4.45	$1CH_3$	2.95	3.35	
T=308 K						
H_h	8.48	8.53	H8	7.77	7.89	23±12
H_d	7.47	8.01	$7CH_3$	3.85	3.95	
He	6.84	7.24	$3CH_3$	3.20	3.54	
H_f	4.24	4.45	$1CH_3$	3.17	3.35	

Thermodynamic parameters of hetero-association

$-\Delta G^0$, kJ/mol	$-\Delta H^0$, kJ/mol	$-\Delta S^0$, J/(mol K)
8.4±0.4	20.3±4.0	40±10

[a] Equilibrium parameters of self-association (and δ_{mY}) of CAF are taken from Ref. [12]: K_Y=11.8±0.3 (T=298 K), K_Y=8.9±0.3 (T=308 K) ΔH^0=− (21.0±0.4) kJ/mol, ΔS^0=− (50±1) J/mol K.

The influence of bulky substituents in the structure of the molecules may also be deduced from analysis of the cooperativity parameter, σ, of the self-association reaction [23]. The σ value calculated for NOR is close to 1 (see Table 1), which indicates that the aggregation of the antibiotic is non-cooperative. Taking the self-association of the acridine dye Proflavine as an example, it has been shown that the cooperativity in the formation of higher order aggregates than dimers ($\sigma < 1$) is due to absence of bulky side groups providing steric hindrance for binding the third and other molecule on the dimer, whereas the cooperativity parameter for Acridine Orange is 0.45 [23]. The presence of large groups in the structure of an aromatic molecule, say, the phenyl ring in Ethidium Bromide, results in an increase of σ up to the unity and in the case of the anthracycline antibiotics, Daunomycin and Nogalamycin, up to 1.3 and higher [26]. Taking these factors into consideration indicates that the $\sigma \approx 1$ for Norfloxacin must result from some mutual compensation of the contribution to cooperativity from the increased hydrophobicity of the molecule and an anti-cooperative contribution to the formation aggregates from the piperazine ring side chain in the NOR structure. This situation is very similar to that observed previously for aggregation of the anticancer antibiotic novatrone [24].

The MM calculated structure of the NOR dimer presented in Fig. 3 shows that the chromophores of the stacked molecules are parallel to each other with a spacing between them 0.34 nm, in which the chromophores are oriented in an anti-parallel arrangement. The structure obtained is characterized by a hydrophobically favourable compact configuration with the possibility of hydrogen-bonding/ionic interactions between the polar/ionic groups of the piperazine rings on adjacent molecules. Hence, if the binding pocket in the gyrase–DNA complex is commensurable to the dimensions of the NOR dimer, it may actually bind with DNA as suggested [6].

3.2. Hetero-association of Norfloxacin with Caffeine

In the mixed solution of NOR with CAF, the experimental ^{1}H NMR curves for NOR (Fig. 4a) show an increase in average shielding of NOR protons upon successive additions of Caffeine in solution measured at constant concentration of NOR. A similar situation has been observed previously for a number of molecules with three fused aromatic rings in hetero-association mixtures with CAF [11,12]. The shielding most likely results from complexation between NOR and CAF occurring via stacking of their aromatic chromophores. It follows that complexation between NOR and CAF is in accord with previous results [8] and does not require divalent metal ions as might be deduced from Ref. [9].

The dynamic equilibrium in solution containing two types of interacting aromatic molecules X and Y may be described using the following general scheme of reactions [27]:

