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General analysis of competitive binding in drug–interceptor–DNA systems

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Abstract A general model of competitive binding in drug–interceptor–DNA systems has been developed in order to quantify both the interceptor and protector mechanisms. The model involves full parameterization of the basic equations governing the mutual competition between drugs binding to DNA and incorporates as partial cases various similar models existing in the literature. The generality of the model results from strict accounting of the statistical effects of the binding of the drug and interceptor with DNA according to the McGhee-von Hippel formalism, and to the strict treatment of hetero-association between the drug and interceptor, which includes formation of all possible types of self- and hetero-complexes in solution. Indirect experimental evidence is provided for the importance of the protector mechanism in drug–caffeine–DNA systems, which is sometimes ignored in the literature because of the small magnitude of the CAF-DNA binding constant.

Keywords DNA complexation · Hetero-association · Competitive binding · Three-component system

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Introduction

The biological synergism of drug molecules administered sequentially or simultaneously into an organism forms the basis of modern combinational chemotherapy of various human diseases and is currently under investigation in terms of the physico-chemical, biochemical and pharmacological interactions. In the majority of cases the underlying mechanism of the drug interactions is not known, which inhibits the development of more effective disease treatment protocols. However, there is an exception for the group of biologically active drugs containing aromatic molecules as a major component and that exert their biological effect primarily by complexation with nuclear DNA. It is known that in the three-component system, drug–interceptor–DNA, which consists of DNA and two different types of aromatic molecules (one is the main DNA-targeting drug and the other is the drug's interceptor aromatic molecule), modulation of the biological activity of the drug may occur by varying the concentration of the interceptor (for a review, see Evstigneev 2010). This effect has been extensively confirmed both in vitro and in vivo for the three main groups of combinations of drug and interceptor aromatic molecules, viz. drug-chlorophyllin (Pietrzak et al. 2008; Osowski et al. 2010), drug-vitamin B_2 (Muñoz et al. 1995; Ramu et al. 2000; Evstigneev et al. 2005) and drug-methylxanthine (Davies et al. 2001; Piosik et al. 2002, 2010; Evstigneev et al. 2006a, b; Hernandez Santiago et al. 2009; Woziwodzka et al. 2011). The efforts of various research groups have led to the conclusion that two basic molecular mechanisms govern the alteration of the biological effect of the drug in the presence of the interceptor (for a review, see Evstigneev 2010), viz.

- protector, i.e. competition of the drug and interceptor for DNA binding sites leading to removal of the drug

from DNA as a result of the interceptor-DNA binding, and
- interceptor, i.e., hetero-association between the drug and interceptor leading to formation of inactive hetero-complexes unable to bind with DNA.

The interceptor mechanism has long been known, and the molecules acting by this mechanism are called 'interceptor molecules.' However, the existence of two molecular mechanisms, operating simultaneously in drug–interceptor–DNA systems, suggests that the term 'interceptor molecule,' used throughout this article, should therefore be understood more broadly.

So far, several protocols have been suggested in order to quantify the three-component system of drug–interceptor–DNA (see the models developed in Pietrzak et al. 2006; Evstigneev et al. 2008), and the link has been established between the observed biological effect of the drug on administration of the interceptor and the relevant equilibrium complexation constants (Evstigneev et al. 2008, 2011a). So far, these developments contain some drawbacks, which commonly fall outside the scope of various scientific papers and are usually omitted as scientific assumptions or simplifications. We shall consider two such problems existing directly or indirectly in most publications in the field of multicomponent competitive binding studies.

Problem 1

The separate protector and interceptor mechanisms are well characterized in biophysical chemistry and can be quantified by various experimental techniques, whereas there is the problem of quantitation for their simultaneous operation in drug–interceptor–DNA systems. The different views on this problem can be divided onto two groups: (1) those that ignore the protector mechanism (see for instance Piosik et al. 2002, 2010; Pietrzak et al. 2006, 2008; Woziwodzka et al. 2011) and (2) those that try to incorporate both mechanisms (see for instance Davies et al. 2001; Evstigneev et al. 2006a, b; Hernandez Santiago et al. 2009). Group 1 bases its view on the fact that the binding constant of the interceptor molecule with DNA is typically orders of magnitude lower than that for the drug-DNA, and often utilizes the UV-Vis technique operating in the low concentration range in order to treat the three-component mixture (as is the case for drug-methylxanthine-DNA systems (Piosik et al. 2002, 2010; Woziwodzka et al. 2011). This assumption is also complicated by the lack of a model able to treat both mechanisms simultaneously and by the fact that for some interceptors direct measurement of the interceptor-DNA binding constant is problematic by UV-Vis because of spectral overlap and the relative insensitivity of this technique to small binding constants measured in the low concentration range. Group 2 treats both interceptor and protector mechanisms simultaneously, but mainly utilizes NMR spectroscopy, where measurements are made at higher concentrations, which are non-physiological, and utilizes non-polymeric DNA in order to simplify the computational scheme. So now the question is whether the protector mechanism is independent of the type of experiment employed for analysis of the biological effects in drug–interceptor–DNA systems. If the answer is yes, is it possible to incorporate the protector mechanism into the UV-Vis-based analysis?

Problem 2

Recently it was shown that using a set of quasi-physiological concentrations and assuming that the drug and interceptor molecules bind with DNA, which is represented in a form of a mixture of independent tetranucleotide fragments (to be further referred to as the 'tetramer' model), enable one to describe the concentration-dependent changes in biological effect of the aromatic drug on addition of the interceptor molecule (Evstigneev et al. 2008, 2011a). Problems with the analysis are the use of DNA fragments to determine the corresponding macroscopic binding constants and ignorance of the differences in sizes of binding sites for different aromatic molecules. The questions now are whether polymeric DNA can be used in such an analysis, and will the result of the quantification of the biological effect depend on microscopic parameters of DNA binding such as microscopic binding constants, binding site sizes, etc.?

In this work we address the two above-mentioned problems.

Materials and methods

Solution preparation and carrying out the measurements

Commercial preparations of the sodium salt of salmon testes DNA (D-1626), ethidium bromide, proflavine (Sigma, USA) and caffeine (Merck, Germany) were used without further purification. The solutions of ethidium bromide (EB), proflavine (PF) and caffeine (CAF) were prepared in phosphate-buffered (0.025 M KH_2PO_4 and 0.025 M Na_2HPO_4) doubly-distilled water (pH = 6.86) by dissolution of accurately weighed compound immediately before carrying out the measurements. A weighed DNA sample was also dissolved in phosphate buffer over 24 h. Weighings were performed using a RADWAG XAS-220/C electronic microbalance. In order to minimize photodegradation of the solutions, their preparation was made in a darkened place lit with weak intensity red light.

