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# Quantitation of the molecular mechanisms of biological synergism in a mixture of DNA-acting aromatic drugs

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**Abstract**

It is suggested that the widely reported biological synergism of a mixture of DNA-targeting aromatic drug molecules both *in vivo* and *in vitro* can be explained, in part, at the molecular level by competition between two basic mechanisms: the 'interceptor' (hetero-association between Drug1 and Drug2) and 'protector' mechanisms (complexation of Drug1 and Drug2 on DNA-binding sites). In the present work a complete analytical methodology has been developed to quantify these processes, providing an estimate of the relative importance of the interceptor/protector mechanisms using just a set of equilibrium association constants. The general methodology may be applied to other molecules with receptors for aromatic drugs.


*Keywords:* Aromatic drugs; Competitive binding; Hetero-association; Interceptor; Protector

## 1. Introduction

Aromatic molecules have been widely utilized in medicine as components of therapeutic drugs effective against various diseases. A good example is a group of aromatic molecules that are currently used as antitumour agents: doxorubicin (DOX), daunomycin (DAU), actinomycin D (AMD), mitoxantrone (NOV), topotecan (TPT), amsacrine (AMSA) [1,2]. It is generally accepted that these drugs affect nucleic acid function in cells, most likely by intercalation into double-stranded DNA and inhibition of DNA synthesis [3–7]. Much effort has been made to elucidate the structure, affinity and specificity of aromatic antibiotics towards DNA molecules, which has led to a number of more potent synthetic drugs successfully introduced into clinical practice [8–10]. Another way to improve the therapeutic index of aromatic drugs is to use them in combination. For example, the combination of DOX+AMD is highly effective against various types of sarcomas [11,12], DOX+NOV is mainly used against breast cancer [13], and DOX+AMSA, NOV+AMSA, NOV+TPT, DAU+TPT are effective against leukaemia [14–17]. In addition, it has also been well-documented that simultaneous administration of these drugs with aromatic molecules from food sources causes significant reduction in total toxicity of the drug: caffeine (CAF) [18–23], chlorophylline (CHL) [24–26] or vitamin $B_2$ (Riboflavin, RBF) [27,28]. There is also much evidence of the antimutagenic potency of CAF, CHL and RBF towards the action of typical aromatic carcinogens and mutagens, which are known to intercalate into DNA: ethidium bromide (EB) [29], benzopyrene [30,31], aflatoxin $B_1$ [30,32,33], quinacrine derivatives [30,34–36]. Although the structural and biological properties of the drugs and mutagens appear to be very different, they all possess at least three similar features: (i) the presence of a planar aromatic chromophore, (ii) the ability to interact with DNA or protein, and (iii) a biological synergism when used with other aromatic drugs.

Currently there is no general understanding of the underlying mechanisms responsible for the observed synergistic effects of aromatic drugs. In each case the mechanism of action is

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developed within the present knowledge of the specific biochemical properties of the particular drugs at the molecular, enzymatic or cellular level. On the other hand, there are many publications on the action of 'food' molecules such as CAF, CHL and RBF on the efficacy of aromatic drugs. It has been demonstrated by various groups that CAF [20,23,35–44], CHL [45–47] and RBF [31,48–51] form non-covalent stacked complexes with aromatic drugs, which lowers the concentration of active monomer species in solution able to interact with a bio-receptor and the trigger biological effect, or may cause chemical destruction of the drug; this 'interceptor' effect is based on the hetero-association of aromatic molecules in aqueous solution (Fig. 1a). In other cases the toxicity or mutagenicity of the aromatic drug is reduced by the action of aromatic 'food' molecules and this has been termed the 'protective' effect [24,38]. Hetero-association is also typical for the interaction of nucleotide derivatives with each other [52,53], aromatic antibiotics with other antibiotics [54,55] and aromatic antibiotics with mutagens [56–58]. It may be concluded that hetero-association (as well as self-association) is a general property for aromatic molecules and the interceptor mechanism may contribute at the molecular level to the biological synergism of any combination of aromatic drugs. However, the suggestion [19–21,23,35–40] that hetero-association is the only mechanism for the protective effect in Drug–Interceptor–Receptor systems is primary based on the assumption that the interceptor molecule does not (or very weakly) interact with the bio-receptor. On the other hand, it has been shown that both CAF [43,59–61] and RBF [50] molecules are able to bind with DNA in a fashion very similar to intercalation or by external binding. A quantitative estimate of the DNA complexation constants gives the same order of magnitude as for the hetero-association constants with various drugs ($\sim 10^2 \ldots 10^3$ $M^{-1}$ for CAF [43] and $\sim 10^3 \ldots 10^4$ $M^{-1}$ for RBF [50]). If the affinities of CAF and RBF to both the drug and DNA molecule are similar, it follows that aromatic molecules are able to 'block' free sites on DNA (via complexation) as well as to 'intercept' free drug species in solution (via hetero-association) with a comparable ability. This process displaces the drug molecules already bound to DNA which results in similar effect as the interceptor mechanism. The complexation of aromatic 'food' molecule with DNA is independent of hetero-association and may be considered as a 'protector' mechanism (or as a competition of drug molecules for receptor binding sites) [43,44,50] (Fig. 1b). It means that the experimentally-observed displacement by 'food' molecule of drug bound to DNA [33,39–41,62,63] reflects the cumulative action of two mechanisms, interceptor and protector. Moreover, in the case

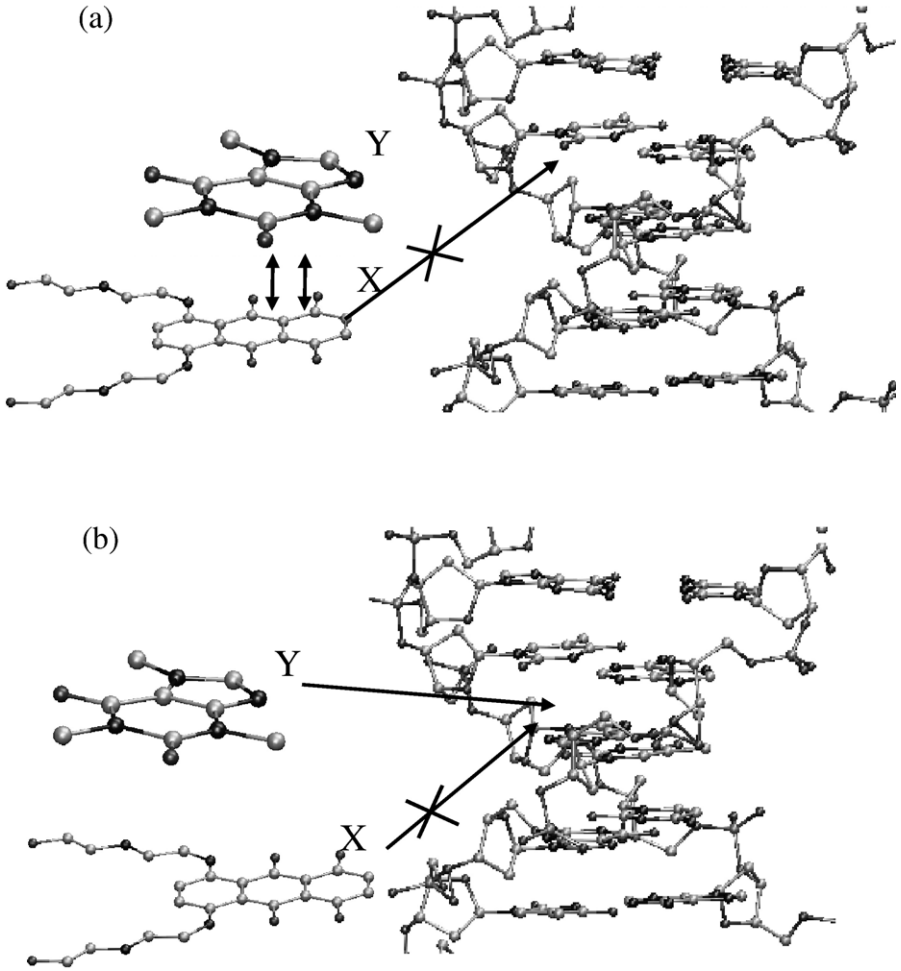


Fig. 1. Schematic representation of interceptor (a) and protector (b) mechanisms.

of two aromatic drugs complexing with DNA with comparable affinities both the 'interceptor' and 'protector' mechanisms should be taken into account.

