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The modulation by xanthines of the DNA-damaging effect of polycyclic aromatic agents Part II. The stacking complexes of caffeine with doxorubicin and mitoxantrone☆

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Abstract

Recently accumulated statistical data indicate the protective effect of caffeine consumption against several types of cancer diseases. There are also reports about protective effect of caffeine and other xanthines against tumors induced by polycyclic aromatic hydrocarbons. One of the explanations of this phenomenon is based on biological activation of such carcinogens by cytochromes that are also known for metabolism of caffeine. In the accompanying paper [Kapuscinski *et al.*, this issue] we provide evidence (flow cytometry and the cell cycle analysis) that the cytostatic effects of caffeine (CAF) on two DNA alkylating agents, which do not require the biological activation, depend on their ability to form stacking (π–π) complexes. In this study, we use physicochemical techniques (computer aided light absorption and microcalorimetry), and molecular modeling to examine previously published qualitative data. This is published both by our and other group's data, indicates that CAF is able to modify the cytotoxic and/or cytostatic action of the two well known antitumor drugs doxorubicin (DOX) and mitoxantrone (MIT). To obtain the quantitative results from the experimental data we used the statistical-thermodynamical model of mixed aggregation, to find the association constants K_{AC} of the CAF–drug interaction (128 ± 10 and 356 ± 21 M^{-1} for DOX–CAF and MIT–CAF complex formation, respectively). In addition, the favorable enthalpy change of CAF–MIT ($\Delta H = -11.3$ kcal/mol) was measured by microcalorimetry titration. The molecular modeling (semi-empirical and force field method) allowed us to obtain the geometry of these complexes, which indicated the favorable energy (ΔE) of complex formation of the protonated drug's molecules in aqueous environment (-7.4 and -8.7 kcal/mol for DOX–CAF·$5H_2O$ and MIT–CAF·$8H_2O$ complex, respectively). The molecular modeling calculation indicates the existence of CAF–drug complexes in which the MIT molecules are intercalated between two CAF molecules ($\Delta E = -29.9$ kcal/mol). These results indicate that the attenuating effect of caffeine on cytotoxic or mutagenic effects of some polycyclic aromatic mutagens cannot be the result of metabolic activation in the cells, but simply is the physicochemical process of the sequestering of aromatic molecules (e.g. carcinogens or mutagens) by formation of the stacking complexes. The caffeine may then act as the ''interceptor'' of potential carcinogens (especially in the upper part of digesting track) where its concentration can reach the mM level). There is, however, no indication, both, in the literature or from our experiments, that the xanthines can reverse the damage to nucleic acids at the point when the damage to DNA has already occurred.

Keywords: Light absorption spectroscopy; Calorimetry; Molecular modeling; Stacking interactions; Thermodynamics

1. Introduction

Caffeine (Fig. 1) has multiple effects on cells. In an accompanying paper [2] we reviewed shortly its effects on the action of some DNA-damaging agents. This review can be summarized as follows:

(i) The CAF potentates the biological action (mutageneticity, cytotoxicity and/or cytostaticity) of several of

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Abbreviations: CAF, caffeine; DOX, doxorubicin; MIT, mitoxantrone; K_D, dimerization association constant; K_{AA}, K_{CC} and K_{AC}, the ''nearest neighbors'' equilibrium constants of association of component A with A, C with C and A with C, respectively; X, ''molecular'' concentration; C_{CC}, C_{AA} and C_{AC}, the ''nearest neighbors'' concentrations; Z, denote partition function equal to the sum of statistical weight of all possible oligomers.

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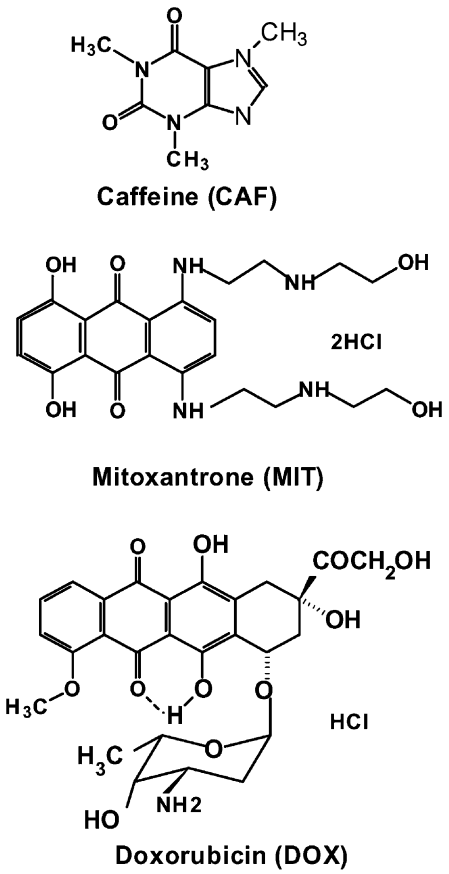

Fig. 1. Chemical structures of doxorubicin, mitoxantrone and caffeine.

such agents (e.g. ionizing radiation or alkylating compounds). This effect is, most likely, the result of CAFs suppression of DNA repair and synthesis. This has been observed generally for inorganic and aliphatic DNA-damaging compounds. The exception to this rule is the suppression of clastogenic effects of dichromate anion by CAF [3], but this may be due to the redox interaction between CAF and dichromate anion.