The measurements were carried out on a UNICO SQ-4802 (USA) double-beam spectrophotometer over a range of 350–600 nm with a scanning step of 0.5 nm. Samples were put into disposable plastic cuvettes with an optical path length of 1 cm. The temperature inside the cuvette section of the spectrophotometer was maintained constant (298 ± 0.25 K) using a thermostat, and immediately before photometry the samples were equilibrated for 10 min.

In all series of spectrophotometric titrations the concentration of caffeine was varied in the range of 0–3 × 10^{-2} M at fixed concentration of the drugs and DNA. The concentrations of the drugs and DNA stock solutions were determined spectrophotometrically using the extinction coefficients $\varepsilon_{480} = 5{,}860\ M^{-1}\ cm^{-1}$ for EB ($C_{EB} = 5 \times 10^{-5}$ M; Bresloff and Crothers 1975), $\varepsilon_{444} = 42{,}000\ M^{-1}\ cm^{-1}$ for PF ($C_{PF} = 1.3 \times 10^{-5}$ M; Schwarz et al. 1970) and $\varepsilon_{260} = 6{,}400\ M^{-1}\ cm^{-1}$ for DNA (the DNA concentration was always expressed in moles of base pairs per liter; Barceló et al. 2002). The DNA concentration in the EB-CAF-DNA system was set to 2.89 × 10^{-5} M, and in the PF-CAF-DNA systems to 1.95 × 10^{-5} M.

Numerical analysis

Numerical analysis of experimental data was performed by means of the MATLAB software (version 7.11). The concentration dependence of optical density of the studied solutions at a single wavelength corresponding to the absorption maximum of the free drug was used as input data. The general algorithm of data analysis includes two main subprograms: (i) the computation of the discrepancy function, Δ (1), and (ii) the solution of a system of non-linear mass balance equations.

$$\Delta = \sum_{i=1}^{m} \left(A_i^{\text{theor}} - A_i^{\text{exper}}\right)^2, \tag{1}$$

where A_i^{theor} and A_i^{exper} are optical densities of the solution, calculated using the model, and those determined experimentally, respectively; m is the number of experimental titrations.

Subprogram (i) searches the global minimum of the discrepancy function by the Levenberg-Marquardt algorithm (Fausett 1999) using the standard MATLAB function '*lsqcurvefit*.' Subprogram (ii) solves the system of non-linear mass balance equations by the 'trust-region dogleg method' (Nocedal and Wright 1999) using the standard MATLAB function '*fsolve*.' Termination of the whole minimization scheme was considered successful when the magnitude of the discrepancy function (1) reached the values 10^{-5}…10^{-4} (corresponding to 0.0001…0.001 deviation between A_i^{theor} and A_i^{exper} per point).

Results and discussion

As discussed in the introductory section there is a problem in quantitation of the protector and interceptor mechanisms, which has not been solved so far for competition between drugs binding to polymeric DNA. Let us assume that both mechanisms are essential for quantitative analysis of experimental observations of drug–interceptor–DNA systems. The principal difficulty is the necessity to take into consideration the full set of interactions among the drug, interceptor and DNA, particularly the statistical distribution of the bound ligands over the DNA sequence. Models solving the three- (or more) component systems of competing drugs are known in the literature, although they all ignore some types of interactions; for example, the model in Wang et al. (1998) takes into account the full set of competitive complexations with a biopolymer, but ignores the hetero-association between the ligands (i.e., ignores the interceptor mechanism); the model in Pietrzak et al. (2006) ignores the competition for the binding sites, but treats indefinite hetero-association (i.e., ignores the protector mechanism); and, finally, the model in Evstigneev et al. (2008) takes into account both protector and interceptor mechanisms, but does it for DNA fragments, i.e., ignoring the statistical effects of ligand binding with polymeric DNA. In this work we developed the general model of competitive binding in drug–interceptor–DNA systems removing all these limitations.

Development of the general model of competitive binding of ligands with DNA

Let the drug be represented by X, the interceptor by Y and DNA by N. The general scheme of reactions between these components in the three-component mixture X-Y-DNA consists of three types of interactions, viz. indefinite self-association of X and Y molecules, indefinite hetero-association between them, and complexation of X and Y with DNA.

The complete and exact treatment of indefinite self-association and hetero-association in the two-component system X-Y has previously been developed using the partition function formalism (Evstigneev et al. 2011b). Following the set of notations in (Evstigneev et al. 2011b) the result is

$$A = \frac{x_1}{1 - K_X x_1}, \quad A_S = \frac{x_1^2}{1 - K_X^2 x_1^2},$$

$$B = \frac{y_1}{1 - K_Y y_1}, \quad B_S = \frac{y_1^2}{1 - K_Y^2 y_1^2},$$

$$Z = \frac{1}{2}\left(\frac{A + 2AK_hB + B}{1 - K_h^2 AB} + \frac{A\left(1 + K_h^2 B_S\right) + B\left(1 + K_h^2 A_S\right)}{1 - K_h^4 A_S B_S}\right), \tag{2}$$

where x_1, y_1 are the concentrations of the monomers of X and Y molecules, respectively; K_X, K_Y are equilibrium constants of self-association of X and Y, respectively, and K_h is the equilibrium hetero-association constant; Z is the grand partition function of the system.