An initial attempt to treat the interceptor and protector action simultaneously has been made [43,57], though that analysis was basically qualitative, because it considered a physiologically-unreasonable concentration range and did not allow any reliable separation of the mechanisms. A method has been suggested to analyse the 'interceptor' and 'protector' mechanisms simultaneously in recent reports on the CAF–Antibiotic [44,64] and Vitamin–Antibiotic [50] systems. In the present work that analysis is generalised for the group of aromatic molecules, which exert their biological effect via complexation with DNA. The method assumes that both the 'interceptor' and 'protector' mechanisms are operative in any given combination of aromatic drugs and therefore may contribute to the observed synergistic effects at the molecular level. The analysis is made for three groups of aromatic drug combinations which complex with DNA, *viz*. Caffeine–Drug, Vitamin–Drug and Drug–Drug, which (as discussed above) are known to exert pronounced synergism *in vitro* or *in vivo*. All the necessary equilibrium parameters have been obtained previously by NMR under similar experimental conditions (buffered aqueous solution, pD 7.0÷7.4, $I \approx 0.1$ M and normalised to $T = 298$ K). Although the analysis is restricted to DNA-complexing agents, the same methodology may be useful for other receptors (such as enzymes) targeted by aromatic ligands.

## 2. Results and discussion

### 2.1. General model of the competitive binding assay in the X–Y-DNA system

Let the $X$ ligand be the main drug exerting its biological effect via complexation with DNA and the $Y$ ligand be the 'interceptor/protector' molecule, which is able to bind non-covalently with both DNA and the $X$ ligand. The hetero-association $X$–$Y$ lowers the concentration of free molecules of $X$ in solution and so the biological response of drug $X$ changes on addition of $Y$. In line with previous work [19–21,38] this hetero-association process is termed the 'interceptor' mechanism. In addition, $Y$ may complex with DNA, block the potential binding sites available for $X$ molecules resulting in lowering of the fraction of $X$–DNA complexes in the overall dynamic equilibrium in solution and cause a change in the biological response of drug $X$; this process is termed the 'protector' mechanism [43,57]. As all possible two-component interactions ($X$–$Y$, $X$–DNA, $Y$–DNA) in the three-component system $X$–$Y$–DNA are being considered, it is also necessary to include one-component interactions in the analysis, *viz*. the self-association of $X$ and $Y$. Such a generalised model does not rely on any limitations in the concentrations of the components and any approximating assumptions in the analysis of any type of drug–drug combinations.

It is necessary to consider the DNA receptor to be used in analysis. One might expect to obtain equilibrium binding parameters of drugs with nucleic acids using polymeric DNA sequences. There are at least two reasons that make this model of a DNA receptor unsuitable for general analysis of competitive binding assay in terms of the basic mechanisms involved. First, there is a very large number of possible binding sites, which requires consideration of the statistical distribution of the ligand making the scheme of analysis very complex but not adding anything to the analysis of the basic mechanisms. The only way to approach such an analysis was to neglect certain interactions or to perform it qualitatively [40,41,47,65]. Second, the DNA receptor in the cell is complexed with histone, which cover at least 50% of the nuclear DNA [66,67]. It follows that only short regions of uncomplexed cellular DNA are actually accessible for ligand binding, hence an oligomeric DNA sequence is physiologically more relevant to study competitive binding process. The present analysis is focused on aromatic drug molecules which follow the 'neighbour exclusion principle' on binding with DNA [68]. If one considers a self-complementary tetrameric DNA sequence as a model DNA receptor, it is expected that just one ligand can be accommodated in it at one time, and so competition between two ligands $X$ and $Y$ can be tracked in its 'pure' form with no influence of second or higher binding processes. Analysis of the literature suggests that the complete set of equilibrium binding constants of different aromatic drugs is only available for the d$(TGCA)_2$ tetranucleotide, ($N$), and so it is used as a model DNA sequence to study the competitive binding of aromatic molecules $X$ and $Y$.

The model can be expressed in Eq. (1) in terms of the dynamic equilibrium between all the complexation reactions in the three-component mixture $X$–$Y$–DNA, taking into account the probability of formation of indefinite aggregates for both self-association and hetero-association reactions:

$$\begin{array}{l}\text{Self}-\text{association}\\ X_1 + X_i \overset{K_X}{\rightleftarrows} X_{i+1}\ (\text{a}) \quad Y_1 + Y_j \overset{K_Y}{\rightleftarrows} Y_{j+1}\ (\text{b})\\ \text{Hetero}-\text{association}\\ X_i + Y_j \overset{K_h}{\rightleftarrows} X_iY_j\ (\text{c}) \quad X_iY_j + X_k \overset{K_h}{\rightleftarrows} X_iY_jX_k\ (\text{d})\\ Y_l + X_iY_j \overset{K_h}{\rightleftarrows} Y_lX_iY_j\ (\text{e})\\ \text{DNA complexation}\\ X + N \overset{K_{XN}}{\rightleftarrows} XN\ (\text{f}) \quad Y + N \overset{K_{YN}}{\rightleftarrows} YN\ (\text{g})\end{array} \tag{1}$$

where $K_X$, $K_Y$ are the self-association constants of $X$ and $Y$, respectively; $K_h$ is the $X$–$Y$ hetero-association constant; $K_{XN}$, $K_{YN}$ are complexation constants of $X$ and $Y$ with the duplex form of the DNA tetramer, respectively. A mathematical description has been made for self-association [69,70], hetero-association [56,71] and Drug-d(TGCA) complexation [72] and summation of these terms gives the system of Eq. (2) for the mass conservation law:

$$\begin{cases} \dfrac{X}{(1-K_XX)^2}\left(1+\dfrac{K_hY}{1-K_YY}+\dfrac{K_h^2Y^2}{2(1-K_YY)^2}\right)+K_{XN}XN = X_0\\ \dfrac{Y}{(1-K_YY)^2}\left(1+\dfrac{K_hX}{1-K_XX}+\dfrac{K_h^2XY}{(1-K_YY)(1-K_XX)}\right)+K_{YN}YN = Y_0\\ N(1+K_{XN}X+K_{YN}Y) = N_0 \end{cases} \tag{2}$$

where $X$, $Y$, $N$ are the monomer (non-complexed) concentrations of $X$, $Y$ and the tetramer, respectively; $X_0$, $Y_0$ are the total concentrations of $X$ and $Y$, respectively; $N_0$ is the total concentration of tetrameric DNA sequences free from histones. Solution of Eq. (2) with respect to $X$, $Y$, $N$ enables the mole fraction of each type of complex to be calculated. Test NMR investigations carried out on the DAU–CAF–TGCA [44] and RBF–DAU–TGCA [50] systems confirmed the physical appropriateness of such an approach to study three-component mixtures. (2) can now be generalised for any combinations of aromatic drugs in the presence of the DNA oligomer.

In order to solve the system of Eq. (2), knowledge of the equilibrium constants of self-association, hetero-association and DNA complexation constants is required. The self-association, hetero-association and complexation constants of a number aromatic drugs with $d(TGCA)_2$ have been studied previously under similar solution conditions (buffered aqueous solution, pD 7.0÷7.4, Ionic strength, $I \approx 0.1$ M) and the equilibrium constants are given in Table 1 at $T=298$ K. So, in principle, a complete quantification of the three-component mixture $X$–$Y$–DNA is possible.

### 2.2. Method for the quantitative estimation of the 'protector' and 'interceptor' mechanisms

As both the $X$–$Y$ hetero-association and the $Y$–DNA complexation processes occur in the same system of $X$–$Y$–DNA components, then the 'interceptor' and 'protector' mechanisms co-exist in the mixed solution. It was thought that the simplest way to distinguish between these two processes would be to calculate the fraction of $X$ in the hetero-complexes ($f_h$) and the complexes of $X$ with DNA ($f_{C2}$) [43,57]. However, both $f_h$ and $f_{C2}$ result from solution of the system of Eq. (2) and therefore they both depend on the effectiveness of $Y$–DNA complexation, $K_{YN}$, and the $X$–$Y$ hetero-association, $K_h$, respectively. This means that the quantities $f_h$ and $f_{C2}$ are interdependent, so a real discrimination between the two processes is not achieved and such an analysis may lead to erroneous results. The two processes could be discriminated by comparison of $f_h$ and $f_{C2}$ in their 'pure' forms, which contain no contribution from the competing process, or just 'switch off' the $Y$–DNA complexation (*i.e.* exclude 'protector' mechanism) or $X$–$Y$ hetero-association (*i.e.* exclude 'interceptor' mechanism), respectively.