(ii) In contrast, the CAF decreases the cytotoxic, cytostatic or mutagenic activity of aromatic DNA-damaging compounds such as polycyclic aromatic hydrocarbons (PAHs), food-born heterocyclic aromatic amines (HAAs), intercalators, and topoizomerase (I and II) blockers. This surprising phenomenon was often explained by the modulation of cytochromes P450 bioactivation of these promutagens by CAF [4]. This hypothesis is, however difficult to accept, because CAF does not suppress, but stimulate the production of cytochromes P450 in liver [4,5], and in view of the abrogation by CAF of cytotoxic effects of some aromatic DNA-damaging compounds which do not require the biological activation, e.g. ethidium bromide [6] or quinacrine mustard [2] and topoizomerase I inhibitors: camptothecine and topotecan [7]. According to us the CAFs (and other xanthines, e.g. pentoxifylline) ability to form stacking (π–π) complexes, with the aromatic compounds provides better explanation of the discrepancies described above. The formation of such complexes lowers the concentration of free aromatic agents available for cells and may shield the procarcinogens against cytochromes biological activation.

(iii) There are also confusing epidemiological reports about the protective effects of caffeine consumption in the form of beverages [8–12]. This effect has been reported for several types of cancer [3,13–16]. It seems that this effect has been different for different types of the diseases and differs for the amount of CAF consumption [8,17]. In general, the higher the amount of CAF consumption the protective effect is more pronounced. In the chemically-induced carcinogenesis this phenomenon was observed exclusively for aromatic carcinogens and totally absent for the aliphatic carcinogens.

(iv) DOX (Adriamycin) (Fig. 1) has been used for more than 30 years in treatment of a variety of malignancies. A number of different mechanisms have been proposed for the cytotoxic and cytostatic action of this drug. Despite the extensive clinical utilization of DOX, its mechanism(s) is uncertain and has been a subject of considerable controversy (reviewed recently in [18]). These mechanisms include intercalation into DNA with consequent inhibition of macromolecular biosynthesis, free radical formation with consequent induction of DNA damage or lipid peroxidation, DNA cross-linking, interference with DNA unwinding or DNA strand separation and helicase activity, direct membrane effects, initiation of DNA damage via inhibition of topoizomerase II, and the induction of the apoptotic cell death [18]. The anthraquinone drugs MIT (novatrone, DHAQ, see Fig. 1) and ametantrone (HQ) toxicity effects are the result of similar mechanism as observed for anthracycline drugs. It should be remembered, however, that drugs interfering with DNA topoisomerases, the DOX and MIT included, which hold great promise for the treatment of cancer, are double-edged sword and may themselves cause mutation, and cancer [19]. The therapy-related myelodesplasia and acute myeloid leukemia has become the most serious long-term complication of cancer therapy [20]. Additionally, both the anthracycline and anthraquinone drugs can denature and condense nucleic acids *in vitro* and in the chromatin [21–23]. Attenuation of both cytostatic ($G_2 + M$ block, proliferation), and cytotoxic (the induction of apoptosis) effects by CAF were observed by us and others for both classes of drugs, as well as for ellipticine, camptothecine, topotecan, amsacrine (*m*-AMSA), etotoposide and ethidium bromide [7,15,24–26]. The most plausible explanation for this phenomenon was the formation of the stacking (π–π) complexes between the CAF (or pentoxifylline) and aromatic DNA-damaging agents. Because of the

complexity of the system (the xanthine molecules can form the oligomers of indeterminate length with or without aromatic drugs (or its oligomers), the earlier (our and others) study of complexes' formation were mostly qualitative [7,15,25]. In the subsequent experiments we adopted the statistic-thermodynamical model of mixed aggregation to calculate the "close neighborhood constants of association" of the system, and compared the experimental data with these calculated by the models. It should be mentioned, however, that some quantitative study of intercalator–CAF has been reported by others [6]. While formation of such complexes has been documented by several physicochemical methods, the mechanism of complexes formation was simplified be neglecting by the authors both intercalator and CAF self-aggregation phenomenon [6]. So far we described quantitative data of the interaction of CAF with acridine orange [27], 4′,6-diamidino-2-phenylidole dichloride (DAPI), ethidium bromide [28], quinacrine mustard (accompanied paper [2]), propidium iodide. In the present study, we provided the physicochemical (light absorption, calorimetry) and molecular modeling study of DOX and MIT complex formation with the caffeine.

2. Materials and methods

2.1. Materials

Caffeine (CAF, Fig. 1) [3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione], DOX (Fig. 1) [1,4 hydroxydaunomycin] and MIT (Fig. 1) 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedi-one (Fig. 1) were purchased from Sigma. CAFs stock solution was prepared by dissolving its weight amount in a buffer containing 5 mM Hepes (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) at 0.1 M NaCl and pH 7.1 (HP buffer). DOX and MIT were dissolved in distilled water as the stock solutions followed by further dilution in HP buffer. The concentrations of solutions were assayed colorimetrically using molar absorption coefficients at the isosbestic point of $E_{542} = 4.5 \times 10^3\ M^{-1}\ cm^{-1}$ and $E_{652} = 8.36 \times 10^3\ M^{-1}\ cm^{-1}$ for DOX and MIT [29,30], respectively. All spectroscopic experiments were performed in the HP buffer at 25 (±0.1)°. The buffers were filtered through a 0.45 μm pore Millex Millipore filters and then degassed by washing with stream of helium.

2.2. Light absorption spectroscopy was measured using Beckman's DU 650 spectrophotometer

The 2 mL aliquot containing drug were placed in a quartz cuvette (1 cm light path) and titrated with CAF solution. The absorption spectra were then measured at 1 nm intervals and stored in digital form. The absorption spectra were corrected for the absorption of the buffer and CAF, which were very low in the measured range, and then expressed in the form of molar absorption coefficient (E_λ, $M^{-1}\ cm^{-1}$).