The total concentrations of X and Y molecules in the two-component X-Y system (i.e., mass balance equations) can be determined directly from Z as

$$\begin{cases} x_{\text{het}} = \dfrac{\partial Z}{\partial \ln x_1} \\ y_{\text{het}} = \dfrac{\partial Z}{\partial \ln y_1} \end{cases}, \tag{3}$$

which further enables the experimentally observed parameter $\xi^{(\text{het})}$ (for instance, chemical shift in NMR, molar absorption in spectrophotometry, etc.) to be written for both molecules in solution (Evstigneev et al. 2011b):

$$\begin{cases} \xi_X^{(\text{het})} = \xi_X^{(m)} - 2\left(\xi_X^{(m)} - \xi_X^{(d)}\right)\dfrac{\partial Z}{\partial \ln K_X}\dfrac{1}{x_{\text{het}}} \\ \quad - \left(\xi_X^{(m)} - \xi_X^{(h)}\right)\dfrac{\partial Z}{\partial \ln K_h}\dfrac{1}{x_{\text{het}}} \\ \xi_Y^{(\text{het})} = \xi_Y^{(m)} - 2\left(\xi_Y^{(m)} - \xi_Y^{(d)}\right)\dfrac{\partial Z}{\partial \ln K_Y}\dfrac{1}{y_{\text{het}}} \\ \quad - \left(\xi_Y^{(m)} - \xi_Y^{(h)}\right)\dfrac{\partial Z}{\partial \ln K_h}\dfrac{1}{y_{\text{het}}} \end{cases}, \tag{4}$$

where $\xi^{(m)}$, $\xi^{(d)}$, $\xi^{(h)}$ are the observed parameters for the molecules in particular environments, viz. in the monomer form, in the homo-dimer and hetero-dimer, respectively.

Equations 3 and 4 provide a complete description of the two-component system taking into account the whole set of intermolecular interactions between the dissolved molecules and thereby enabling the most exact treatment of the interceptor mechanism. Further introduction of the polymeric DNA (and hence the protector mechanism) can be accomplished by using the standard approach of McGhee-von Hippel (McGhee and von Hippel 1974) assuming that the X and Y ligands bind with DNA as monomers in non-cooperative manner.

Consideration of the statistical effects of ligand distribution over the DNA lattice in the three-component system X-Y-DNA may be accomplished by using binding densities, θ_X, θ_Y, of each DNA site for two types of competing ligands, X and Y, respectively (McGhee and von Hippel 1974):

$$\begin{cases} \theta_X = K_{XN}x_1(1 - n_X\theta_X - n_Y\theta_Y) \\ \quad \times \left(\dfrac{1 - n_X\theta_X - n_Y\theta_Y}{1 - (n_X - 1)\theta_X - (n_Y - 1)\theta_Y}\right)^{n_X - 1} \\ \theta_Y = K_{YN}y_1(1 - n_X\theta_X - n_Y\theta_Y) \\ \quad \times \left(\dfrac{1 - n_X\theta_X - n_Y\theta_Y}{1 - (n_X - 1)\theta_X - (n_Y - 1)\theta_Y}\right)^{n_Y - 1} \end{cases}, \tag{5}$$

where n_X, n_Y are the numbers of DNA base pairs engaged by X and Y molecules, respectively (the binding site sizes); K_{XN}, K_{YN} are equilibrium binding constants of X and Y with DNA.

The system of mass balance equations for the three-component system can now be written by merging Eqs. 3 and 5 together, viz.

$$\begin{cases} x_0 = x_{\text{het}} + \theta_X N_0 = \dfrac{\partial Z}{\partial \ln x_1} + \theta_X N_0 \\ y_0 = y_{\text{het}} + \theta_Y N_0 = \dfrac{\partial Z}{\partial \ln y_1} + \theta_Y N_0 \end{cases}, \tag{6}$$

where N_0 is the total DNA concentration in base pairs.

The equations for the experimentally observed parameter for X and Y molecules can be obtained by analogy from Eq. 4 by adding the term $\xi_X^{(b)}\theta_X\frac{N_0}{x_0}$ or $\xi_Y^{(b)}\theta_Y\frac{N_0}{y_0}$ which represents the contribution of DNA bound ligands to the total observed signal ξ:

$$\begin{cases} \xi_X = \xi_X^{(m)} - 2\left(\xi_X^{(m)} - \xi_X^{(d)}\right)\dfrac{\partial Z}{\partial \ln K_X}\dfrac{1}{x_0} \\ \quad - \left(\xi_X^{(m)} - \xi_X^{(h)}\right)\dfrac{\partial Z}{\partial \ln K_h}\dfrac{1}{x_0} - \left(\xi_X^{(m)} - \xi_X^{(b)}\right)\theta_X\dfrac{N_0}{x_0} \\ \xi_Y = \xi_Y^{(m)} - 2\left(\xi_Y^{(m)} - \xi_Y^{(d)}\right)\dfrac{\partial Z}{\partial \ln K_Y}\dfrac{1}{y_0} \\ \quad - \left(\xi_Y^{(m)} - \xi_Y^{(h)}\right)\dfrac{\partial Z}{\partial \ln K_h}\dfrac{1}{y_0} - \left(\xi_Y^{(m)} - \xi_Y^{(b)}\right)\theta_Y\dfrac{N_0}{y_0} \end{cases}. \tag{7}$$

The systems of Eqs. 6 and 7 are the key relations of the general model of non-cooperative competitive binding in X-Y-DNA systems enabling the full analysis of the interceptor and protector mechanisms without any limitations introduced by the analogous models existing in the literature. By setting $K_{YN} = 0$ ($\theta_Y = 0$), Eqs. 6 and 7 can be transformed to the case when the protector mechanism is excluded, which was discussed in (Pietrzak et al. 2006, 2008; Piosik et al. 2010). Note that the final equations in the cited works are different from those obtained from Eqs. 6 and 7 because of non-strict treatment of the hetero-association in Weller's model used as a basis for development of the three-component model in Pietrzak et al. (2006) (for more discussion of the problem see Evstigneev et al. 2011b). Further introduction of a cooperativity of X and/or Y binding with DNA can be accomplished by substitution of Eq. 5 with corresponding equations, taking into account the cooperativity (see, for example, Nechipurenko 1984). It is also worth mentioning that the basic approach for derivation of Eqs. 6 and 7 is rather flexible as it allows the substitution of Z and θ_X (θ_Y) with any equations depending on a particular set of limitations introduced by an investigator to the basic reaction scheme in the X-Y-DNA mixture. We shall

consider one such particular case that, in practice, may find more applicability for the description of spectrophotometric data than the general model considered above.