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

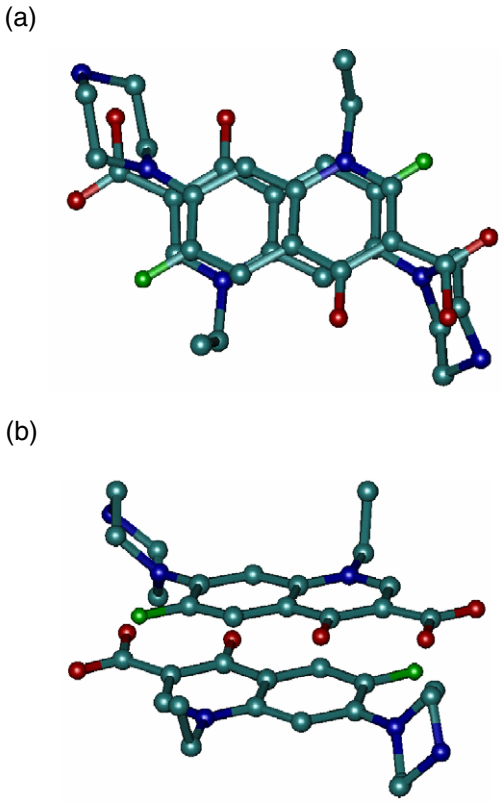

Fig. 3. MM calculated spatial structure of the Norfloxacin dimer: (a) view from the top, (b) side view.

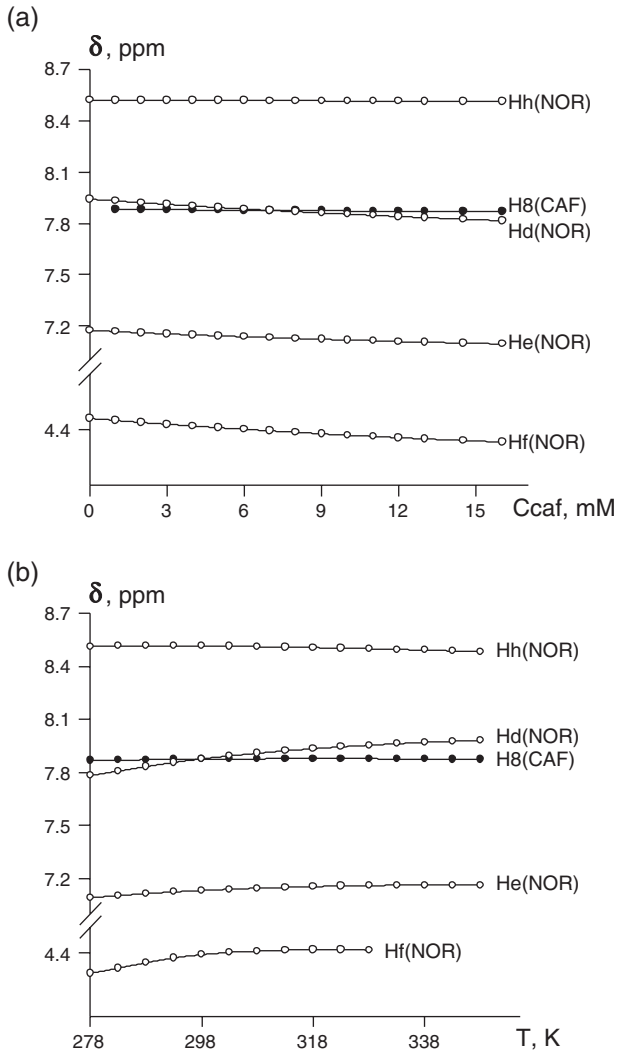

Fig. 4. Experimental dependence of ^{1}H NMR chemical shifts of Norfloxacin and Caffeine in 0.1 M Na–phosphate buffer, pD 7.1 on: (a) CAF concentration, T=298 K, C_{NOR}=1.13 mM; (*b*) temperature, C_{NOR}=1.13 mM, C_{CAF}=7 mM.

where X_1 and Y_1 correspond to the monomers of NOR and CAF, and X_i, X_k, Y_j, Y_l are the aggregates containing i, k monomers of NOR and j, l monomers of CAF, respectively; K_X, K_Y are equilibrium self-association constants for X and Y and K_h is the hetero-association constant. As in previous work [12] the reaction (3e) may be safely neglected for CAF–drug systems, which simplifies analytical expressions for the observed dependence of proton chemical shifts of both X and Y components to the form [12]

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

where x_0 and x_1, y_1 are the total and the monomer concentrations of the drug and CAF molecules in solution; δ_m, δ_d, δ_h are chemical shifts of X or Y protons in the monomer, dimer and in the hetero-complex, respectively. The values of δ_m, δ_d and the equilibrium self-association constants are known from self-association studies of NOR and CAF (Tables 1 and 2). The monomer concentrations x_1 and y_1 may be derived from the solution of the mass concentration law for scheme (3) [12]. It follows that the model (4) is a function of two unknown quantities, δ_h and K_h, which may be determined from the concentration dependences of δ (Fig. 4a) using the numerical procedure described previously [12,27].