2.3. Calculation of the neighborhood association constant of DOX or MIT with CAF was carried out using statistical-thermodynamical model of mixed aggregation

In this model, there are two types of molecules: A (i.e. DOX or MIT), and C (CAF), which can form different self- and mixed-aggregates of a type [27]

$$\ldots (C)_i(A)_j(C)_i(A)_j \ldots,$$

where $i = 0, 1, 2 \ldots$ and $j = 0, 1, 2$

Both DOX and MIT form dimers in solution and, therefore, parameter *j* is reduced to value 0, 1 or 2. According to the statistical-thermodynamical model the neighborhood dimerization constant $K_{AA} = 2K_D$, where K_D is the classical (molecular) dimerization constant [31–33]. CAF however can form indefinite complexes ($i = 0, 1, 2 \ldots$), the process characterized by the "neighborhood association constant" K_{CC}. This type is called an isodesmic association ([31,32] and the references cited therein). The neighborhood concentration of the components in indefinite possible oligomers are calculated using the partition function *Z*, which is obtained by adding the statistical weight of all oligomers (the statistical weight of an oligomer is a number proportional to the frequency of occurrence of this oligomer in the mixture of all possible oligomers) [27,28,32,34]

$$Z = y + \frac{C_C(1 + K_{AC}y)^2}{1 - C_C(K_{CC} + K_{AC}^2 y)}, \quad (1)$$

where

$$y = C_A + \tfrac{1}{2}K_{AA}C_A^2. \quad (2)$$

Terms C_A and C_C denote the concentration of isolated (i.e. free) A and isolated C molecules in solution. K_{CC}, K_{AC} and K_{AA} denote the nearest neighbor's equilibrium constants of association of C with C, A with C, and A with A, respectively. The total "molecular" concentration of A (C_{TA}) and C (C_{TC}), can be expressed in form of equations

$$C_{TA} = C_A(1 + K_{AA}C_A)\left[\frac{1 - C_C(K_{CC} - K_{AC})}{1 - C_C(K_{CC} + K_{AC}^2 y)}\right]^2, \quad (3)$$

and

$$C_{TC} = C_C\left[\frac{1 + K_{AC}y}{1 - C_C(K_{CC} + K_{AC}^2 y)}\right]^2. \quad (4)$$

The Eqs. (3) and (4) enable one to calculate the unknown concentration C_C, constant K_{AC}, with the known K_{CC} [32], K_{AA} (obtained from separate experiment), C_A, C_{TA}, and

C_{TC} (results of spectroscopy titration). The solution of the Eqs. (3) and (4) has to be found numerically. Using the properties of the partition function Z [28], it is possible to find the unknown "concentrations of neighborhoods" AA, CC, and AC

$$C_{AA} = K_{AA}\frac{\partial Z}{\partial K_{AA}} = \frac{1}{2}C_A^2\left[\frac{1 - C_C(K_{CC} - K_{AC})}{1 - C_C(K_{CC} + K_{AC}^2 y)}\right]^2, \quad (5)$$

$$C_{CC} = K_{CC}\frac{\partial Z}{\partial K_{CC}} = K_{CC}\left[\frac{C_C(1 + K_{AC}y)}{1 - C_C(K_{CC} + K_{AC}^2 y)}\right]^2, \quad (6)$$

and

$$C_{AC} = K_{CC}\frac{\partial Z}{\partial K_{AC}} = K_{AC}\frac{2C_C y(1 + K_{AC}y)[1 - C_C(K_{CC} - K_{AC})]}{[1 - C_C(K_{CC} + K_{AC}^2 y)]^2}, \quad (7)$$

C_{AA}, C_{CC}, and C_{AC} are not molar concentrations of AA, CC, and AC. They take into account all neighborhoods in all possible oligomers, respectively. However, if free component concentrations of the system (e.g. C_A and C_C) are known, it is possible to calculate the molar concentrations of components A (X_{AA}) in A dimers, and molar concentrations of C (X_{CC}) in C oligomers [27]

$$X_{AA} = K_{AA}C_A^2, \quad (8)$$

and

$$X_{CC} = \frac{K_{CC}C_{CC}^2}{1 - K_{CC}C_C}\left[2 + \frac{K_{CC}C_C}{1 - K_{CC}C_C}\right]. \quad (9)$$

The molecular concentrations of A and C bound in mixed oligomers (X_{BA} and X_{BC}) can be calculated based on mass conservation law

$$X_{BA} = C_{TA} - C_A - X_{AA}, \quad (10)$$

and

$$X_{BC} = C_{TC} - C_C - X_{CC}. \quad (11)$$

For calculation of the parameter of interaction of the component, we used the method of non-linear regression included in the Mathcad Plus 6 (MathSoft Inc.) software, based on Marqardt–Levenberg method, which fits the experimental data of colorimetric titration with Eqs. (3)–(11).

2.4. Microcalorimetry

Heat of MIT–CAF interaction was measured using differential Omega (Microcal Inc., Northampton, MA) Titration Microcalorimeter [35], 10 portions of the titrant (MIT), 10 μL each, were added to 1.3 mL of the solution of CAF, and the heat of the process was measured as a function of time (μcal/s). Data were corrected for CAF and MIT heat of dilution (which included the heat of the component depolymerization.