The concentration range of the absorbing compound used in UV-Vis or spectrofluorimetry typically falls in the micro- or nanomole range, which precludes any self-association of such molecules. It is, therefore, a common practice in spectrophotometry to ignore the self-association of the absorbing compound (in the context of the present work the self-association of the drug X). On the other hand, the second non-absorbing compound Y may reach concentrations that allow indefinite self-association. This particular case matches the situation frequently met in the drug–interceptor–DNA system, i.e., the in vitro concentrations of the drug are typically small (μM or nM), whereas the concentrations of the interceptor may reach the mM range (as is, for example, the case for the methylxanthines (Traganos et al. 1991, 1993). In such a case the general model (6, 7) for analysis of the spectrophotometric data may be reduced to the '1:n model,' which takes into account only indefinite self-association of the non-absorbing compound Y, and only one monomer of compound X may be present in a complex with Y. There are only three types of complexes in such systems, viz. Y_i, XY_i and Y_iXY_j, which can be characterized by the partition function

$$Z^{(1:n)} = x_1 + B + K_h x_1 B + K_h^2 x_1 B^2. \tag{8}$$

The fourth term in Eq. 8 must be subjected to a correction for the 'reflected' complexes of type $Y_iXY_j \equiv Y_jXY_i$ (i.e., non-symmetrical with respect to the molecule X complexes that give twofold contributions to the partition function) in the form $\left(K_h^2 x_1 B^2 + K_h^2 x_1 B_S\right)/2$ (see for more discussion of the problem in Evstigneev et al. 2011b):

$$Z_{\text{corr}}^{(1:n)} = x_1 + \frac{y_1}{1 - K_Y y_1} + \frac{K_h x_1 y_1}{1 - K_Y y_1} + \frac{K_h^2 x_1 y_1^2}{(1 - K_Y y_1)^2 (1 + K_Y y_1)}. \tag{9}$$

Equation 9, when substituted into Eqs. 3 and 4, yields the mass balance equations:

$$\begin{cases} x_{\text{het}}^{(1:n)} = \dfrac{\partial Z_{\text{corr}}^{(1:n)}}{\partial \ln x_1} = x_1 \left(1 + \dfrac{K_h y_1}{1 - K_Y y_1} + \dfrac{K_h^2 y_1^2}{(1 - K_Y y_1)^2 (1 + K_Y y_1)}\right) \\ y_{\text{het}}^{(1:n)} = \dfrac{\partial Z_{\text{corr}}^{(1:n)}}{\partial \ln y_1} = \dfrac{y_1}{(1 - K_Y y_1)^2} \left(1 + K_h x_1 + \dfrac{K_h^2 x_1 y_1}{1 - K_Y y_1} + \dfrac{K_h^2 x_1 y_1}{(1 + K_Y y_1)^2}\right) \end{cases} \tag{10}$$

and the experimentally observed parameter for compound X (note that self-associates of X are absent in the 1:n model and thereby $\frac{\partial Z}{\partial \ln K_X} = 0$) in the two-component system X-Y:

$$\xi_X^{(\text{het})} = \xi_X^{(m)} - \left(\xi_X^{(m)} - \xi_{XY}^{(h)}\right) \frac{K_h y_1}{1 - K_Y y_1} \left(1 + \frac{2 K_h y_1}{1 - K_Y^2 y_1^2}\right) \frac{x_1}{x_{\text{het}}^{(1:n)}}. \tag{11}$$

Equations 10 and 11 can now be used to get the mass balance in Eq. 12 and the experimentally observed parameter (13) for the three-component system containing DNA according to Eqs. 6 and 7:

$$\begin{cases} x_0 = x_{\text{het}}^{(1:n)} + \theta_X N_0 = \dfrac{\partial Z_{\text{corr}}^{(1:n)}}{\partial \ln x_1} + \theta_X N_0 \\ y_0 = y_{\text{het}}^{(1:n)} + \theta_Y N_0 = \dfrac{\partial Z_{\text{corr}}^{(1:n)}}{\partial \ln y_1} + \theta_Y N_0 \end{cases} \tag{12}$$

$$\xi_X = \xi_X^{(m)} - \left(\xi_X^{(m)} - \xi_X^{(h)}\right) \frac{K_h x_1 y_1}{(1 - K_Y y_1) x_0} \left(1 + \frac{2 K_h y_1}{1 - K_Y^2 y_1^2}\right) - \left(\xi_X^{(m)} - \xi_X^{(b)}\right) \theta_X \frac{N_0}{x_0}. \tag{13}$$

Equations 12 and 13 form the basis of the 1:n model, which may find more specific application to describe spectrophotometric data in the drug–interceptor–DNA system. Equation 13, when multiplied by x_0, yields the optical density, A, of compound X measured in the experiment at a given wavelength. The quantities, $\xi_X^{(m)}$, $\xi_X^{(h)}$ and $\xi_X^{(b)}$, are replaced by extinction coefficients ε_m, ε_h and ε_b, for the molecule in monomer state, in a hetero-complex with Y and in complex with DNA, respectively. Both the general model (6) and (7) and the 1:n model (12) and (13) may be used to reconstruct the concentration dependences of optical density, A, in a spectrophotometric titration experiment by minimizing the discrepancy function (1) as described in the "Materials and methods" section.

As a final point in the development of the general model of competitive binding, it is necessary to link it to the literature models of the drug–interceptor–DNA system. As discussed in the introductory section, two main views exist concerning the description of the three-component systems with competing drugs, viz. in view (i) polymeric DNA is used but the complexation of Y with DNA is ignored (i.e., the protector mechanism), and in view (ii) both the protector and interceptor mechanisms are considered, but sequences of DNA fragments (e.g., tetramers) are used. The general model (6) and (7) developed in this work accounts in a strict way for the protector and interceptor mechanisms with respect to polymeric DNA, which means that this model incorporates as a partial case the models suggested in view (i) (for example, those used in Pietrzak et al. 2006, 2008; Piosik et al. 2010). It is not difficult to show that the 'tetramer' model used within view (ii) can

also be derived after some reduction of the equations of the general model. Assuming that polymeric DNA consists of non-overlapping sites of equal length (4 nucleotides) for the drug and the interceptor, it is necessary to put $n_X = 1$, $n_Y = 1$ in Eq. 5, which yields the standard linear Scatchard equations (Zimmermann 1986):

$$\begin{cases} \theta_X = \dfrac{K_{XN}x_1}{1 + K_{XN}x_1 + K_{YN}y_1} = \dfrac{K_{XN}x_1N_1^{\text{tetr}}}{N_0^{\text{tetr}}} \\ \theta_Y = \dfrac{K_{YN}y_1}{1 + K_{XN}x_1 + K_{YN}y_1} = \dfrac{K_{YN}y_1N_1^{\text{tetr}}}{N_0^{\text{tetr}}} \end{cases}, \quad (14)$$

where N_1^{tetr} is the concentration of ligand-free tetramer fragments. When Eq. 14 is substituted into Eqs. 6 and 7, the resultant relations will contain the DNA-related terms that are identical to those appearing in models within view (ii), for example, those used in (Evstigneev 2010; Evstigneev et al. 2005, 2006a, b; Hernandez Santiago et al. 2009). Note that the terms relating to hetero-association in these references are different from those appearing in Eqs. 6 and 7, which is due to utilization of a less general description of hetero-association in previous work compared to the present work. For more discussion of the problem, see Evstigneev et al. 2011b). Hence, the 'tetramer' model is equivalent to a simple linear Scatchard approach, where the concentrations must be scaled by a factor of 4, i.e., $N_0^{\text{tetr}} = N_0/4$, in order to match the concentrations expressed in base pairs in the general model and the concentrations expressed in tetramer fragments in the 'tetramer' model.