The thermodynamical parameters, enthalpy ($\Delta H°_h$) and entropy ($\Delta S°_h$), were obtained from the observed temperature dependences of the proton chemical shifts of NOR and CAF (Fig. 4b) using the van't-Hoff's formalism (2) as described above for the self-association of NOR. The calculated equilibrium, δ_h and K_h, and thermodynamical parameters, $\Delta H°_h$ and $\Delta S°_h$, are summarised in Table 2.

It is seen from Table 2 that the equilibrium hetero-association constant K_h, as well as the enthalpy/entropy values, range between those for self-association of NOR (e.g.

K_X) and CAF (e.g. K_Y) analogous to the results for complexation of other aromatic molecules (acridine and phenanthridine dyes, antibiotics) with Caffeine reported previously [11,12,24], where it was shown that stacking interactions, which includes both dispersive and hydrophobic interactions, play a major role in stabilisation of the sandwich-type hetero-complexes of aromatic chromophores in solution. The structure of the NOR–CAF hetero-association complex presented in Fig. 5 clearly demonstrates the co-planarity of the aromatic chromophores of NOR and CAF, which supports the conclusion about the stacking as a major stabilizing force in the given system. The degree of aromaticity of the two-ring NOR chromophore is apparently lower than that of the three fused-ring aromatic molecules studied previously [12]. The concomitant weakening of π–π interactions in NOR–CAF hetero-complex with respect to the three-ring aromatic molecules is reflected in the lower magnitude of K_h and thermodynamical parameters (Table 2) as compared to those found previously for aromatic molecules with three fused rings [12].

3.3. Complexation of norfloxacin with the DNA fragment 5′-d (TpGpCpA)

The scheme of dynamic equilibrium reactions of NOR and TGCA in solution is the same as in previous work [12,26]; viz. formation of 1:1, 1:2, 2:1 and 2:2 complexes of NOR (X) with the single-(N) and double-stranded (N_2) forms of the DNA oligomer in solution with the corresponding complexation constants, K_{11}, K_{12}, K_{21}, K_{22}, respectively, and the dimerization constant for the tetramer, K_N (K_N=110 M^{-1} [26], reaction (5b)):

$$X + X \overset{K_X}{\longleftrightarrow} X_2 \text{ (a) } N + N \overset{K_N}{\longleftrightarrow} N_2 \text{ (b)}$$
$$X + N \overset{K_{11}}{\longleftrightarrow} XN \text{ (c) } X + N_2 \overset{K_{12}}{\longleftrightarrow} XN_2 \text{ (d) } X + XN \overset{K_{21}}{\longleftrightarrow} X_2N \text{ (e)}$$
$$X + XN_2 \overset{K_{22}}{\longleftrightarrow} X_2N_2 \text{ (f)}. \quad (5)$$

Incorporation of other reactions (say, associated with an alternative mode of NOR binding to the tetramer duplex) will make the number of search parameters too big for reliable description of the data available.

Normally, the complexation of aromatic drug molecules with short DNA sequences occurs with a binding constant of 10^3–10^5 M^{-1} [12,26], which is greater than the self-association constants for the drug by at least an order of magnitude. Hence, a simple dimerization of NOR is sufficient for such analysis rather than the indefinite self-association given above. The observed concentration dependence of the proton chemical shifts of NOR (Fig. 6a) is given by

$$\delta = \frac{x_1}{x_0}(\delta_{mX} + 2K_X x_1 \delta_{dX} + K_{11} N \delta_{11} + K_N K_{12} N^2 \delta_{12} + 2K_{11} K_{21} x_1 N \delta_{21} + 2K_N K_{12} K_{22} D N^2 \delta_{22}), \quad (6)$$

where δ_{11}, δ_{12}, δ_{21}, δ_{22} are proton chemical shifts of NOR when complexed with the tetramer. The parameter K_N is known from self-association studies of d(TGCA) tetramer [12]. Eq. (6) thus provides 8 unknown quantities (K_{11} … K_{22} and δ_{11} … δ_{22}). Both the derivation of Eq. (6) and the numerical procedure for determination of the unknown