2.5. Molecular modeling we calculated using HyperChem (Hypercube Inc.) software

The molecular mechanics (MM+), and then semi-empirical method (PM3) allowed us to calculate electronic properties, charge distribution, optimized geometry, and obtain the energy minimum of complex formation. To take into consideration both, the hydrophobic and the hydrophilic interactions, we "put" our molecular system into periodic box containing several dozens of molecules of water. The periodic boundary conditions simulate a continuous system with a constant density of molecules.

3. Results

3.1. The absorption spectra and the calculation of the parameters of substrate interaction

The light absorption spectra of DOX and MIT solutions are presented in Fig. 2A and B. The increase of drug's concentration in the sample results in the maximum peak(s) shift to the longer wavelength (bathochromic effect) and the presence of the isosbestic points. As indicated by this spectra's changes, there are only two components present in the mixture. These are namely monomer and dimer forms of the drug tested. The spectra are consistent with those reported previously by us [36] and others [29,37]. Using numerical methods developed previously [27] we obtained the spectra of monomer (the extrapolation of spectra to the infinite dilution of the drug) calculated the spectra of the drugs' dimers, and the dimerization constants of the drugs. The results of these calculations are presented in Fig. 2 and Table 1. One can notice (taking into consideration the difference of ionic strength of the buffers) that there is a very good correlation between the calculated DOX dimerization constants (K_D) and this is reported in the literature [29]. The K_D calculated in this work is most likely more accurate than those reported by us before [36], due to improvement of numerical calculation [27] (all points of the spectrum were used to obtain K_D and MIT dimer spectrum (Fig. 2B), rather than single wave-

Table 1
Dimerization constants (K_D) of doxorubicin and mitoxantrone and caffeine isodesmic association constant

Compounds	($K_D \pm SE$) $\times 10^{-4}$ M^{-1}	
	Literature	This paper
Doxorubicin	1.7 ± 2[a]	1.30 ± 0.03[b]
	1.3[c]	
Mitoxantrone	3.0 ± 0.4[d]	5.48 ± 0.06[b]
Caffeine	0.00113[e]	–

[a] 0.15 M NaCl, 50 mM Tris–HCl, pH 7.0, 22° [29].
[b] 0.1 M NaCl, 5 mM Hepes, pH 7.1, 25°.
[c] [37].
[d] 0.15 M NaCl, 5 mM Hepes, pH 7.0, 25° [36].
[e] Isodesmic "neighborhood association constant" (K_{AA}) [32].

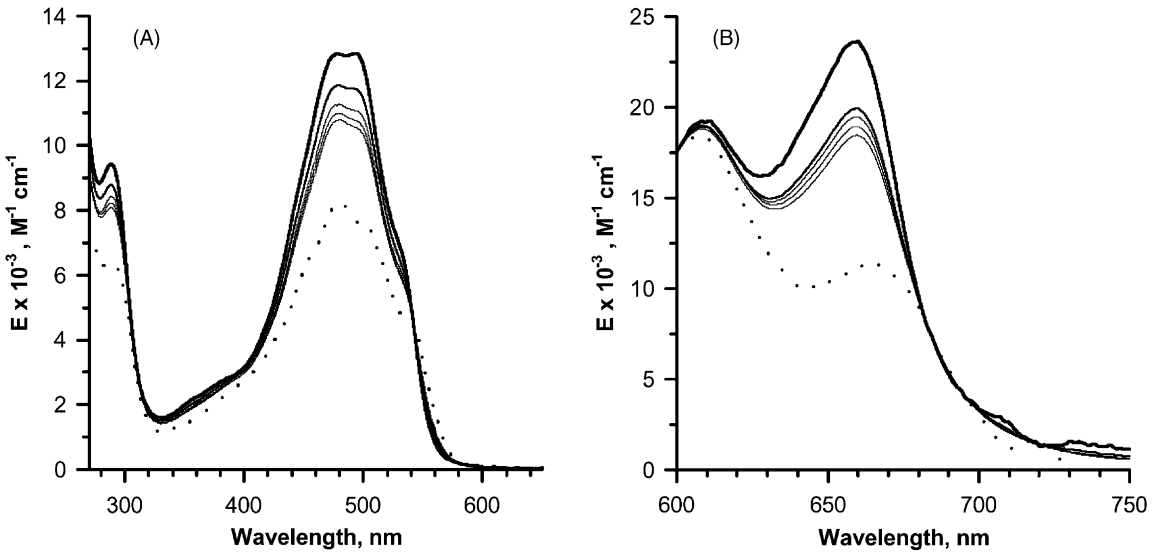

Fig. 2. Spectrophotometric spectra of the drugs. (A) Spectrum of doxorubicin (concentration, thin lines from top: 0.16, 0.66, 1.47, 2.6 mM); spectrum of doxorubicin monomer, and the dimer, bold solid line (top) and dashed line (bottom), respectively. (B) Spectrum of mitoxantrone (concentration, thin lines from top: 5.44, 7.01, 8.96, 10.7 mM); spectrum of mitoxantrone monomer, and the dimer, bold solid line (top), and dashed line (bottom), respectively.