The 'protector' and 'interceptor' mechanisms may be quantified using the criterion, $R_D$, which is the relative decrease in proportion of $X$–DNA complexes on addition of the ligand $Y$. A similar criterion was used previously [44,50,64] to analyse the competitive binding to DNA of aromatic drugs with either Caffeine or Vitamin $B_2$, if the vitamin or CAF are considered as 'interceptors/protectors' of the drugs.

$R_D$ may be considered for two limiting circumstances:

(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.* $f^X_{C2(C)}$, 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.* $f^X_{C2(h)}$:

$$R_D = \frac{f^X_{C2(0)} - f^X_{C2(C)}}{f^X_{C2(0)} - f^X_{C2(h)}}, \tag{3}$$

where $f^X_{C2(0)}$ 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 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 $Y$,

$$A_D = \frac{f^X_{C2(0)} - f^X_{C2}}{f^X_{C2(0)}}, \tag{4}$$

where $f^X_{C2}$ is the relative amount of drug $X$ bound to DNA in the presence of $Y$ when both mechanisms are operative. It has been suggested above that the drug $X$ exerts its biological action mainly via complexation with DNA, and so it is reasonable to

Table 1
Equilibrium self-association, hetero-association and $d(TGCA)_2$ complexation constants ($l \cdot mol^{-1}$) for various aromatic drugs in buffered aqueous-salt solution, pH 7.0–7.4, $I \approx 0.1$ M, $T=298$ K [a]

| Drug | CAF | FMN | DAU | DOX | NOG | NOV | AMD | PF | EB | $d(TGCA)_2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| CAF | 11.8 [43] | – | 72 [43] | 208 [44] | 210 [44] | 324 [42] | 246 [43] | 160 [43] | 62 [43] | 246 [43] |
| FMN | | 265 [50] | 453 [50] | – | 965 [79] | 49000 [77] | 890 [78] | 920 [51] | 640 [51] | 8000 [50] |
| DAU | | | 720 [72] | – | 3400 [79] | 15000 [54] | 2800 [55] | – | – | 560000 [72] |
| DOX | | | | 1800 [44] | – | – | – | – | – | 800000 [44] |
| NOG | | | | | 7400 [44] | 12000 [79] | 4600 [79] | – | – | 1200000 [44] |
| NOV | | | | | | 28600 [42] | 2500 [55] | – | – | 850000 [80] |
| AMD | | | | | | | 1420 [69] | – | – | 1000000 [79] |
| PF | | | | | | | | 700 [69] | – | 15000 [43] |
| EB | | | | | | | | | 305 [69] | 72000 [43] |

[a] Details of the experimental procedure used for obtaining the equilibrium constants are given in the cited papers and are similar for all the molecular combinations studied.

associate the relative displacement of drug from DNA, $A_D$, with the change in biological response of drug $X$ on addition of ligand $Y$. In further analysis $A_D$ quantity will be used as an indication of the biological effect of the drug $X$ in the three-component system, $X$–$Y$–DNA.

The mole fractions $f^X_{C2}, f^X_{C2(0)}, f^X_{C2(h)}, f^X_{C2(C)}$ can be calculated from the solution of Eq. (2) as $\frac{K_{XN}XN}{X_0}$ under the conditions formulated above, once there is some assessment of the concentrations of the drugs $X_0$, $Y_0$ and the tetrameric regions free-from-histones on cellular DNA, $N_0$, to be used in the analysis.

### 2.3. Choice of concentrations for the three-component mixture

The concentration $Y_0$ of the 'interceptor' molecule $Y$ is taken to be a variable quantity ranging from zero up to millimolar concentrations, in order to cover all possible experimental circumstances reported for various 'interceptor' molecules in the Introduction section.

In order to decide on an appropriate concentration for drug $X$, it has been taken into account that the majority of experimental data on the synergistic effect of the $X$–$Y$ combination on DNA has been reported for anthracycline drugs (or its derivatives) in the mixture with Caffeine. The peak concentration of an anthracycline drug in human blood plasma under a standard chemotherapeutic regimen reaches few micromolars [73,74], though the intracellular concentration of the drug acting on cellular DNA may exceed the plasma concentration hundreds of times [75,76]. In order to represent the physiological condition, it is thought reasonable to take an intermediate concentration of $X$ drug, $X_0 \approx 10$ μM, to be used in the numerical analysis.

The choice of the concentration of the free-from-histones tetrameric regions of cellular DNA, $N_0$, is quite difficult, because no relevant experimental data is available. It has been shown previously [44] that the $A_D$ factor calculated for the removal of anthracycline drugs from DNA on addition of CAF in the millimolar concentration reaches dozens of percents under the condition that $N_0 = 0.01$ mM. A similar percentage of detoxification due to drug removal is observed experimentally in *in vitro* systems on addition of mM concentrations of CAF to anthracycline-containing cell cultures [20]. Hence, a value of $N_0 = 0.01$ mM is suggested as a reasonable estimate of the physiological concentration of free tetrameric regions on cellular DNA.

### 2.4. Analysis of the protector/interceptor action in the Drug–Caffeine–DNA system

The $R_D$ factor has been calculated over a wide concentration range of CAF ($Y_0$) as summarised in Fig. 2. As the 'protector' effect of CAF on aromatic drug molecules is commonly observed *in vitro* in the mM concentration range [19,20], it is seen in Fig. 2 that at such concentrations of CAF the 'protector' mechanism ($R_D > 1$) is dominant over the 'interceptor' mechanism for the combinations CAF with EB, DAU or PF, and that both mechanisms are of comparable efficacy for the combinations CAF with NOG, DOX, AMD or NOV. In previous work on the effect of CAF on the complexation of aromatic drugs with DNA, the complexation of CAF with DNA had been neglected, which had resulted in the erroneous conclusion that DOX and NOV act solely via the 'interceptor' mechanism [20,23,38]. It is shown in this work that both mechanisms should always be taken into consideration in analysing the complexation of aromatic drugs with DNA in the presence of an aromatic molecule such as CAF.

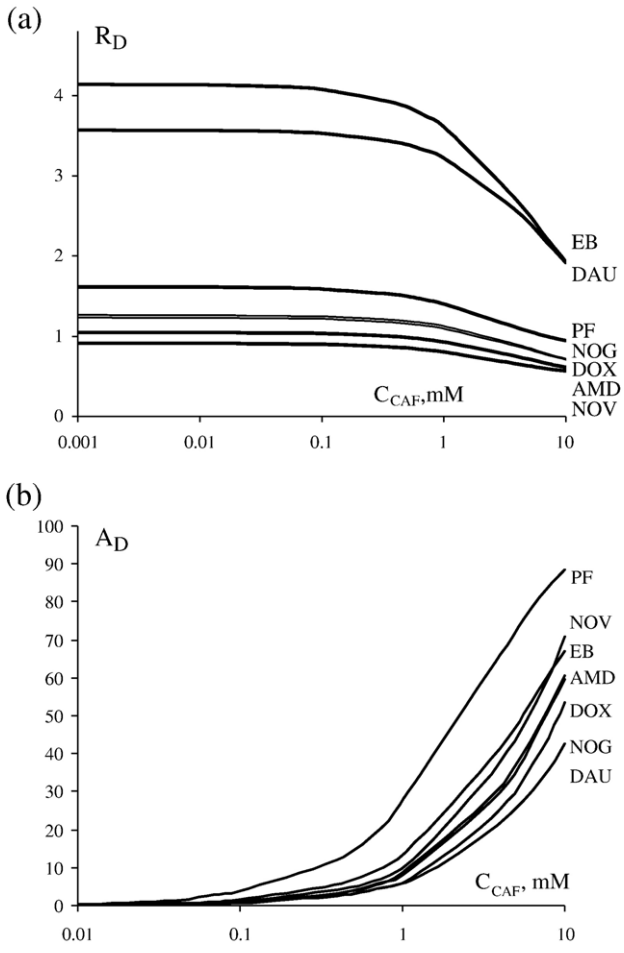


Fig. 2. Calculated from Eqs. (3), (4): (a) $R_D$ and (b) $A_D$ factors in Drug–CAF–DNA systems at $X_0$, $N_0 = 0.01$ mM, $Y_0$-*var*.

Analysis of the $R_D$ curves in Fig. 2 shows that the intersect on the $R_D$ axis at $Y_0 \to 0$ for all the systems studied reveals an unambiguous correlation between $R_D$ and the hetero-association constant $K_h$ in the form

$$R_D \approx \frac{C}{K_h} \tag{5}$$

where $C \approx 250$ is a constant (to be justified later).