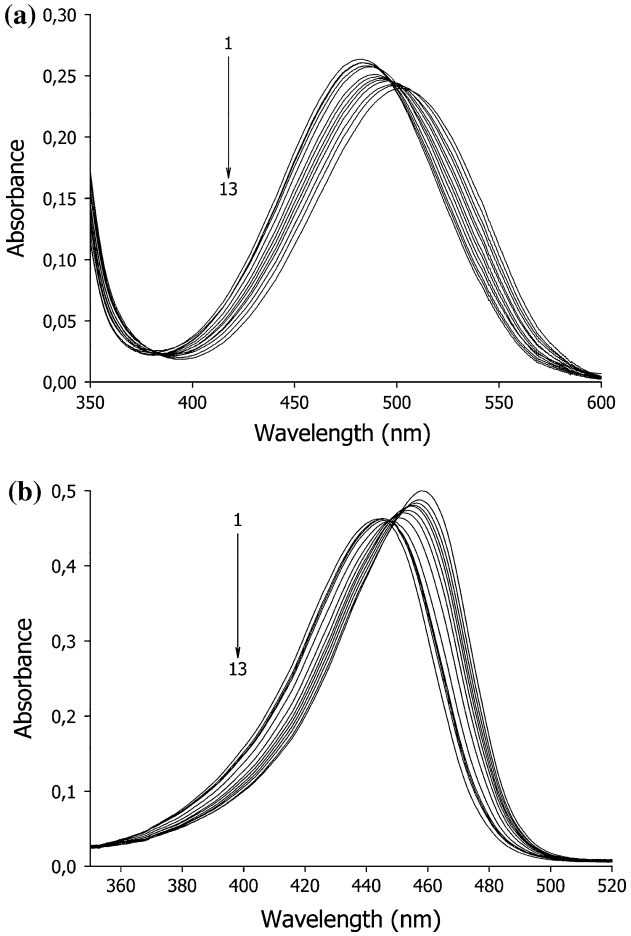

Fig. 1 Absorption spectra of EB-CAF-DNA (**a**) and PF-CAF-DNA (**b**) solutions. The concentration of caffeine: 0 (*1*), 0.45 (*2*), 0.75 (*3*), 1.5 (*4*), 3 (*5*), 4.5 (*6*), 6 (*7*), 7.5 (*8*), 9 (*9*), 12 (*10*), 15 (*11*), 21 (*12*), 30 (*13*) mM

Test of the appropriateness of the general model for competitive binding

In order to test whether the general model developed above is appropriate for the analysis of drug–interceptor–DNA systems, it needs to be verified with experimental data in three-component systems. In the present work the model is tested against the UV-Vis data for two systems, EB-CAF-DNA (Fig. 1a) and PF-CAF-DNA (Fig. 1b), where the two mutagenic dyes, ethidium bromide and proflavine, act as the drug and caffeine acts as the interceptor molecule. Both EB and PF have been well characterized in the past by various experimental techniques in terms of their binding with polymeric DNA and their hetero-association with CAF (for a review, see Evstigneev 2010). The in vitro biological effect of EB is also known to be altered on addition of caffeine via the interceptor/protector mechanisms (Johnson et al. 2003). The experimental conditions were selected to be similar to those expected for drug–interceptor–DNA systems in vitro (Evstigneev 2010; Traganos et al. 1991, 1993), viz. variation of the concentration of caffeine over a wide range from μM up to mM at constant (μM or lower) concentrations of the drug and DNA (the changes are monitored by measurements of the drug's spectrum in the visible range not overlapped by DNA and CAF).

Such a test was previously used to check the appropriateness of the 'tetramer' model for NMR data of various drug–interceptor–DNA systems (Evstigneev et al. 2005, 2006a) and is based on the fact that the general model (6) and (7) of the three-component drug–interceptor–DNA system contains only the parameters of interactions (equilibrium constants and observed ξ in particular complexes), which may be obtained from preliminary experiments in one-component (i.e., self-association of the drug and interceptor molecules) and two-component (i.e., drug–interceptor hetero-association, and drug-DNA and interceptor-DNA complexations) systems. It means that if the model is correct and no non-linear effects of interactions are expected in the three-component system, the calculated dependence of ξ_X on the concentration of Y (i.e., caffeine)

from Eq. 7 must be identical to the experimental dependence of ξ_X on y_0 (Fig. 2), allowing minimal variation of the input parameters within the error of their determination. The full set of parameters corresponding to the one- and two-component interactions in EB-CAF-DNA and PF-CAF-DNA systems (Baranovsky et al. 2008, 2009; Table 1), and obtained using similar solution conditions and the method of data analysis, is available in the literature, except for the PF-CAF system. The latter was measured and analyzed in the present work following the common procedure used for analysis of the EB-CAF system in Baranovsky et al. (2009; the calculated parameters of PF-CAF hetero-association are presented in Table 1).