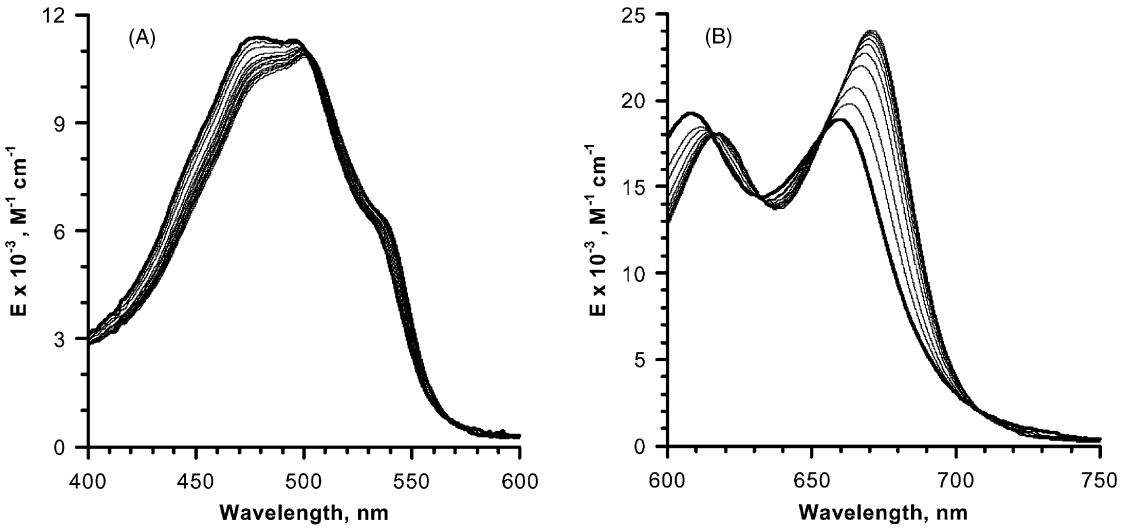

Fig. 3. Spectrophotometric titrations of the drugs with caffeine. The changes in the electronic spectra of caffeine are not shown. (A) Doxorubicin: thin lines from the top to the bottom. Concentrations of the components in the mixtures are given in Table 1. The upper thick solid line on the top represents the spectrum of free doxorubicin (sample #0 in Table 1). (B) Mitoxantrone: thin lines from the top to the bottom. Concentrations of the components in the mixture are given in Table 2. The upper thick solid line on the top represents the spectrum of free mitoxantrone.

length as used by us before). This is supported by much smaller standard error of estimation (see Table 1).

The absorption spectra's changes of DOX and MIT titrated with CAF is presented in Fig. 3. The range of wavelength has been chosen in such a way to reflect only changes in structure of drugs, as CAF does not absorb over 350 nm. Hypochromic and bathochromic effects are noticeable, but there are no isosbestic points because more than two components are present in the mixture. Using the method we described before [27], we tried to decompose these spectra by non-linear regression (three-component, e.g. monomer, dimer and monomer complexed with CAF) analysis (not shown). The errors of such procedure were considerable so it indicated the presence of at least one more component in the solution. By the error analysis, described by us previously [27], we calculated the spectrum of such complexes. This data suggests that the dimer of drugs is most likely complexed with CAF. The spectra of all four component presented in the mixture (except free CAF, which does not absorb in measured wavelength range) are presented in Figs. 4 and 5.[1] This precise

[1] It should be mentioned that the changes in light absorption spectra's of several fluorochromes were induced by CAF, as it has been observed before. According to us, these have been interpreted incorrectly, as a cationic dye "monomerization" [38], because one can observe the evident differences between spectra of the monomer of the dye, and the dyes—CAF mixtures presented in this paper (Fig. 2) and other publications, e.g. [2,25,27,28].

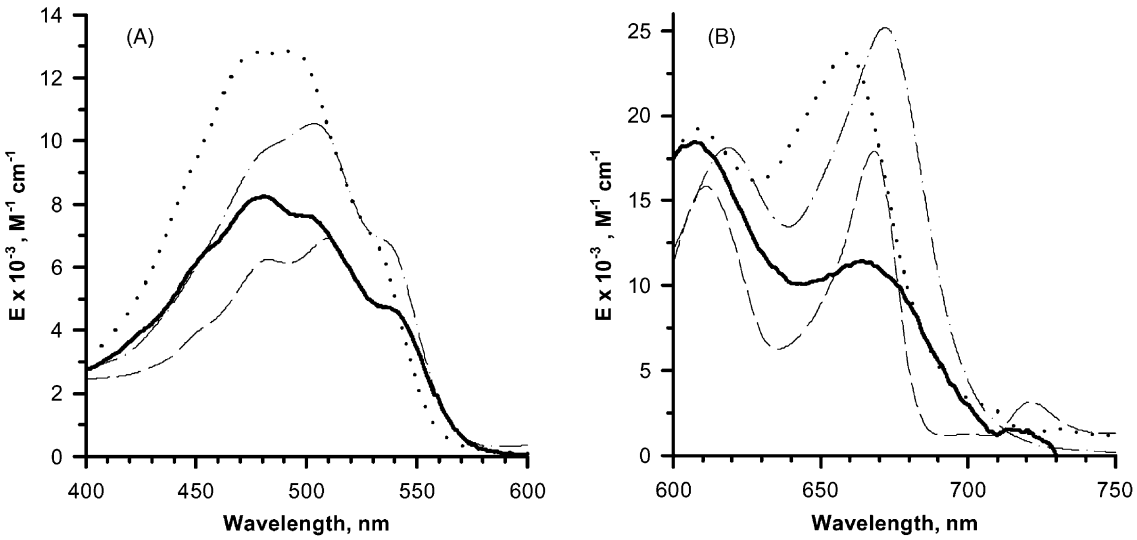

Fig. 4. Spectra of the calculated numerically (as described in Section 2) components of the drugs and their complexes with caffeine. (A) Doxorubicin's spectra maxima from the top to the bottom DOX monomer, DOX monomer–CAF complex, DOX dimer and DOX dimer–CAF complex. (B) Mitoxantrone; maxima from the top to the bottom: MIT monomer–CAF complex, MIT monomer, MIT dimer–CAF complex, and MIT dimer.