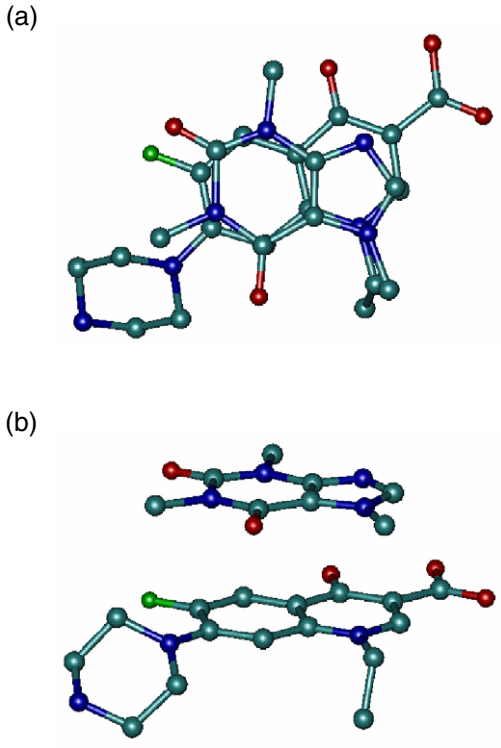

Fig. 5. MM calculated spatial structure of the NOR–CAF 1:1 hetero-complex: (a) view from the top, (b) side view.

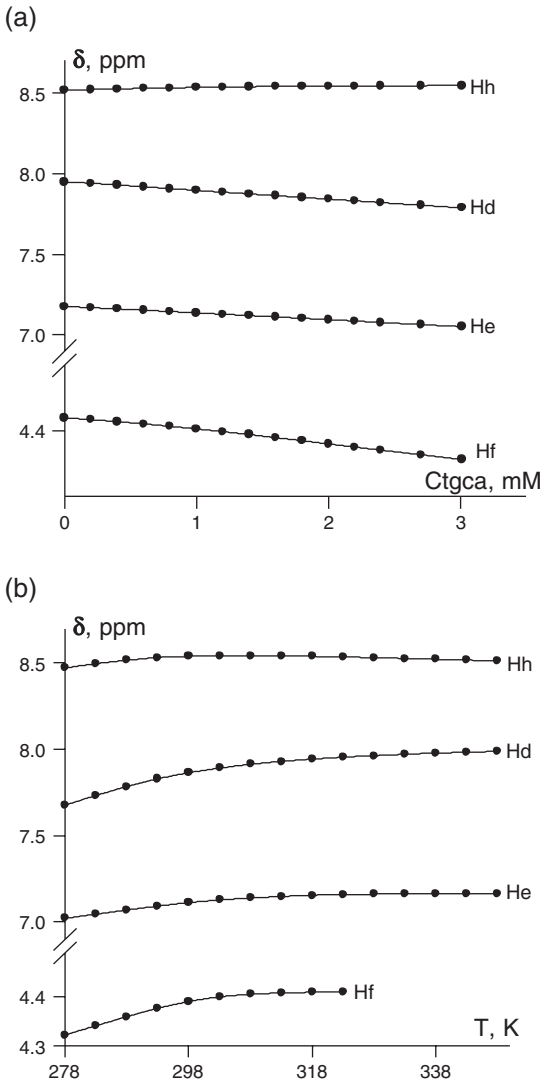

Fig. 6. Experimental dependence of ^{1}H NMR chemical shifts of Norfloxacin in 0.1M Na–phosphate buffer, pD 7.1 on: (a) TGCA concentration, T=298 K, C_{NOR}=1.02 mM; (b) temperature, C_{NOR}=1.02 mM, C_{TGCA}=1.6mM.

quantities is described in detail [28]. The thermodynamical parameters, enthalpies and entropies, of the complexation reaction (5) have been determined from experimental temperature dependences of NOR proton chemical shifts (Fig. 6b) using the approach similar to that for the self- and hetero-association, described above.