The $R_D$ factor remains practically constant up to millimolar concentrations of CAF as seen from Fig. 2a. It is also found (not shown) that $R_D$ changes insignificantly (within 10%) on varying $N_0$ or $X_0$ by an order of magnitude. Hence Eq. (5) may be used to estimate the interrelation between protector/interceptor mechanisms in any $X$–CAF combination with DNA

using just the measured equilibrium hetero-association constant for *X*–CAF.

Analysis of the $A_D$ factor (Fig. 2b) enables the drugs to be categorised in descending order for their sensitivity to the addition of CAF at mM concentrations:

i.e. PF>NOV ≈ EB>AMD ≈ DOX>NOG>DAU.

It means that the largest synergistic effect on addition of CAF at mM concentrations is expected for the mutagen PF and the lowest change for the anti-cancer drug DAU with DNA. Analysis also shows that the sequence of drugs presented does not depend on $N_0$ and $X_0$. Moreover, that sequence is in good agreement with the sequence of numbers calculated using an empirical relation (see Table 2)

$$A_D \equiv \frac{K_h^2}{K_{XN}} \tag{6}$$

The approximation is good for the two extreme cases (PF and DAU) and for the group of molecules in the intermediate cases (NOV,EB,AMD,DOX), though the approximation is not perfect for the individual molecules in the intermediate cases, because the synergistic effects of these drugs differ from each other by no more than 10% in $A_D$ units and *ca*.20% in $K_h^2/K_{XN}$ units (see Fig. 2b and Table 2). Nevertheless, using just values of the equilibrium hetero-association constant for *X*–*Y* and the *X*–DNA complexation constant, Eq. (6) can be used to predict within a 20% accuracy the *relative* change in synergistic effect (with respect to a known change in biological effect of a reference drug) of an aromatic drug *X* on addition of CAF. In fact, Eq. (6) also provides a rationale for the direct regulation of biological activity in Drug–CAF systems by varying the equilibrium hetero-association and complexation constants in different systems.

### 2.5. Analysis of the protector/interceptor action in the Drug–Vitamin–DNA system

The calculated factors, $R_D$ and $A_D$, for various Drug–Vitamin $B_2$ systems are presented in Fig. 3. Vitamin $B_2$ is not very soluble so for the experimental observations the more soluble physiological derivative of Vitamin $B_2$, Flavin-mononucleotide (FMN) was used [50,51,77,78]. It can be seen from Fig. 3a that for most of the drugs studied the 'protector' mechanism is dominant ($R_D$>1) for the effect of drug binding with DNA of the FMN Vitamin at physiological concentrations ($Y_0$<0.1 mM), except for the NOV–FMN system which is characterised by a predominance of the 'interceptor' mechanism ($R_D$<1). These results can be explained by the much larger value of the hetero-association constant for the NOV–FMN system compared to all the other systems (Table 2). It has previously been shown that the hetero-complexes NOV–FMN are additionally stabilized by intermolecular H-bonds [77], which considerably increases the hetero-association constant in solution and therefore produces the remarkable 'interceptor' effect upon binding with DNA.

The $R_D$ value at $Y_0 \to 0$ (Fig. 3a) and the sequence of drugs by $A_D$ factor, NOV>PF>EB>AMD ≈ NOG>DAU (Fig. 3b) exactly follow the empirical relations Eq. (5) and (6) (Table 2) and do not significantly depend on $X_0$ and $N_0$. Again, as found for CAF-containing systems, some regulation of the effect of an aromatic drug on addition of Vitamin $B_2$ is also possible in Vitamin-containing systems.

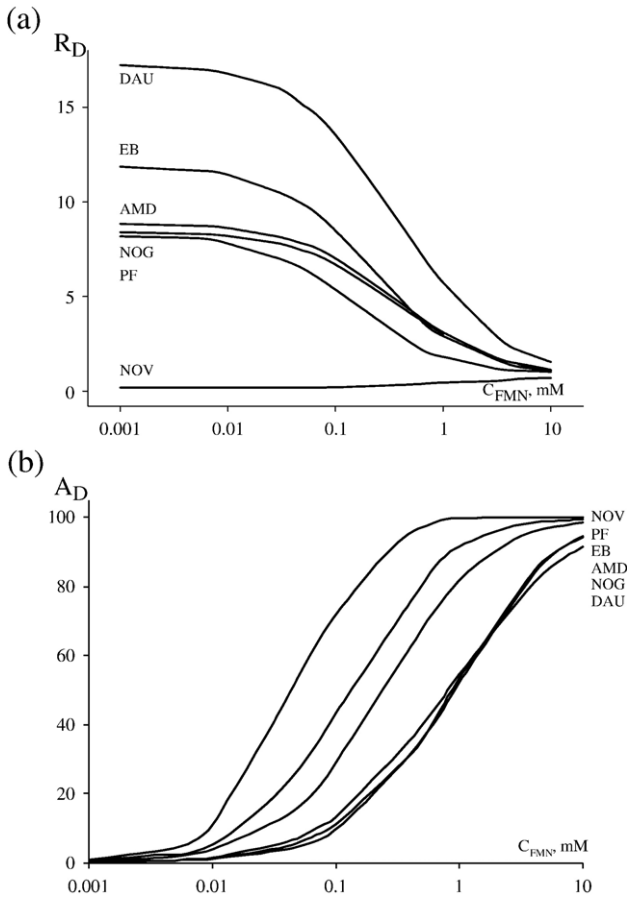


Fig. 3. Calculated from Eqs. (3), (4): (a) $R_D$ and (b) $A_D$ factors in Drug–FMN–DNA systems at $X_0$, $N_0$=0.01 mM, $Y_0$-*var*.

Table 2
Calculation of the $A_D$ factor in various *X*–*Y*–DNA systems

| Drug–CAF–DNA systems | | | | | | | |
|---|---|---|---|---|---|---|---|
| Sequence of $A_D$ curves | PF | NOV | EB | AMD | DOX | NOG | DAU |
| $A_D \equiv K_h^2/K_{XN}$ | 1.71 | 0.12 | 0.05 | 0.06 | 0.05 | 0.04 | 0.01 |

| Drug–FMN–DNA systems | | | | | | |
|---|---|---|---|---|---|---|
| Sequence of $A_D$ curves | NOV | PF | EB | AMD | NOG | DAU |
| $A_D \equiv K_h^2/K_{XN}$ | 2825 | 56 | 5.7 | 0.8 | 0.77 | 0.37 |

| Drug–Drug–DNA system (an example when NOV acts as an interceptor molecule) | | | |
|---|---|---|---|
| Sequence of $A_D$ curves | DAU | NOG | AMD |
| $A_D \equiv K_h^2/K_{XN}$ | 402 | 120 | 6 |

### 2.6. Analysis of the protector/interceptor action in Drug–Drug–DNA systems

The drug–drug system is different from the *X*–*Y*–DNA systems so far considered, because both aromatic drugs exert their biological effect via complexation with DNA and therefore either of them could be considered as the 'interceptor molecule'. In the present analysis, however, we shall formally treat a combination of drugs as an *X*–*Y* system, in which *X* is the main drug and *Y* is the putative 'interceptor' drug.

All possible pairwise combinations of drugs (within the set DAU, NOG, NOV and AMD) have been analyzed such that, in each pair, each drug was considered as the *X* or *Y* ligand. An example of the calculated values of the $R_D$ and $A_D$ factors for the particular case when NOV acts as the 'interceptor' ligand is shown in Fig. 4, though the curves are qualitatively similar for all other drug combinations analysed. It is seen that in the Drug–Drug–DNA system there is a predominance of the 'protector' mechanism at physiological concentration range of $Y_0 < 0.1$ mM. On the other hand, as seen from Fig. 4b, an $A_D$ factor of 20% and above can be reached only at concentrations of drug *Y* greater than 0.01 mM It follows that under typical physiological concentrations of drugs used in clinical practice (in the micromolar range), the protector/interceptor mechanisms are unlikely to govern the biological synergism in drug–drug systems. Nevertheless, analysis shows that $R_D$ and $A_D$ factors for the set of aromatic drugs studied are in full agreement with Eqs. (5) and (6) (as an example, see the results for the Drug–NOV–DNA system in Table 2), which suggests that the empirical Eqs. (5) and (6) are of general applicability for any *X*–*Y* combination of aromatic drug molecules and that this originates from the general property of aromatic molecules to complex with DNA and with each other in solution.