analysis of the spectra indicated the presence of complex of the drug's dimer with CAF is not in the agreement with a simple, 1:1 model, of the stacking complex formation of the CAF with aromatic compounds, proposed before [6]. While the drugs dimerization is dependent on the ionic strength, and the experiments described in [6] were performed in solution not containing NaCl (only 5 mM Hepes), the self-aggregation is not occurring [27]. The agreement of the association constant K for acridine orange—CAF claimed by the authors of [6] with K_{AC} value reported by us [27] is coincidental, because K is classical "molecular" association constant, while the K_{AC} denotes statistical-thermodynamic "neighborhood" association constant, and these two constants cannot be compared. The collected spectra of the drug–CAF mixtures (Fig. 3) were, therefore, decomposed into a weight sum of these four component spectra, by non-linear regression analysis as described before [27]. The example of this procedure performed for DOX–CAF (Table 2, sample #5), and MIT–CAF (Table 3, sample #9) are presented in

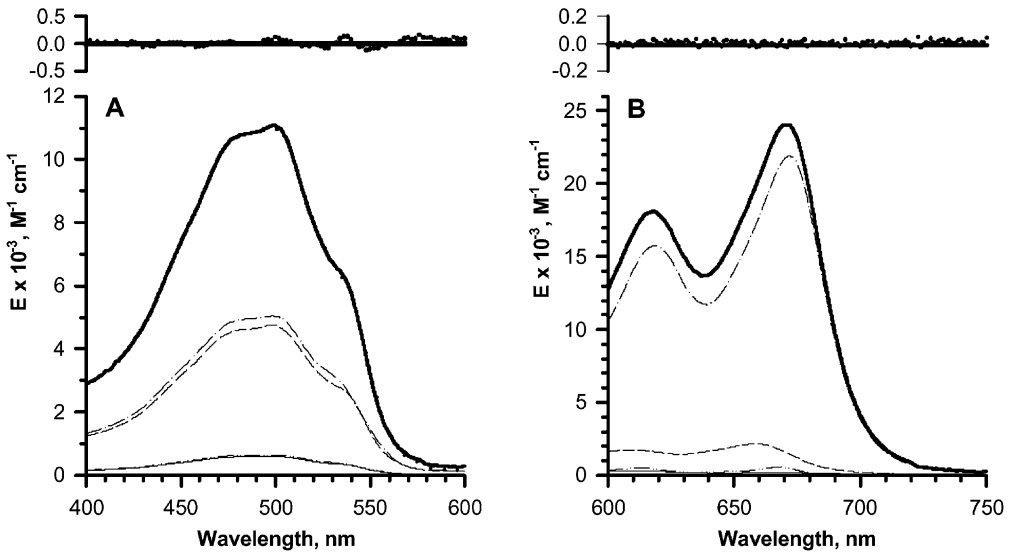

Fig. 5. The example of four-parameter (e.g. spectra shown in Fig. 4) analysis of doxorubicin–, and mitoxantrone–caffeine mixtures. On the top of the pictures the residuals (notice the magnification of scale) of the measured spectrum and weighted sum of components spectra (presented in Fig. 4, multiplied by their molar fractions in the mixture) are shown. (A) Doxorubicin–caffeine mixture (composition see sample #5, Table 2); at top is the spectrum of the sample #5, next to the bottom: component weighted spectra: DOX monomer–CAF, DOX monomer, and overlapped spectra DOX dimer and DOX dimer–CAF. (B) Mitoxantrone–caffeine mixture (composition see sample #9, Table 3). At top is the spectrum of the sample #9, next to the bottom: component weighted spectra: MIT monomer–CAF, MIT monomer, MIT dimer and MIT dimer–CAF.

Table 2
Titration of doxorubicin with caffeine in 5 mM Hepes, 0.1 M NaCl, pH 7.1 at 25°

Sample #	C_{TC} (mM)	C_{TA} (μM)	C_C (mM)	C_A (μM)	$C_{A'}$ (μM)	C_{CC} (mM)	C_{AA} (pM)	C_{AC} (μM)	X_{CC} (mM)	X_{AA} (μM)	X_{BA} (μM)	K_{AC} (M^{-1})
0	0.00	11.91	0.00	9.54	9.54	0.00	45.5	0.00	0.00	2.37	0.00	–
1	0.49	11.85	0.48	8.12	8.58	0.00	41.6	1.27	0.00	1.92	1.35	200.7
2	0.98	11.79	0.95	7.31	7.77	0.01	38.1	2.38	0.02	1.57	2.45	170.0
3	1.93	11.68	1.85	6.24	6.47	0.04	32.4	4.23	0.08	1.09	4.11	142.1
4	2.87	11.56	2.69	5.49	5.49	0.09	27.8	5.70	0.17	0.78	5.28	128.3
5	3.79	11.45	3.49	4.91	4.74	0.15	24.1	6.89	0.29	0.58	6.13	120.3
6	4.70	11.34	4.25	4.35	4.14	0.23	21.2	7.86	0.44	0.44	6.76	118.8
7	5.60	11.24	4.96	4.08	3.65	0.31	18.8	8.67	0.61	0.35	7.23	109.8
8	7.30	11.03	6.29	3.55	2.93	0.52	15.0	9.90	1.00	0.22	7.87	101.2
9	9.00	10.83	7.50	3.10	2.42	0.76	12.3	10.77	1.45	0.15	8.26	97.2
10	10.60	10.63	8.60	2.82	2.04	1.03	10.3	11.40	1.95	0.11	8.48	91.5
11	12.90	10.36	10.00	2.46	1.64	1.47	8.1	12.05	2.76	0.07	8.65	86.7