On proceeding with the computations the magnitudes of the complexation parameters for the formation of 2:1 (5e) and 2:2 (5f) complexes appeared to be very unreliable, which is due to the relatively small changes in the experimental proton chemical shifts with both concentration and temperature, as compared with three-ring intercalators with the same tetramer [28]. Hence, we have excluded reactions 2:1 and 2:2 from scheme (5) and re-made all calculations using just a 4-parameter set. The calculated complexation parameters for 1:1 and 1:2 reactions are given in Table 3, which shows that the equilibrium constant for 1:2 complexation is higher than that of 1:1, i.e. NOR binds more tightly to the double-stranded form of the oligonucleotide in solution. Even so the complexation parameters for this reaction (K_{12} and enthalpy/entropy) are much smaller in absolute value compared with the three-ring intercalators [12,26] but much larger than that calculated above for self-

Table 3
Equilibrium parameters of the complexation of Norfloxacin (NOR) with 5′-d(TpGpCpA) in 0.1M Na–phosphate buffer, pD 7.1

Proton NOR	H_h	H_d	H_e	H_f
δ_{11}, ppm	8.66	7.70	6.99	4.37
δ_{12}, ppm	8.49	7.42	6.75	4.16
T, K	298		308	
K_{11}, l/mol	300±50		200±50	
K_{12}, l/mol	2000±500		1000±300	
$-\Delta H°_{11}$, kJ/mol	21±4			
$-\Delta S°_{11}$, J/(mol K)	25±7			
$-\Delta H°_{12}$, kJ/mol	32±4			
$-\Delta S°_{12}$, J/(mol K)	45±10			

Equilibrium constant K_{12} for complexation of CAF with 5′-d(TGCA) at T=298 K is given as K_{12}=246±18 M^{-1} [12].

association of NOR and its hetero-association with CAF (Tables 1 and 2). Firstly, these results indicate that NOR complexes with the double-stranded form of the tetramer by an intercalation-type binding between the antibiotic and adjacent base pairs of the tetramer duplex. The observed shielding of all the aromatic protons of NOR in the 1:2 complex ($\delta_m-\delta_{12}>0$, see Tables 1 and 3), which is known to be a distinctive feature of intercalation [26,28,29], supports this conclusion. Moreover, a systematic shielding pattern in the 1:2 complex suggests that intercalation is likely to be a dominant mechanism of binding if an alternative mode of binding (say, groove-binding) is also presumed. Secondly, the decrease in magnitude of the complexation parameters is apparently due to a smaller contribution from π–π stacking arising when the two fused-ring NOR chromophore is intercalated between the adjacent bases along the oligomer sequence. It was not possible from the data available to deduce the site of preferential binding of NOR with the tetramer as well as to build the structure of the complex. In addition, no intermolecular cross-peaks were observed in 2D-NOESY spectra, which was also reported previously [21]. It should be noted that an intercalation-type binding of NOR with double-stranded DNA has also been proposed [21] and the magnitude of interaction has been reported to be of the order of 10^3 M^{-1} (though under low ionic strength conditions) [30], which is similar to that obtained in the current study under high ionic strength conditions.

Special examination of the δ_{11} values (Table 3) is required, because the average shielding over all aromatic protons in the 1:1 complex ($\Delta\delta_{11}=\delta_m-\delta_{11}=0.13$ ppm) is much smaller than that in the 1:2 complex ($\Delta\delta_{12}=\delta_m-\delta_{12}=0.35$ ppm) and in the NOR–CAF hetero-complex ($\Delta\delta_h=\delta_m-\delta_h=0.29$ ppm). Moreover, in contrast to the other protons, the H_h proton is deshielded moving downfield (to high frequency) on increasing the concentration of TGCA (see Fig. 6a), which is also quite different from that observed previously for the three fused-ring intercalators [26,28]. This result suggests a non-intercalative mode of binding of NOR with the single-stranded form of the tetramer. It worth noting that both types of interaction, intercalative and non-intercalative, may occur between the antibiotic and DNA as suggested previously [21].