As the importance of Eq. (6) for the prediction of a change of biological activity in the *X*–*Y* system was discussed above, it is necessary to compare the criterion Eq. (6) against published literature data on *in vitro* action of 'interceptor molecules' on the biological response of aromatic drugs in cultured cell lines.

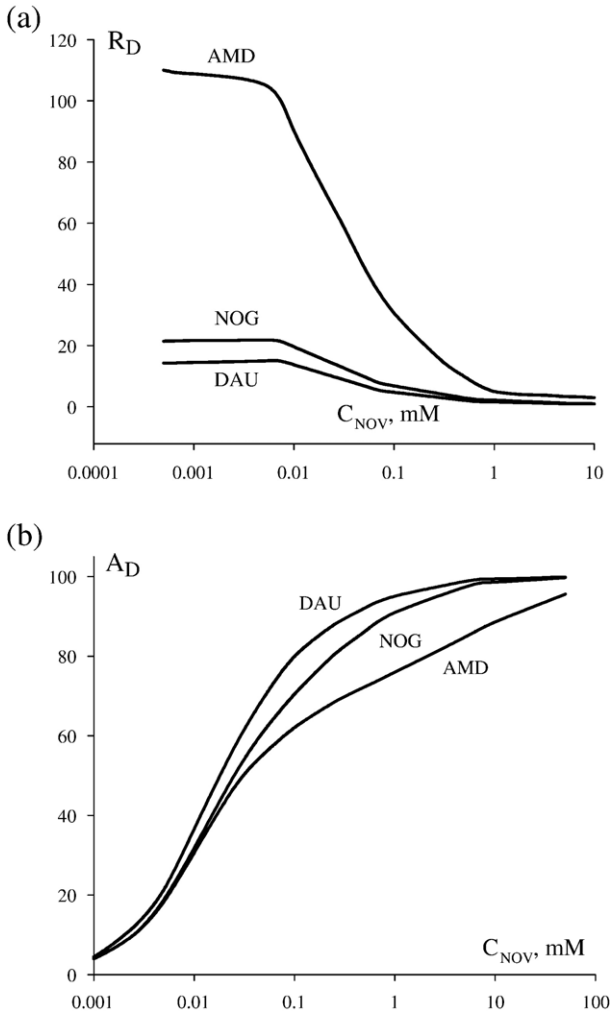


Fig. 4. Calculated from Eqs. (3), (4): (a) $R_D$ and (b) $A_D$ factors in Drug–Drug–DNA systems at $X_0$, $N_0 = 0.01$ mM, $Y_0$-*var*.

### 2.7. A protocol for estimating the relative change in biological activity in the X–DNA system on addition of aromatic molecule Y

First, it is important to note that the comparison should only be made utilizing data obtained under similar experimental conditions. For the set of drugs whose NMR results have been considered in this work, a straightforward comparison can only be made for published data [20] on the effect of CAF on the action of the drugs NOV and DOX. Second, it is necessary to point out that superposition of the $A_D$ factors for different drugs on a single plot (as in Figs. 2b–4b) is convenient for comparison of the effects of different drugs but it depends on the assumption that the intrinsic biological activities of the drugs in the absence of an interceptor molecule are similar. Of course, that assumption is not correct for the aromatic drugs studied in this work and so the $A_D$ factor for any given drug should be normalised with respect to its intrinsic biological activity.

A simple protocol is suggested for estimating the relative change in biological activity in the *X*–DNA system on addition of aromatic molecule *Y*. Using published data on the drugs NOV and DOX, as an example, it was reported [20] that the suppression of cell growth by NOV and DOX amounts to $I_{NOV} = 52\%$ and $I_{DOX} = 74\%$, respectively, when compared to the control cell culture (intrinsic biological activity). Hence, the drug DOX is *ca*. 1.4 times ($I_{DOX}/I_{NOV} = 74/52$) more potent than NOV in that cell culture. On addition of CAF the cell growth suppression decreases to $I_{NOV-CAF} = 17\%$ and $I_{DOX-CAF} = 35\%$. If DOX is taken as the reference drug, then the change in the biological effect of the drugs in the CAF-containing system, corrected for the intrinsic activity of the drug, can be expressed as

$$A_D(\mathrm{NOV}) = \frac{I_{DOX}}{I_{NOV}}(I_{NOV} - I_{NOV-CAF}) = 50\%,$$
$$A_D(\mathrm{DOX}) = I_{DOX} - I_{DOX-CAF} = 39\%.$$

It is concluded that NOV is more sensitive than DOX to addition of CAF, which is in full agreement with the results of

calculations in Fig. 2b and the empirical estimation of $A_D$ using Eq. (6). Using the same protocol, it is also possible to solve the more important reverse task, *i.e.* having a set of theoretically-calculated $A_D$ curves, one can scale them to experimentally-predicted $A_D$s. This situation can be generalised for any combination of aromatic drug molecules.

Let $R$ be the reference drug and $X$ is a drug whose change in biological activity on addition of drug $Y$ needs to be predicted relative to the same action of $Y$ on the $R$-containing system. Let $I_X$ and $I_R$ be the quantitative measures (for instance, suppression of cell growth, apoptotic activity, mutagenicity *etc.*) of the intrinsic biological effect of $X$ and $R$ drugs, respectively, in the given system in the absence of $Y$. Hence, it is necessary to calculate and compare the quantities $A_D{}^X$ and $A_D{}^R$ for the drugs $X$ and $R$, respectively:

$$\text{i.e.}\quad A_D^X \equiv \frac{K_{XY}^2}{K_{XN}} \cdot \frac{I_X}{I_R}, \quad A_D^R \equiv \frac{K_{RY}^2}{K_{RN}}. \tag{7}$$

where $K_{XY}$ and $K_{RY}$ are the hetero-association constants of $X$ and $R$ with $Y$, respectively; $K_{XN}$ and $K_{RN}$ are the complexation constants of $X$ and $R$ with the DNA tetramer. If $A_D^X > A_D^R$ then one can expect a greater biological effect on addition of $Y$ to the $X$-system compared to the $R$-system, or *vice versa*.

The accuracy of predictions based on $A_D$ using Eq. (7) needs to take into account that the basic empirical relation Eq. (6) gives a 20% uncertainty in determining the sequence of $A_D$ curves for various drugs. In addition, equilibrium constants measured under *in vitro* solution conditions (Table 1) may be different from those in 'real' biological system and there is the limitation of using the $d(TGCA)_2$ sequence as a model of free tetrameric regions on cellular DNA. Nevertheless, Eq. (7) does not reflect the absolute value of a biological effect but just a relative one. It follows that, in terms of a calculated relation in biological activities of different drugs, the actual magnitudes of equilibrium constants lose their influence on the error of estimation, if the equilibrium constants are measured under similar solution conditions. In summary, it is thought that an accuracy of 20% is a reasonable expectation of the accuracy of Eq. (7).

### 2.8. Theoretical analysis of protector/interceptor actions in X–Y–DNA system

An accurate determination of the $R_D$ and $A_D$ factors can be made by the solution of the general system of Eq. (2). As an analytical solution of that system is unlikely to be possible due to the complexity of the mathematics, a numerical solution is expected to remain the better choice of quantifying the interceptor/protector actions in three-component systems. Nevertheless, under certain circumstances, it is possible to reach an analytical solution of Eq. (2).

If self-association of $X$ and $Y$ molecules can be excluded from the analysis and only 1:1 hetero-association considered, then the system of Eq. (2) may be simplified. Such assumptions are reasonable because at physiological concentrations (μM) the self-association of aromatic drugs as well as high order hetero-association are not significant and so Eq. (2) reduce to Eq. (8)

$$\begin{cases} X_0 = X + K_{XN}XN + K_hXY \\ Y_0 = Y + K_{YN}YN + K_hXY \\ N_0 = N + K_{YN}YN + K_{XN}XN \end{cases} \tag{8}$$

under the set of conditions

$$\left\{ N_0 = X_0, \quad X_0 \ll \frac{1}{K_h}, \quad N_0 \ll \frac{1}{K_{YN}} \right\}. \tag{9}$$

A more detailed analysis of Eq. (8) (see Appendix) with respect to $R_D$ and $A_D$ enables the complete set of analytical equations to be derived in the following form

$$\begin{cases} R_D = \dfrac{(1+c)^{1/2}+(1+4a+c)^{1/2}}{(1+b)^{1/2}+(1+4a+b)^{1/2}} \cdot \dfrac{(1+4a)^{1/2}(1+b)^{1/2}-(1+4a+b)^{1/2}}{(1+4a)^{1/2}(1+c)^{1/2}-(1+4a+c)^{1/2}} \\ A_D(R_D \ll 1) = \dfrac{2}{(1+4a)^{1/2}-1} \cdot \dfrac{(1+4a)^{1/2}(1+c)^{1/2}-(1+4a+c)^{1/2}}{(1+c)^{1/2}+(1+4a+c)^{1/2}} \\ A_D(R_D \gg 1) = \dfrac{2}{(1+4a)^{1/2}-1} \cdot \dfrac{(1+4a)^{1/2}(1+b)^{1/2}-(1+4a+b)^{1/2}}{(1+b)^{1/2}+(1+4a+b)^{1/2}} \end{cases} \tag{10}$$

where $a = K_{XN}X_0$, $b = K_{YN}Y_0$, $c = K_hY_0$.