C_{TC}: total CAF concentration; C_{TA}: total DOX concentration; C_C: concentration of CAF monomer; C_A: concentration of monomer DOX (measured spectrophotometrically); $C_{A'}$: concentration of monomer DOX; C_{CC}: concentration of neighborhoods CAF–CAF complexes; C_{AA}: concentration of neighborhoods DOX–DOX complexes; C_{AC}: concentration of neighborhoods DOX–CAF complexes; X_{CC}: concentration of CAF–CAF complexes; X_{AA}: concentration of DOX dimers; X_{BA}: concentration of DOX–CAF complexes; K_{AC}: association constant DOX–CAF; mean $K_{AC} = 128.3 \pm 10$ (SE).

Fig. 4. The weight coefficients are equal to the molar fraction of drug in the particular component (e.g. drug monomer, dimer and their complexes with CAF). Knowing these fractions and the total ''molecular'' concentration of drug (C_{TA}) and CAF (C_{TC}) we calculated ''molecular'' concentration of the free, and dimer concentration ($X_A = C_A$ and X_{AA}, respectively), and the concentration of drug monomer and dimer associated with CAF (X_{CA} and X_{AA}, respectively).

Knowing the association constant of nearest neighborhood of drug ($K_{AA} = 2K_D$), CAF (K_{CC}) associations (Table 1), measured concentration of free drug (C_A), and using Eqs. (3)–(7), we were able to calculate numerically nearest neighbors equilibrium constant of the drug–CAF association (K_{AC}) and concentrations of all possible neighborhoods between molecules of the drugs in the samples (Tables 2 and 3, the latter presented in the abbreviated form). Having calculated values of K_{AC} and using Eqs. (8)–(11) we calculated molar concentrations of components of the drug–CAF interaction, as described above (calculations). The comparisons of the measured and calculated ''molecular'' concentrations of components presented in the drug–CAF mixtures are shown in Fig. 6. There is a good correlation between these data, in both cases. As the CAF concentration in the solution is rising, the concentration of DOX and MIT molecules (in monomer or dimer form) is decreasing, and the concentration of bound DOX and MIT is increasing.

3.2. Microcalorimetric titration of CAF with MIT

Fig. 7 indicates the favorable enthalpy ($\Delta H = -11.3$ kcal/mol) of MIT–CAF complex formation. This result is the next proof that intercalator–CAF complex formation involves interaction of polycyclic aromatic rings of the reactants. The details of the experiment and calculation are given in Section 2.4 and in the legend to Fig. 7. The possibility of (π–π) complex formation between Q and CAF was also supported by molecular modeling.

Table 3
Titration of mitoxantrone (MIT) with caffeine (CAF) in 5 mM Hepes, 0.1 M NaCl, pH 7.1 at 25°

Sample #	C_{TC} (mM)	C_{TA} (mM)	C_C (mM)	C_A (μM)	$C_{A'}$ (μM)	X_{CC} (mM)	X_{AA} (μM)	X_{BA} (μM)	K_{AC} (M^{-1})
0	0.00	9.93	0.00	5.99	5.99	0.00	3.94	0.00	–
1	0.49	9.88	0.48	4.23	4.73	0.00	2.45	2.70	543.4
2	0.97	9.83	0.95	3.62	3.84	0.02	1.62	4.38	411.1
3	1.93	9.74	1.84	2.69	2.69	0.08	0.79	6.25	356.4
4	2.87	9.64	2.69	2.08	2.00	0.17	0.44	7.20	340.0
5	3.78	9.55	3.48	1.66	1.55	0.29	0.26	7.73	333.0
6	4.69	9.46	4.24	1.33	1.24	0.44	0.17	8.05	334.6
7	5.57	9.37	4.95	1.10	1.02	0.60	0.11	8.23	335.4
8	6.44	9.28	5.63	0.87	0.86	0.79	0.08	8.34	352.7
9	7.29	9.20	6.28	0.76	0.73	1.00	0.06	8.41	344.2

Total concentrations of CAF (C_{TC}) and MIT (C_{TA}); concentrations of monomers of CAF (C_C) and MIT: measured spectrophotometricaly (C_A), calculated based on mean K_{AC} an Eqs. (2)–(8): ($C_{A'}$); concentrations of CAF-CAF complexes (X_{CC}), MIT dimers (X_{AA}) and MIT complexed with CAF (X_{BA}); association constant of mixed aggregation CAF with MIT (K_{AC}), mean $K_{AC} = 356.4 \pm 21$ (SE)