3.4. Complexation of norfloxacin with the DNA fragment 5′-d (TpGpCpA) in the presence of CAF

The general scheme of analysis of the three-component mixture of NOR, CAF and d(TGCA) should comprise all possible interactions between the dissolved molecules, i.e. the self-association of NOR, CAF and d(TGCA), the hetero-association NOR–CAF and the complexation of NOR and CAF with d(TGCA). It was suggested previously [14] that in order to conform DNA binding in the nuclear chromatin in a cell, the competitive binding analysis should consider just the tetramer duplex N_2 (if a ligand exerts a predominant affinity to a double-stranded DNA) so the basic scheme of reactions describing the complex equilibrium of NOR, CAF and the DNA tetramer in solution is a compilation of self-association (3a,b), hetero-association (3c,d,e) and complexation (5c,d) reactions discussed above. Each reaction adds its own term to the corresponding mass conservation law which may be written as

$$\begin{cases} \dfrac{x_1}{(1-K_X x_1)^2}\left(1+\dfrac{K_h y_1}{1-K_Y y_1}+\dfrac{K_h^2 y_1^2}{2(1-K_Y y_1)^2}\right)+x_1 N_2 K_{12X}=x_0 \\ \dfrac{y_1}{(1-K_Y y_1)^2}\left(1+\dfrac{K_h x_1}{1-K_X x_1}+\dfrac{K_h^2 x_1 y_1}{(1-K_X x_1)(1-K_Y y_1)}\right)+y_1 N_2 K_{12Y}=y_0, \\ N_2(1+K_{12X}x_1+K_{12Y}y_1)=N_0 \end{cases} \tag{7}$$

where N_2 is the double-stranded form of the tetramer that is not complexed with either NOR or CAF. The equilibrium parameters K_X, K_Y, K_h, K_{12X}, K_{12Y} for both NOR and CAF are known (see Tables 1–3). The system of Eq. (7) is central in the analysis of the competitive binding assay as it enables the determination of the monomer concentrations x_1, y_1 and N_2 for any given set of total concentrations x_0, y_0 and N_0. These quantities can be further used to calculate the relative fraction of each type of complex in the mixed solution.

It is known that the hetero-association of an aromatic drug with CAF decreases the fraction of the monomer form of the drug available for binding with DNA in solution [11–13], and that competition between the drug and CAF for binding with DNA results in displacement of the bound drug with DNA [12,13]. Both processes may act simultaneously and, in either case, a decrease in binding of drug with DNA should affect the biological activity in the given system [12,13]. In order to estimate the influence of the hetero-association (‘*interceptor*’ action of CAF) and DNA complexation (‘*protector*’ action of CAF) on the complexation affinity of NOR with DNA in the presence of Caffeine separately, the relative decrease of NOR-d $(TGCA)_2$ complexes is calculated for two circumstances: (i) under the ‘switched off’ hetero-association NOR–CAF and ‘switched on’ complexation of CAF with $d(TGCA)_2$ ($K_h=0$, $K_{12Y}\neq 0$), i.e. $f^X_{C2(C)}$, and (ii) under the ‘switched on’ hetero-association NOR–CAF and ‘switched off’ complexation of CAF with $d(TGCA)_2$ ($K_h\neq 0$, $K_{12Y}=0$), i.e. $f^X_{C2(H)}$. R_D, Eq. (8), is a relation between these two quantities that enables one to discriminate between and to quantify the effect of hetero-association and DNA-complexation on the decrease of NOR binding with DNA, i.e. between the interceptor and protector actions of CAF:

$$R_D=\frac{f^X_{C2(0)}-f^X_{C2(C)}}{f^X_{C2(0)}-f^X_{C2(H)}}, \tag{8}$$

where $f^X_{C2(0)}$ is the mole fraction of NOR-$d(TGCA)_2$ complexes with a ‘switched off’ hetero-association and CAF–DNA complexation. A similar scheme has also recently been used for analysis of simultaneous binding of the antibiotic Daunomycin and a Vitamin B_2 analogue with $d(TGCA)_2$ [14]. The range of $R_D>1$ corresponds to the predominance of CAF–DNA complexation over NOR–CAF hetero-association (i.e. the protector action of CAF) and $R_D<1$ means a major contribution of hetero-association to the displacement of NOR molecules from DNA (i.e. the interceptor action of CAF).