Using the equilibrium constants given in Table 1, it is simple to verify that for the physiological concentrations used in this work, $X_0, N_0 \le 0.01$ mM, the set of conditions Eq. (9) always applies for any Drug–CAF–DNA and Drug–FMN–DNA systems but may not be applicable for Drug–Drug–DNA systems. The most limiting condition in Eq. (9) is the one stating that the affinity of an interceptor molecule towards DNA, $K_{YN}$, should be less than 10,000 l mol$^{-1}$.

In the extreme limit of a great excess of interceptor molecules in solution, *i.e.* $Y_0 \to \infty$, evaluation of the $\lim_{Y_0 \to \infty} R_D = 1$ is unity, which means that there is an equal contribution of the interceptor/protector mechanisms.

Another extreme limit, $\lim_{Y_0 \to 0} R_D$, yields the relation between equilibrium constants that

$$R_D = \frac{K_{YN}}{K_h}, \tag{11}$$

which is in good agreement with Eq. (5) derived empirically for particular $X$–$Y$–DNA systems. It is now possible to formulate the physical meaning of the constant $C$ in the numerator of Eq. (5) as the equilibrium constant of complexation of $Y$–DNA. It is seen from Figs. 2a and 3a that the $R_D$ factor remains practically unchanged up to millimolar concentrations of $Y_0$, hence, Eq. (11) can be safely used to estimate the interrelation between the 'interceptor' and 'protector' mechanisms in any $X$–$Y$–DNA mixture up to millimolar concentrations of an interceptor molecule. The link between the analytical (Eq. (10)) and empirical (Eq. (6)) forms of the $A_D$ factor is being studied in our laboratory.

## 3. Conclusions

It is suggested that the widely reported biological synergism, both *in vivo* and *in vitro*, of a mixture of DNA-acting aromatic

drug molecules can be explained, in part, at the molecular level by the competition between two basic mechanisms: the 'interceptor' (*i.e.* hetero-association between Drug1 and Drug2) and the 'protector' (competition of Drug1 and Drug2 on DNA binding sites) mechanisms. Both mechanisms act via removal from DNA of the main drug (Drug1) on addition of a second drug (Drug2) thus leading to a change in the biological response of Drug1. As aromatic drug molecules have a pronounced tendency for non-covalent intermolecular association with DNA or with other aromatic molecules, it is expected that the two mechanisms are *always* operative in any Drug1–Drug2 combination, if the main drug (Drug1) exerts its biological effect via complexation with DNA.

Two parameters have been developed, an $A_D$ factor (Eq. (7)) which indicates the relative change in biological effect in any Drug1–Drug2–DNA system and an $R_D$ factor (Eq. (11)) which gives a qualitative estimation of the relative importance of the interceptor and protector mechanisms. The two factors, $A_D$ and $R_D$, only include equilibrium constants for hetero-association and DNA-complexation, which are measured in physicochemical experiments. Hence, knowledge of the equilibrium constants enables the synergistic effect of drugs to be estimated and to suggest how the biological effect in any mixture of aromatic drug molecules may be modified. A more complete analysis includes the concentrations of molecular components in solution to give accurate analytical forms of the factors $A_D$ and $R_D$ (Eq. (10)). The development of a general method for quantitative analysis of a three-component system of aromatic molecules, which includes DNA as a bio-receptor in this case, could be adapted with minor modification for other receptors for aromatic drugs.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2007.11.001.

## References

[1] E. Chu, V.T. DeVita, Physicians' cancer chemotherapy drug manual, Jones and Bartlett Publ., London, 2003.
[2] I.H. Stockley, Stockley's Drug Interactions, 6th ed., Pharm. Press, London, 2002.
[3] S. Neidle, M.J. Waring, Molecular aspects of Anti-cancer drug action, Macmillan, London, 1983.
[4] B. Pullman, Molecular mechanism of specifity in DNA-antitumor drug interactions, Adv. Drug Res. 18 (1989) 2–112.
[5] D.E. Graves, L.M. Velea, Intercalative binding of small molecules to nucleic acids, Curr. Org. Chem. 4 (2000) 915–928.
[6] M.F. Brana, M. Cacho, A. Gradillas, B. de Pascual-Teresa, A. Ramos, Intercalators as anticancer drugs, Curr. Pharm. Des. 7 (2001) 1745–1780.
[7] S. Yao, D. Murali, P. Seetharamulu, K. Haridas, P.N.V. Petluru, D.G. Reddy, F.H. Hausheer, Topotecan lactone selectively binds to double- and single-stranded DNA in the absence of Topoisomerase I. Cancer Res. 58 (2001) 3782–3786.
[8] F. Arcamone, F. Animati, G. Capranico, P. Lombarda, G. Pratesi, S. Manzini, R. Supino, F. Zunino, New developments in antitumor anthracyclines, Pharmacol. Ther. 76 (1997) 117–124.
[9] J.B. Chaires, Drug–DNA interactions, Curr. Opin. Struct. Biol. 8 (1998) 314–320.
[10] C. Monneret, Recent developments in the field of antitumour anthracyclines, Eur. J. Med. Chem. 36 (2001) 483–493.
[11] L.H. Baker, J. Frank, G. Fine, S.P. Balcerzak, R.L. Stephens, W.J. Stuckey, S. Rivkin, J. Saiki, J.H. Ward, Combination chemotherapy using adriamycin, DTIC, cyclophosphamide, and actinomycin-D for advanced soft-tissue sarcomas: a randomized comparative trial. A phase-III, Southwest-Oncology-Group-Study, J. Clin. Oncol. 5 (1987) 851–861.
[12] J. Dunst, S. Ahrens, M. Paulussen, C. Rube, W. Winkelmann, A. Zoubek, D. Harms, H. Jurgens, Second malignancies after treatment for Ewing's sarcoma: a report of the CESS-studie, Int. J. Radiat. Oncol. Biol. Phys. 42 (1998) 379–384.
[13] M. Bontenbal, A.S.T. Planting, C.J. Rodenburg, A. Dees, J. Verweij, C.C.M. Bartels, J. Alexievafigusch, W.L.J. Vanputten, G.J.M. Klijn, Weekly low-dose mitoxantrone plus doxorubicin as 2nd-line chemotherapy for advanced breast-cancer, Breast Cancer Res. Treat. 21 (1992) 133–138.
[14] Y. Kano, K. Suzuki, M. Akutsu, K. Suda, Effects of mitoxantrone in combination with other anticancer agents on a human leukemia–cell line, Leukemia 6 (1992) 440–445.
[15] M. Schaich, T. Illmer, W. Aulitzky, H. Bodenstein, M. Clemens, A. Neubauer, R. Repp, U. Schakel, S. Soucek, H. Wandt, G. Ehninger, Intensified double induction therapy with high dose mitoxantrone, etoposide, m-amsacrine and high dose ara-C for patients aged 61–65 years with acute myeloid leukemia, Haematologica 87 (2002) 808–815.
[16] B. Hochsmann, M. Griesshammer, L. Bergmann, A new combination therapy with topotecan, cytarabine and mitoxantrone in patients with refractory or relapsed acute myeloid leukemia or blast crisis of chronic myeloid leukemia, Blood 94 (1999) 230B–230B (Suppl. 1).
[17] C.G. Taylor, A.W. Elgie, C.J. Williamson, J.M. Sargent, The combination of topotecan and daunorubicin shows striking synergism *in vitro* in Aml. Leukemia. 15 (2001) 492–492.
[18] G. Iliakis, M. Nusse, R. Ganapathi, J. Egner, A. Yen, Differential reduction by caffeine of adriamycin induced cell killing and cell cycle delays in Chinese hamster V79 cells, Int. J. Radiat. Oncol. Biol. Phys. 12 (1986) 1987–1995.
[19] F. Traganos, B. Kaminska-Eddy, Z. Darzynkiewicz, Caffeine reverses the cytotoxic and cell kinetic effects of Novatrone (mitoxantrone), Cell Prolif. 24 (1991) 305–319.
[20] F. Traganos, J. Kapuscinsky, Z. Darzynkiewicz, Caffeine Modulates the Effects of DNA-intercalating Drugs in Vitro: A Flow Cytometric and Spectrophotometric Analysis of Caffeine Interaction with Novatrone, Doxorubicin, Ellipticine, and the Doxorubicin Analogue AD198, Cancer Res. 51 (1991) 3682–3689.
[21] F. Traganos, J. Kapuscinski, J.P. Gong, B. Ardelt, R.J. Darzynkiewicz, Z. Darzynkiewicz, Caffeine prevents apoptosis and cell-cycle effects induced by camptothecin or topotecan in HL-60 cells, Cancer Res. 53 (1993) 4613–4618.
[22] C. Perez, F. Pelayo, N.E. Vilaboa, P. Aller, Caffeine attenuates the action of amsacrine and etoposide in U-937 cells by mechanisms which involve inhibition of RNA-synthesis, Int. J. Cancer 57 (1994) 889–893.
[23] J. Piosik, M. Zdunek, J. Kapuscinski, The modulation by xanthines of the DNA-damaging effect of polycyclic aromatic agents. Part II. The stacking complexes of caffeine with doxorubicin and mitoxantrone, Biochem. Pharmacol. 63 (2002) 635–646.
[24] P.E. Hartman, D.M. Shankel, Antimutagens and Anticarcinogens — a survey of putative interceptor molecules, Environ. Mol. Mutagen. 15 (1990) 145–182.
[25] G.M. Gentile, S. Rahimi, J. Zwiesler, G.J. Gentile, L.R. Ferguson, Effect of selected antimutagens on the genotoxicity of antitumor agents, Mutat. Res. 402 (1998) 289–298.
[26] Y.A. Amara-Mokrane, M.P. Lehucher, G. Balansard, G. Dumenil, A. Botta, Protective effects of α-hedrin, chlorophyllin and ascorbic acid towards the induction of micronuclei by doxorubicin in cultured human lymphocytes, Mutagenesis 11 (1996) 161–167.
[27] G.B. Raiczyk, R.S. Rivlin, J. Pinto, Enhancement of adriamycin-induced mortality during riboflavin administration and riboflavin deficiency in rats, Proc. Soc. Exp. Biol. Med. 188 (1988) 495–499.
[28] S. Shinozawa, Y. Gomita, Y. Araki, Protective effects of various drugs on adriamycin (doxorubicin)-induced toxicity and microsomal lipid-peroxidation in mice and rats, Biol. Pharm. Bull. 16 (1993) 1114–1117.