In order to perform the approximation of the experimental concentration dependences of molar absorption of the drugs (see Fig. 2a, b), we used two strategies, viz. computation of the discrepancy function (1) when (i) all input parameters are fixed, and (ii) when the parameters

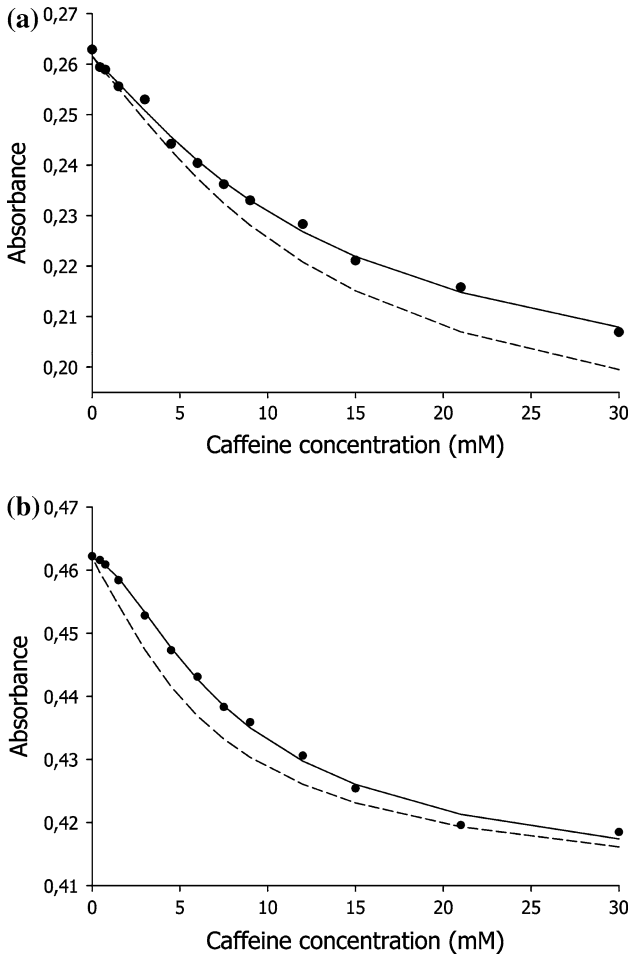

Fig. 2 Dependence on the caffeine concentration of the optical density of **a** ethidium bromide in the EB-CAF-DNA system at $\lambda = 480$ nm and **b** proflavine in the PF-CAF-DNA system at $\lambda = 444$ nm. *Solid* and *dashed curves* represent the model including and excluding the protector mechanism, respectively

responsible for hetero-association of the drugs (K_h, ε_h) and the drug-DNA binding (K_{XN}, ε_b), are varied (all other parameters remain fixed). We used both the general model (Eqs. 6 and 7) and the 1:n model (Eqs. 12 and 13), which yielded nearly the same results of the approximation outlined in Table 1. We also performed the same set of computations using the Scatchard equations (14) substituted into Eqs. 6, 7 and 12, 13 instead of θ_X (θ_Y) with the total DNA concentration N_0 divided by the factor 4, thereby mimicking the 'tetramer' model discussed above. In addition to the computational protocol formulated above, variation of the coefficient 4 over the range 3…5 was adopted with all other parameters fixed, in order to confirm that the choice of the DNA concentration, $N_0/4$, in the Scatchard model results in the best approximation of the experimental data.

The most important result summarized in Table 1 is that, when all input parameters are fixed, the value of the discrepancy Δ is in the range 10^{-4}…10^{-5}, which means that the general model gives a satisfactory approximation to the experimental data (see the quality of approximation by the solid line in Fig. 2), suggesting that this model is appropriate for the description of drug–interceptor–DNA systems. This conclusion is confirmed by the fact that variation of the parameters with an unrestrained minimum search leads to insignificant change in the magnitudes of these parameters within the typical 10–20% error of their determination. Another important feature of the data in Table 1 is that utilization of the Scatchard approach in both strategies (with fixed and varied parameters) gives similar sets of discrepancies and parameters as in the general model with the strict McGhee-von Hippel treatment of ligand binding. Hence, for simple DNA intercalators without branched side chains, such as EB and PF, the use of the Scatchard approach and the 'tetramer' model (see discussion above) does not lead to significant error in description of the dynamic equilibrium in drug–interceptor–DNA systems. It is important, however, to stress that in the general case the use of the 'tetramer' model must always be checked for appropriateness for each particular system under investigation, as it does not take into account any specific features of the drug and the interceptor binding with DNA.

Based on the conclusion that the general model is appropriate for the description of drug–interceptor–DNA systems and incorporates as partial cases models of the three-component systems known from the literature, it is now possible to address the question of whether the protector mechanism is important. Setting $K_{YN} = 0$ in Eqs. 6 and 7 (i.e., switching off the interceptor-DNA binding and thereby excluding the protector mechanism) and performing the approximation of the experimental data with all parameters fixed according to strategy (i), one gets the

Table 1 Equilibrium parameters used in computations for EB-CAF-DNA and PF-CAF-DNA systems

	General model		Scatchard model	
	Fixed	Varied	Fixed	Varied
EB-CAF-DNA				
n_X	3.60	–	3.60	–
ε_b (M^- cm^{-1})	2,500	2,081	2,500	2,099
ε_h (M^{-1} cm^{-1})	4,665	4,591	4,665	4,592
K_{XN} (M^{-1})	232,000	232,000	232,000	232,001
K_h (M^{-1})	96.5	81.4	96.5	74.8
Δ	3.42×10^{-5}	1.29×10^{-5}	4.99×10^{-5}	1.35×10^{-5}
PF-CAF-DNA				
n_X	2.44	–	2.44	–
ε_b (M^{-1} cm^{-1})	27,300	28,942	27,300	21,703
ε_h (M^{-1} cm^{-1})	34,689	34,403	34,689	34,413
K_{XN} (M^{-1})	41,000	40,825	41,000	42,141
K_h (M^{-1})	192.4	183.9	192.4	177.6
Δ	1.66×10^{-4}	7.03×10^{-6}	4.85×10^{-4}	1.74×10^{-5}

lowest discrepancy corresponding to the dashed curve in Fig. 2. It is seen that the curve apparently goes lower than the experimental curve, which corresponds to a significant underestimation of the dynamic equilibrium in the three-component systems studied. Allowing the parameters of the binding to vary according to strategy (ii), there is a much better approximation of the experimental curve, although in this case the resultant values of the varied parameters deviate from their initial values, obtained from independent one- and two-component studies, much more than the error of their determination (data not shown). Although indirect, this result provides the first experimental evidence of the importance of the protector mechanism in analyzing drug–interceptor–DNA systems in the spectrophotometric concentration range. The development of the general model of competitive binding in drug–interceptor–DNA systems and the evidence of the importance of the protector mechanism provides an answer to Problem 1 outlined in the introductory section.