Competition between NOR and CAF for DNA binding sites of chromatin in the cell is likely to occur in the vicinity of regions of DNA free from proteins [14]. Assuming that the physiological order of concentration of Norfloxacin (x_0) is about 1 μM and that the concentration of the free DNA fragments in chromatin is much greater than that of the antibiotic (N_0=10 mM), the R_D profile in Fig. 7 is shown for the variation in concentration of CAF over the vast range from 0 to 100 mM. It is seen that $R_D>1$ over the full concentration range of CAF, which indicates the predominance of the protector action of CAF on DNA over the interceptor action; this conclusion results from the relatively small value of the hetero-association constant for NOR–CAF [$K_{hNOR-CAF}$=30 M^{-1} (Table 2)] and concomitantly the small contribution of hetero-association reactions to the overall dynamic equilibrium in solution. In fact the same conclusion has also been drawn for a number other aromatic DNA intercalators being complexed with the DNA oligomer in the presence of Caffeine [12]. On the other hand, previous work on the competition between Daunomycin (DAU) and Vitamin B_2, an approximately equal contribution of the interceptor and protector action of the vitamin to the decrease of DAU binding with DNA has been reported [14]. In comparison with the NOR–CAF–TGCA system, the results for DAU–Vitamin B_2–TGCA may now be explained by relatively large hetero-association constant (K_h=453±28 M^{-1}, T=298 K) for the DAU–Vit. system [14].

It can be seen in Fig. 7 that the R_D profile has a maximum, which has also been observed in DAU–Vit–TGCA system [14]. At small concentrations of CAF, it cannot effectively displace NOR molecules that are bound to $d(TGCA)_2$, because the concentration of NOR in solution is small compared to the tetramer. Nevertheless, the hetero-association between NOR and CAF occurs in all cases. It follows that the protector and interceptor actions of CAF appear to be commensurate up to millimolar concentration of CAF. In this range, CAF starts to saturate the tetramer (see the C_2 curve in Fig. 7), which at the very beginning accentuates the role of CAF–DNA complexation (protector action) and then, after saturation, transfers the equilibrium towards hetero-association.

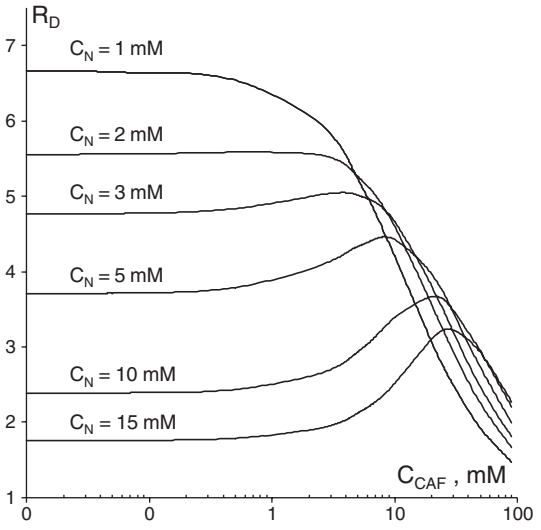

Fig. 8. Relation between R_D, a measure of the effectiveness of the interceptor and protector action of Caffeine, and the concentration of CAF for different concentrations of nucleotide (C_N).

The effect of variation of the tetramer concentration on the R_D profile is presented in Fig. 8. The importance of hetero-association gradually increases on increasing the concentration of nucleotide, N_0. In fact if an estimate of N_0 is available, Fig. 8 may be used to discriminate between the effect of CAF–NOR hetero-association and CAF–DNA complexation on the decrease of NOR binding with DNA in the given cell system.

And finally, the relative amount of NOR molecules, removed from DNA upon addition of CAF, A_D ($A_D=(f^X_{C2(0)}-f^X_{C2})/f^X_{C2(0)}$ where the f^X_{C2} is relative amount of NOR bound to $d(TGCA)_2$ in the presence of CAF) is presented in Fig. 9 for different concentrations of the tetramer. The curves reflect the cumulative effect of the interceptor and protector actions of CAF and exhibit a saturation profile resulting from a finite amount of