[29] H. Kimura, T. Aoyama, Decrease in sensitivity to ethidium bromide by caffeine, dimethylsulfoxide or 3-aminobenzamide due to reduced permeability, J. Pharmacobiodyn 12 (1989) 589–595.
[30] S. Arimoto, S. Fukuoka, C. Itome, H. Nakano, H. Rai, H. Hayatsu, Binding of polycyclic planar mutagens to chlorophyllin resulting in inhibition of the mutagenic activity, Mutat. Res. 287 (1993) 293–305.
[31] A.W. Wood, J.M. Sayer, H.L. Newmark, H. Yagi, D.P. Michaud, D.M. Jerina, A.H. Conney, Mechanism of the inhibition of mutagenicity of a benzo[a]pyrene 7,8-diol 9,10-epoxide by riboflavin 5′-phosphate, Proc. Natl. Acad. Sci. U.S.A. 79 (1982) 5122–5126.
[32] R. Dashwood, T. Negishi, H. Hayatsu, V. Breinholt, J. Hendricks, G. Bailey, Chemopreventive properties of chlorophylls towards aflatoxin $B_1$: a review of the antimutagenicity and anticarcinogenicity data in rainbow trout, Mutat. Res. 399 (1998) 245–253.
[33] R.P. Webster, M.D. Gawde, R.K. Bhattacharya, Modylation of carcinogen-induced DNA damage and repair enzyme reactivity by dietary riboflavin, Cancer Lett. 98 (1996) 129–135.
[34] B. Ardelt, J. Kunicki, F. Traganos, Z. Darzynkiewicz, Chlorophyllin protects cells from the cytostatic and cytotoxic effects of quinacrine mustard but not of nitrogen mustard, Int. J. Oncol. 18 (2001) 849–853.
[35] J. Kapuscinski, B. Ardelt, J. Piosik, M. Zdunek, Z. Darzynkiewicz, The modylation of the DNA-damaging effect of polycyclic aromatic agents by xanthines. Part I. Reduction of cytostatic effects of quinacrine mustard by caffeine, Biochem. Pharmacol. 63 (2002) 625–634.
[36] J. Piosik, K. Ulanowska, A. Gwizdek-Wisniewska, A. Czyz, J. Kapuscinski, G. Wegrzyn, Alleviation of mutagenic effects of polycyclic aromatic agents (quinacrine mustard, ICR-191 and ICR-170) by caffeine and pentoxifylline, Mutat. Res. 530 (2003) 47–57.
[37] M. Zdunek, J. Piosik, J. Kapuscinski, Thermodynamical model of mixed aggregation of ligands with caffeine in aqueous solution. Part II. Biophys. Chemist. 84 (2000) 77–85.
[38] R.W. Larsen, R. Jasuja, R. Hetzler, P.T. Muraoka, V.G. Andrada, D.M. Jameson, Spectroscopic and molecular modelling studies of caffeine complexes with DNA intercalators, Biophys. J. 70 (1996) 443–452.
[39] M.B. Lyles, I.L. Cameron, H.R. Rawls, Structural basis for the binding affinity of xanthines with the DNA intercalator acridine orange, J. Med. Chem. 44 (2001) 4650–4660.
[40] M.B. Lyles, I.L. Cameron, Interactions of the DNA intercalator acridine orange, with itself, with caffeine, and with double stranded DNA, Biophys. Chemist. 96 (2002) 53–76.
[41] M.B. Lyles, I.L. Cameron, Caffeine and other xanthines as cytochemical blockers and removers of heterocyclic DNA intercalators from chromatin, Cell Biol. Int. 26 (2002) 145–154.
[42] D.B. Davies, D.A. Veselkov, M.P. Evstigneev, A.N. Veselkov, Self-association of the antitumour agent novatrone (mitoxantrone) and its hetero-association with caffeine, J. Chem. Soc., Perkin Trans. 2 (2001) 61–67.
[43] D.B. Davies, D.A. Veselkov, L.N. Djimant, A.N. Veselkov, Hetero-association of caffeine and aromatic drugs and their competitive binding with a DNA oligomer, Eur. Biophys. J. 30 (2001) 354–366.
[44] M.P. Evstigneev, V.V. Khomich, D.B. Davies, Complexation of anthracycline drugs with DNA in the presence of caffeine, Eur. Biophys. J. 36 (2006) 1–11.
[45] N. Tachino, D. Guo, W.M. Dashwood, S. Yamane, R. Larsen, R. Dashwood, Mechanisms of the *in vitro* antimutagenic action of chlorophyllin against benzo[a]pyrene: Studies of enzyme inhibition, molecular complex formation and degradation of the ultimate carcinogen, Mutat. Res. 308 (1994) 191–203.
[46] M. Pietrzak, Z. Wieczorek, A. Stachelska, Z. Darzynkiewicz, Interactions of chlorophyllin with acridine orange, quinacrine mustard and doxorubicin analyzed by light absorption and fluorescence spectroscopy, Biophys. Chemist. 104 (2003) 305–313.
[47] M. Pietrzak, Z. Wieczorek, J. Wieczorek, Z. Darzynkiewicz, The 'interceptor' properties of chlorophylline measured within the three-component system: intercalator-DNA-chlorophylline, Biophys. Chemist. 123 (2006) 11–19.
[48] M.A. Munoz, C. Carmona, J. Hidalgo, P. Guardado, M. Balon, Molecular associations of flavins with betacarbolines and related indoles, Bioorg. Med. Chem. 3 (1995) 41–47.
[49] A. Ramu, M.M. Mehta, J. Liu, I. Turyan, A. Aleksic, The riboflavin-mediated photooxidation of doxorubicin, Cancer Chemother. Pharmacol. 46 (2000) 449–458.
[50] M.P. Evstigneev, Yu.V. Mykhina, D.B. Davies, Complexation of daunomycin with a DNA oligomer in the presence of an aromatic vitamin ($B_2$) determined by NMR spectroscopy, Biophys. Chemist. 118 (2005) 118–127.
[51] M.P. Evstigneev, Yu.V. Mukhina, D.B. Davies, $^1$H NMR study of the hetero-association of flavin-mononucleotide with mutagenic dyes: ethidium bromide and proflavine, Mol. Phys. 104 (2006) 647–654.
[52] N.I. Nakano, S.J. Igarashi, Molecular interactions of pyrimidines, purines, and some other heteroaromatic compounds in aqueous media, Biochemistry 9 (1970) 577–583.
[53] F. Aradi, A. Foldesi, Effect of methylation on the association ability of purine and pyrimidine in aqueous solutions. A $^1$H NMR chemical shift study, Magn. Reson. Chem. 27 (1989) 592–598.
[54] A.N. Veselkov, M.P. Evstigneev, A.O. Rozvadovska, A.A. Hernandez Santiago, O.V. Zubchenok, L.N. Djimant, D.B. Davies, $^1$H NMR structural and thermodynamical analysis of the hetero-association of daunomycin and novatrone in aqueous solution, J. Mol. Struct. 701 (2004) 31–37.
[55] D.B. Davies, M.P. Evstigneev, D.A. Veselkov, A.N. Veselkov, Hetero-association of anti-cancer antibiotics in aqueous solution: NMR and molecular mechanics analysis, Biophys. Chemist. 117 (2005) 111–118.
[56] D.B. Davies, D.A. Veselkov, V.V. Kodintsev, M.P. Evstigneev, A.N. Veselkov, $^1$H NMR investigation of the hetero-association of aromatic molecules in aqueous solution: factors involved in the stabilization of complexes of daunomycin and acridine drugs, Mol. Phys. 98 (2000) 1961–1971.
[57] D.B. Davies, D.A. Veselkov, A.N. Veselkov, NMR determination of the hetero-association of phenanthridines with daunomycin and their competitive binding to a DNA oligomer, Eur. Biophys. J. 31 (2002) 153–162.
[58] D.A. Veselkov, L. Karawajew, A.N. Veselkov, D.B. Davies, $^1$H NMR investigation of the hetero-association of phenanthridine dyes with daunomycin: effect of substitution of amino with azido groups in the dye chromophore, Mol. Phys. 102 (2004) 1933–1942.
[59] L.-S. Kan, P.N. Borer, D.M. Cheng, P.O.P. Ts'o, $^1$H- and $^{13}$C-NMR studies on caffeine and its interaction with nucleic acids, Biopolymers 19 (1980) 1641–1654.
[60] H. Fritzsche, I. Petri, H. Schitz, K. Weller, P. Sedmera, H. Lang, On the interaction of caffeine with nucleic acids. III. $^1$H NMR studies of caffeine-5′-adenosine monophosphate and caffeine-poly(riboadenylate) interactions, Biophys. Chemist. 11 (1980) 109–119.
[61] S. Tornaletti, P. Russo, S. Parodi, A.M. Pedrini, Studies on DNA binding of caffeine and derivatives: evidence of intercalation by DNA-unwinding experiments, Biochim. Biophys. Acta 1007 (1989) 112–115.
[62] J. Pangrekar, K. Krishnaswamy, V. Jagadeesan, Effects of riboflavin deficiency and riboflavin administration on carcinogen-DNA binding, Food Chem. Toxicol. 31 (1993) 745–750.
[63] I.M. Johnson, S.G. Bhuvan Kumar, R. Malathi, De-intercalation of ethidium bromide and acridine orange by xanthine derivatives and their modulatory effect on anticancer agents: a study of DNA-directed toxicity enlightened by time correlated single photon counting, J. Biomol. Struct. Dyn. 20 (2003) 677–685.
[64] M.P. Evstigneev, K.A. Rybakova, D.B. Davies, Complexation of norfloxacin with DNA in the presence of caffeine, Biophys. Chemist. 121 (2006) 84–95.
[65] H. Wang, H. Zou, Y. Zhang, Quantitative study of competitive binding of drugs to protein by microdialysis/high-performance liquid chromatography, Anal. Chem. 70 (1998) 373–377.
[66] R.F. Itzhaki, Studies on the accessibility of deoxyribonucleic acid in deoxyribonucleoprotein to cationic molecules, Biochem. J. 122 (1971) 583–592.
[67] R.F. Itzhaki, The arrangement of proteins on the deoxyribonucleic acid in chromatin, Biochem. J. 125 (1971) 221–224.
[68] D.M. Crothers, Calculation of binding isotherms for heterogeneous polymers, Biopolymers 6 (1968) 575–584.
[69] D.B. Davies, L.N. Djimant, A.N. Veselkov, $^1$H NMR investigation of self-association of aromatic drug molecules in aqueous solution, J. Chem. Soc., Faraday Trans. 92 (1996) 383–390.