Method for quantitative analysis of competitive binding in drug–interceptor–DNA systems

The method for quantitation of the biological effect in drug–interceptor–DNA systems has only previously been developed for fragments of DNA sequences (i.e., Problem 2). We shall now adapt this method to polymeric DNA within the framework of the general model.

It was suggested (Evstigneev 2010; Evstigneev et al. 2005, 2006a, 2008) that the 'protector' and 'interceptor' mechanisms could be quantified using the criterion, R_D, which is the relative decrease in proportion of *X*-DNA complexes on addition of the ligand *Y* as summarized in Eq. 15. R_D is calculated for two limiting situations:

(i) the condition of 'switched off' *X*-*Y* hetero-association and 'switched on' complexation of *Y* with DNA ($K_h = 0$, $K_{YN} \neq 0$), *i.e.* $f^X_{C(C)}$, and

(ii) the condition of 'switched on' *X*-*Y* hetero-association and 'switched off' complexation of *Y* with DNA ($K_h \neq 0$, $K_{YN} = 0$), i.e., $f^X_{C(h)}$:

$$R_D = \frac{f^X_{C(0)} - f^X_{C(C)}}{f^X_{C(0)} - f^X_{C(h)}}, \tag{15}$$

where $f^X_{C(0)}$ is the mole fraction of *X*-DNA complexes with 'switched off' hetero-association and *Y*-DNA complexation. The range of $R_D > 1$ corresponds to the predominance of *Y*-DNA complexation over *X*-*Y* hetero-association (i.e., the 'protector action' of ligand *Y*), and $R_D < 1$ corresponds to hetero-association being the major contribution to the displacement of drug molecules from DNA (i.e., the 'interceptor action' of ligand *Y*).

An estimate of the amount of drug *X* displaced from DNA due to the presence of *Y* ligand can be made using the quantity, A_D, which corresponds to the relative amount of *X* molecules removed from DNA on addition of *Y*,

$$A_D = \frac{f^X_{C(0)} - f^X_{C(hC)}}{f^X_{C(0)}}, \tag{16}$$

where $f^X_{C(hC)}$ is the relative amount of drug *X* bound to DNA in the presence of *Y* when both mechanisms are 'switched on', i.e., $K_h \neq 0$, $K_{YN} \neq 0$.

If drug *X* exerts its biological action mainly via complexation with DNA, then the quantity A_D may be used to

indicate the biological effect of drug X in the three-component system, X-Y-DNA, commonly expressed in percents.

The computation of the f_C^X factors in Eqs. 15 and 16 could be performed from knowledge of the amount of drug X bound to DNA, x_C, computed with the corresponding set of the 'switched on' and 'switched off' equilibrium constants, i.e.,

$$f_C^X = \frac{x_C}{x_0}, \tag{17}$$

Following the standard definition of the binding density, θ, in Eq. 5 as the number of moles of the ligand bound to DNA per mole of DNA base pairs, $\theta_X = \frac{x_C}{N_0}$ (McGhee and von Hippel 1974), Eq. 17 can now be modified in terms of the definitions of the general model:

$$f_C^X = \theta_X \frac{N_0}{x_0}. \tag{18}$$

It follows from Eq. 18 that the modified equations for the computation of the R_D, A_D factors within the framework of the general model take the form

$$R_D = \frac{\theta_X^{(0)} - \theta_X^{(C)}}{\theta_X^{(0)} - \theta_X^{(h)}}, \quad A_D = \frac{\theta_X^{(0)} - \theta_X^{(hC)}}{\theta_X^{(0)}}, \tag{19}$$

where θ_X comes from the solution of the system of Eq. 6 [or Eq. 12 in the 1:n model] with the corresponding set of the 'switched on' and 'switched off' equilibrium constants.

Note that the computation of the R_D, A_D factors and further application of the general model to biological systems does not require the equation for the observed experimental parameter (Eqs. 7 and 13), because the target function now is the A_D factor, which can be measured in biological experiments as a function of a change in biological effect of the drug X (percent of apoptosis, mutagenicity, etc.) on concentration of the interceptor molecule.

Parameterization of the general model

In order to make the general model applicable for analysis of the competitive binding under in vitro conditions in biological systems, it is necessary to select appropriate concentrations of the components x_0, y_0, N_0, applying the common assumption in such analyses that the equilibrium binding constants appearing in Eqs. 6 and 12 and measured in independent physico-chemical experiments are the same when in biological systems. The set of such concentrations was previously termed quasi-physiological (Evstigneev 2010; Evstigneev et al. 2008, 2011a):

$$x_0 = 10\,\mu\text{M}, \quad y_0 - \text{var}, \quad N_0^{\text{tetr}} = 10\,\mu\text{M}. \tag{20}$$

It is important to note that the concentrations (20) were previously obtained from in vitro data on the reduction in toxicity of the antitumour antibiotics Doxorubicin (DOX) and Novatrone (NOV) in human leukemia cell lines on addition of caffeine using the 'tetramer' model (Evstigneev 2010; Evstigneev et al. 2006a, 2008) and recently confirmed in quantification of the apoptosis-concentration curve for the anti-cancer agent Topotecan (Evstigneev et al. 2011a). As shown above, the 'tetramer' model is a particular case of the general model, and for simple aromatic drugs no significant error in quantification of the drug–interceptor–DNA system is expected when switching between the general and the 'tetramer' models. It follows that applying the scaling factor of 4 to the DNA concentration $N_0^{\text{tetr}} = N_0/4$ the set of quasi-physiological conditions for polymeric DNA may be summarized as

$$x_0 = 10\,\mu\text{M}, \quad y_0 - \text{var}, \quad N_0 = 40\,\mu\text{M}, \tag{21}$$

where N_0 is expressed in units of base pairs (or phosphates).