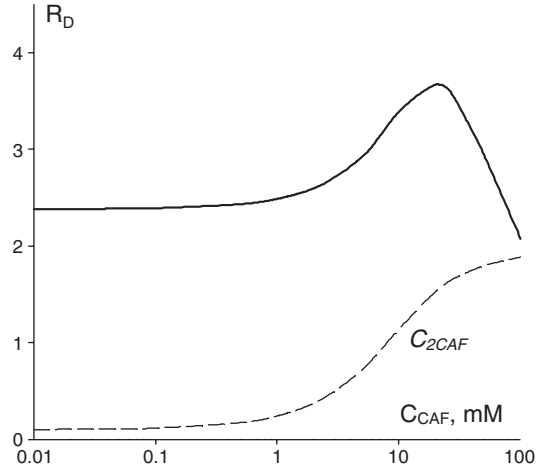

Fig. 7. Relation between R_D, a measure of the effectiveness of the interceptor and protector action of Caffeine, and the concentration of CAF; $C_{2(CAF)}$ is a schematic depiction of the absolute concentration of CAF–tetramer complexes.

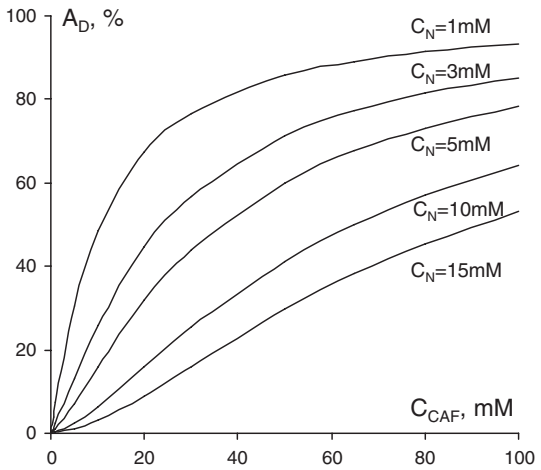

Fig. 9. Fraction of bound NOR removed from DNA (A_D%) upon addition of Caffeine as a function of CAF concentration at different concentrations of nucleotide (C_N).

NOR available in the system ($x_0 = 1$ μM). The biological action of the NOR itself originates from the fraction of the molecules which are bound to DNA (f_{C2}^{X}). So the relative displacement of NOR from DNA, A_D, may be related to the change in cytotoxicity of NOR upon addition of CAF. Hence, if an estimation of N_0 is available, Fig. 9 may be used to anticipate the effect of added CAF on the biological activity of antibiotic Norfloxacin.

3.5. Conclusions

In the present work we have studied the self-association of the antibiotic Norfloxacin, its hetero-association with Caffeine and complexation with the DNA tetramer, 5′-d(TpGpCpA) under similar solution conditions (0.1 M Na–phosphate buffer). The equilibrium parameters obtained in each separate experiment were used to quantify the effect of competitive binding of NOR with the tetramer in the presence of CAF.

Analysis of the self-association parameters of NOR has revealed that the tendency of NOR molecules to aggregate in solution is driven by the stacking of aromatic chromophores with a sandwich-type arrangement and that formation of the stacked species is hydrophobically favourable. If all steric conditions are satisfied, it thus seems possible for the NOR dimer to intercalate into the DNA–gyrase binding pocket as suggested previously [6]. The analysis of the hetero-association of NOR with CAF has shown that Norfloxacin molecules can complex with purine derivatives without presence of divalent ions as suggested previously [9] and that hetero-association of the aromatic molecules results from stacking interactions stabilized mainly by dispersive interactions of the aromatic chromophores of NOR and CAF. Analysis of the complexation of NOR with d(TGCA) has shown that intercalative binding of the antibiotic occurs with double-stranded DNA but non-intercalative binding also occurs with the single-stranded DNA form.

Finally, analysis of the competitive binding of NOR and CAF with DNA in the three-component mixture NOR–CAF–d$(TGCA)_2$ has been able to discriminate between hetero-association (CAF–NOR) and CAF–DNA complexation and to quantify the effect of both processes on the decrease in binding of NOR with DNA. It has been concluded that at likely physiological concentrations of NOR (μM) and CAF (mM) the dominant mechanism influencing the binding affinity of NOR with DNA is displacement of bound NOR molecules from DNA due to CAF–DNA complexation (i.e. the protector action of Caffeine).

Acknowledgements

We thank the Royal Society for a NATO Fellowship (MPE).

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