[70] R.B. Martin, Comparisons of indefinite self-association models, Chem. Rev. 96 (1996) 3043–3064.
[71] A.N. Veselkov, M.P. Evstigneev, D.A. Veselkov, D.B. Davies, A generalized NMR-derived statistical-thermodynamical model of hetero-association of aromatic molecules in aqueous solution, J. Chem. Phys. 115 (2001) 2252–2266.
[72] D.B. Davies, R.J. Eaton, S.F. Baranovsky, A.N. Veselkov, NMR investigation of the complexation of daunomycin with deoxytetranucleotides of different base sequence in aqueous solution, J. Biomol. Struct. Dyn. 17 (2000) 887–901.
[73] J.M. Collins, D.S. Zaharko, R.L. Dedrick, B.A. Chabner, Potential roles for preclinical pharmacology in Phase I clinical trials, Cancer Treat. Rep. 70 (1986) 73–80.
[74] L. Gianni, L. Vigano, A. Surbone, D. Ballinari, P. Casali, C. Tarella, J.M. Collins, G. Bonadonna, Pharmacology and clinical toxicity of 4′-iodo-4′ deoxydoxorubicin: an example of successful application of pharmacokinetics to dose escalation in Phase I trials, J. Natl. Cancer Inst. 82 (1990) 469–477.
[75] B. Sundman-Engberg, U. Tidefelt, A. Gruber, C. Paul, Intracellular concentrations of mitoxantrone in leukemia cells *in vitro* vs *in vivo*, Leuk. Res. 17 (1993) 347–352.
[76] A. Feofanov, S. Sharonov, F. Fleury, I. Kudelina, I. Nabiev, Quantitative confocal spectral imaging analysis of mitoxantrone within living K562 cells: intracellular accumulation and distribution of monomers, aggregates, naphtoquinoxaline metabolite, and drug-target complexes, Biophys. J. 73 (1997) 3328–3336.
[77] A.N. Veselkov, M.P. Evstigneev, A.O. Rozvadovska, Yu.V. Mukhina, D.B. Davies, A structural and thermodynamic analysis of novatrone and flavin mononucleotide heteroassociation in aqueous solution by $^{1}$H NMR spectroscopy, Russ. J. Bioorgan. Chem. 31 (2005) 453–459.
[78] A.N. Veselkov, M.P. Evstigneev, A.O. Rozvadovskaya, Yu.V. Mukhina, K.A. Rybakova, D.B. Davies, $^{1}$H NMR analysis of the complex formation of aromatic molecules of antibiotic and vitamin in aqueous solution: Heteroassociation of actinomycin D and flavin mononucleotide, Biophysics 50 (2005) 20–27.
[79] M.P. Evstigneev, Intermolecular interactions of biologically active aromatic compounds and DNA in aqueous solution. DrSci thesis (Doctor of Physical and Mathematical Sciences), Kharkov National University, Ukraine, 2007.
[80] A.N. Veselkov, M.P. Evstigneev, S.A. Vysotskii, D.A. Veselkov, D.B. Davies, Thermodynamic analysis of the antibiotic mitoxantrone with the deoxytetranucleotide 5′-d(TpGpCpA) in aqueous solutions, based on $^{1}$H NMR spectroscopy data, Biophysics 47 (2002) 459–466.