It should be noted that in previous analyses (Evstigneev 2010; Evstigneev et al. 2006a, 2008) derivation of the conditions (20) was performed using binding constants of drugs to DNA tetramers derived from NMR measurements. Drug-oligonucleotide equilibrium binding constants are commonly different from the microscopic binding constants to polymeric DNA obtained using the McGhee-von Hippel or Scatchard treatment, which are supposed to be used in the general model. Hence, it is necessary to re-derive the quasi-physiological conditions (20) using the microscopic drug-DNA binding constants to polymeric DNA (see Table 1) within the framework of the general model. In order to accomplish this we shall follow the protocol used by Evstigneev et al. (2005, 2006a, 2008) for obtaining Eq. 20. At 5 mM concentration of caffeine added into the cell line, the change in percent of apoptotic cells normalized to the intrinsic biological activity of the same concentration of DOX in the same cell line amounts to $A_D^{\text{DOX}} = 39\%$ and $A_D^{\text{NOV}} = 50\%$ for antibiotic NOV (Evstigneev et al. 2008). Prediction of the exact magnitudes of the A_D factor measured in the biological experiment using a purely physico-chemical model is considered to be impossible within the current approach, although a value of the A_D factor of ca. 50% enables adjustment of the order of magnitude of the quasi-physiological concentrations (i.e., parameterize the general model) to be further used in prediction of a *relative change* in A_D for various drug–interceptor–DNA systems (see Evstigneev 2010; Evstigneev et al. 2008 for further discussion of the R_D, A_D factors). Substitution of the concentrations (21) into Eqs. 6 and 19 yields the calculated value of the A_D factor close to 50%, which confirms the appropriateness of conditions (21) for polymeric DNA and the general model of drug–interceptor–DNA system with the microscopic binding parameters as the input data (i.e., binding constants and

binding site sizes). The independent variation of x_0 within the range 1…20 μM and N_0 within the range 20…60 μM using the Monte-Carlo approach (1,000 random generations of x_0/N_0 pairs) resulted in less than 10% change in the A_D factor, which corresponds to the typical error of the in vitro biological experiment. It means that the error in using the quasi-physiological conditions (21) for description of the in vitro data of aromatic drugs in the general model should not be significant. This conclusion is in accord with similar results obtained by Evstigneev et al. (2005, 2006a, 2008, 2011a) within the framework of the 'tetramer' model and provides the solution to Problem 2 outlined in the introductory section.

Using Eqs. 6 and 19 of the general model and the input parameters from Eq. 21 and Table 2, computation of the R_D, A_D factors for various drug–caffeine–DNA systems has been accomplished (Fig. 3). As seen from the figure, the pattern of the calculated dependences is similar to that obtained in (Evstigneev et al. 2006a, 2008; see also the interpretation of the R_D, A_D dependences on y_0 in these references). The sequence of A_D curves PF → NOV → EB → DOX → DAU, representing the sensitivity of the given drug to the addition of caffeine, is also similar to that reported by Evstigneev et al. (2008).

Conclusions

The principal outcome of this work is the development of the general model of competitive binding in drug–interceptor–DNA systems with full parameterization of the basic equations, incorporating as partial cases similar models developed by various research groups in the past. The generality of this model is due to strict accounting of the statistical effects of the drug and interceptor binding with DNA according to the McGhee-von Hippel formalism (cooperative or non-cooperative), and to the strict treatment of hetero-association between the drug and interceptor, which includes the formation of all possible types of self- and hetero-complexes in solution. It follows that the general model accounts for the two basic molecular processes governing the mutual binding of competing drugs with DNA, viz. the protector and interceptor mechanisms. Another important feature of the general model is the utilization as input quantities of the microscopic binding parameters (equilibrium binding constants and binding site sizes) derived from independent one- and two-component physico-chemical experiments, such as the self-association of the drug and interceptor, their hetero-association and their complexation with DNA separately. The general model may be used to analyze three-component competitive binding systems independent of the experimental method used. A link to the biological in vitro experiment was also established within the framework of the developed model.

Another important outcome of this work is the indirect experimental evidence for the importance of the protector

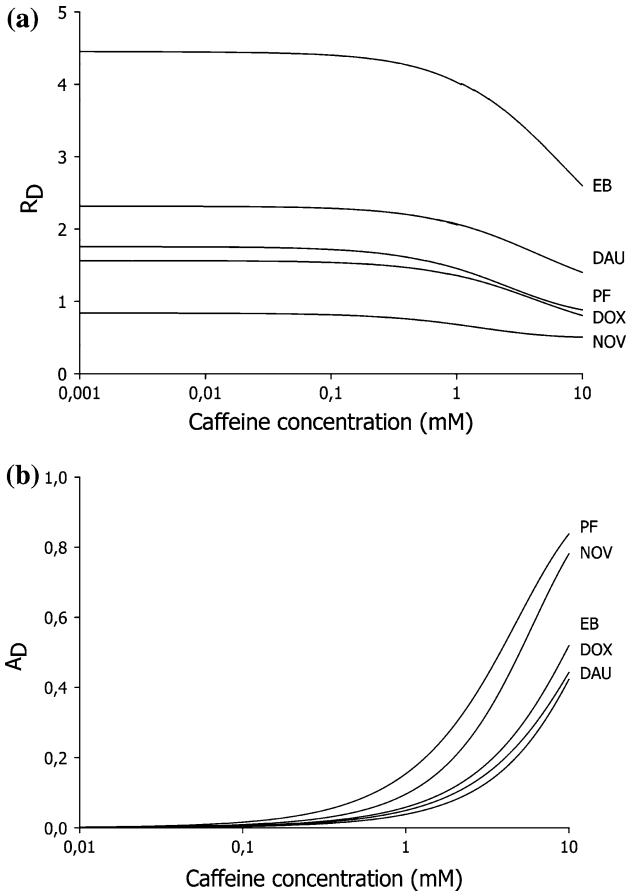

Fig. 3 **a** R_D and **b** A_D factors as a function of caffeine concentration under quasi-physiological concentrations (21)

Table 2 Equilibrium parameters of complexation of aromatic ligands with caffeine and DNA

Drug	K_h (M^{-1})	$K_{XN} \times 10^5$ (M^{-1})	n_X
PF	192.4[a]	0.41[b]	2.4[b]
NOV	356.4[c]	2.52[d]	2.6[d]
EB	96.5[e]	2.32[e]	3.6[e]
DOX	128.3[c]	2.90[f]	1.8[f]
DAU	148.5[g]	6.70[h]	3.3[h]

[a] This work

[b] Baranovsky et al. (2008)

[c] Piosik et al. (2002)

[d] Kapuscinski and Darzynkiewicz (1985)

[e] Baranovsky et al. (2009)

[f] Pietrzak et al. (2006)

[g] Piosik et al. (2005)

[h] Chaires (1990)

mechanism in quantification of competitive binding in drug–caffeine–DNA systems, which sometimes is ignored in the literature based on the small magnitude of the CAF-DNA binding constant. Direct quantification of the separate interceptor and protector effects in three component systems was not possible in the present work and is a matter for further investigation.

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