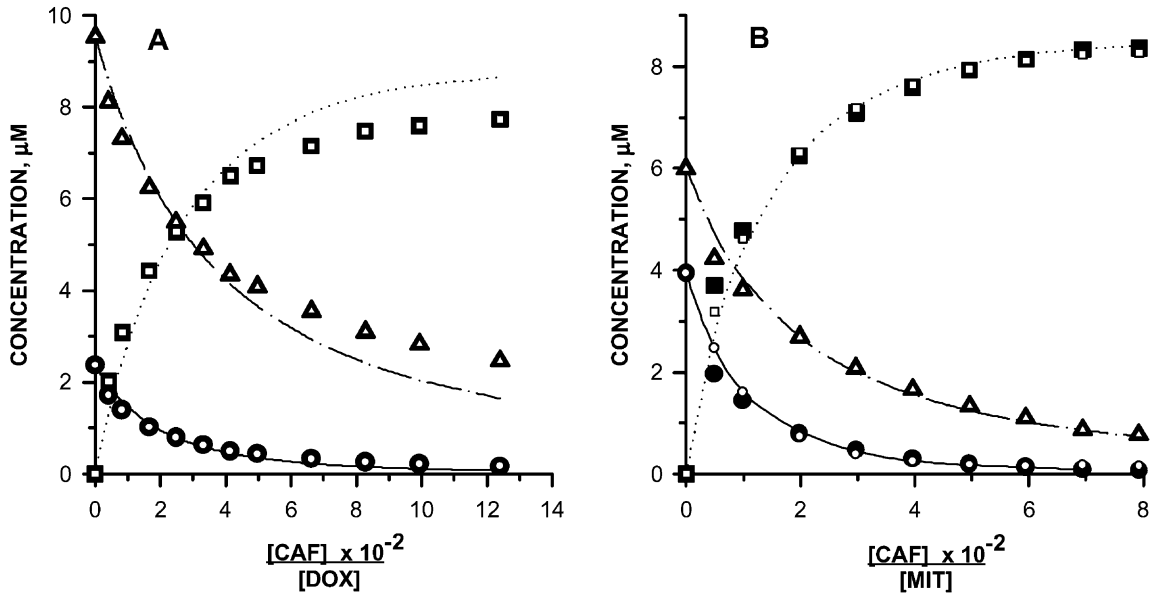

Fig. 6. The comparison of the results of four-parameter analysis of the mixtures doxorubicin or mitoxantrone with caffeine. Lines represent concentrations calculated using Eqs. (2)–(11), the mean values of K_{AC} showed in Tables 2 and 3, and the concentrations of free monomer of the drug C_A measured spectrophotometrically and the known C_{TA}, C_{TC} values (listed in Table 2) for doxorubicin (A), and mitoxantrone (Table 3) (B). Dotted lines represent concentrations of molecules of the drugs in mixed aggregates, dash-dotted line represents concentrations of free monomer, and solid line represents concentrations of free dimer of the drugs, respectively. Closed symbols represent concentrations calculated using data (C_{TA}, C_{TG} and C_A listed in Tables 2 and 3) for doxorubicin and mitoxantrone, respectively. Squares represent concentrations of the drugs bound in complex with caffeine; triangles represent concentrations of free monomer of the drug and circles represent concentrations of free dimer of the drug. Opened symbols represent the same concentrations as described above calculated using four-parameter analysis of the spectra (an example of such analysis is given in Fig. 5).

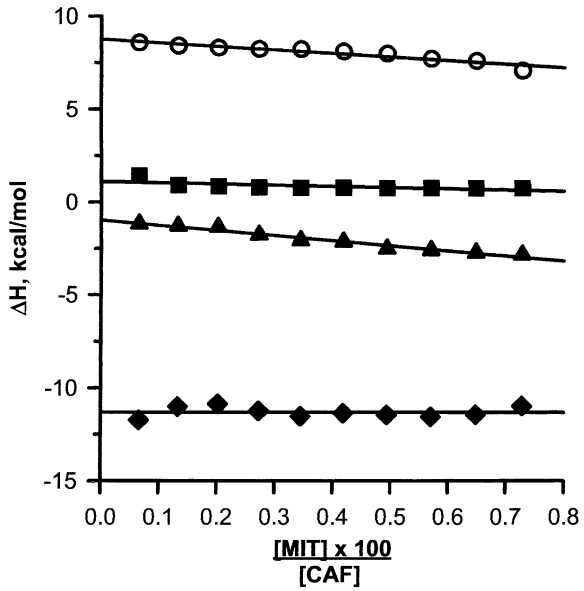

Fig. 7. Microcalorimetric titration of caffeine with the buffer (○), buffer with mitoxantrone (■) and caffeine with mitoxantrone (▲) calculated as kcal/mol of titrant injected (with exception of the titration of caffeine with buffer, which has been corrected for the heat of titration of the buffer with buffer, and expressed as a heat of dilution per injection). The enthalpy change (ΔH, ◆) of mitoxantrone–caffeine interaction was calculated from the above data by subtracting the sum of the first two sets from the latter and the extrapolation of experimental points ([mitoxantrone]/caffeine]) → 0 (lines).

3.3. Molecular modeling calculations

In the aqueous solution at pH ≅ 7 the DOX molecules, like other anthracycline drugs containing amino sugar moiety daunosamine ($pK_a = 8.4$, [39]), are predominantly protonated ($DOXH^+$). The MIT was commercially obtained in the form of dihydrochloride. In our earlier studies [30] we have shown, that at neutral pH, it is the mixture of about equimolar mono- and di-protonated molecules in the solution. For molecular modeling calculation of the drug–CAF interaction we used mono-protonated $DOXH^+$ and $MITH^+$ in the presence of large amount of water molecules. The result of these semi-empirical calculations is presented in Fig. 8. It should be mentioned that there are eight and four possible structures for 1:1 complexes of CAF with $DOXH^+$ and $MITH^+$, respectively. Only one of each (most likely) conformer structures is presented in these drawings. There is, of course, possibility of other than 1:1 stechiometry CAF–drug complex formation. For example, the result of optimization of geometry calculation of the $MITH^+$–$(CAF)_2$ is presented in Fig. 8E and F. The favorable energy formation of this complex is the one more proof for the correctness of the mixed aggregation of CAF with the aromatic polycyclic compounds used in this work. More details of the geometry and the energy formation of 1:1 and 1:2 complexes are given in the legend to Fig. 8.

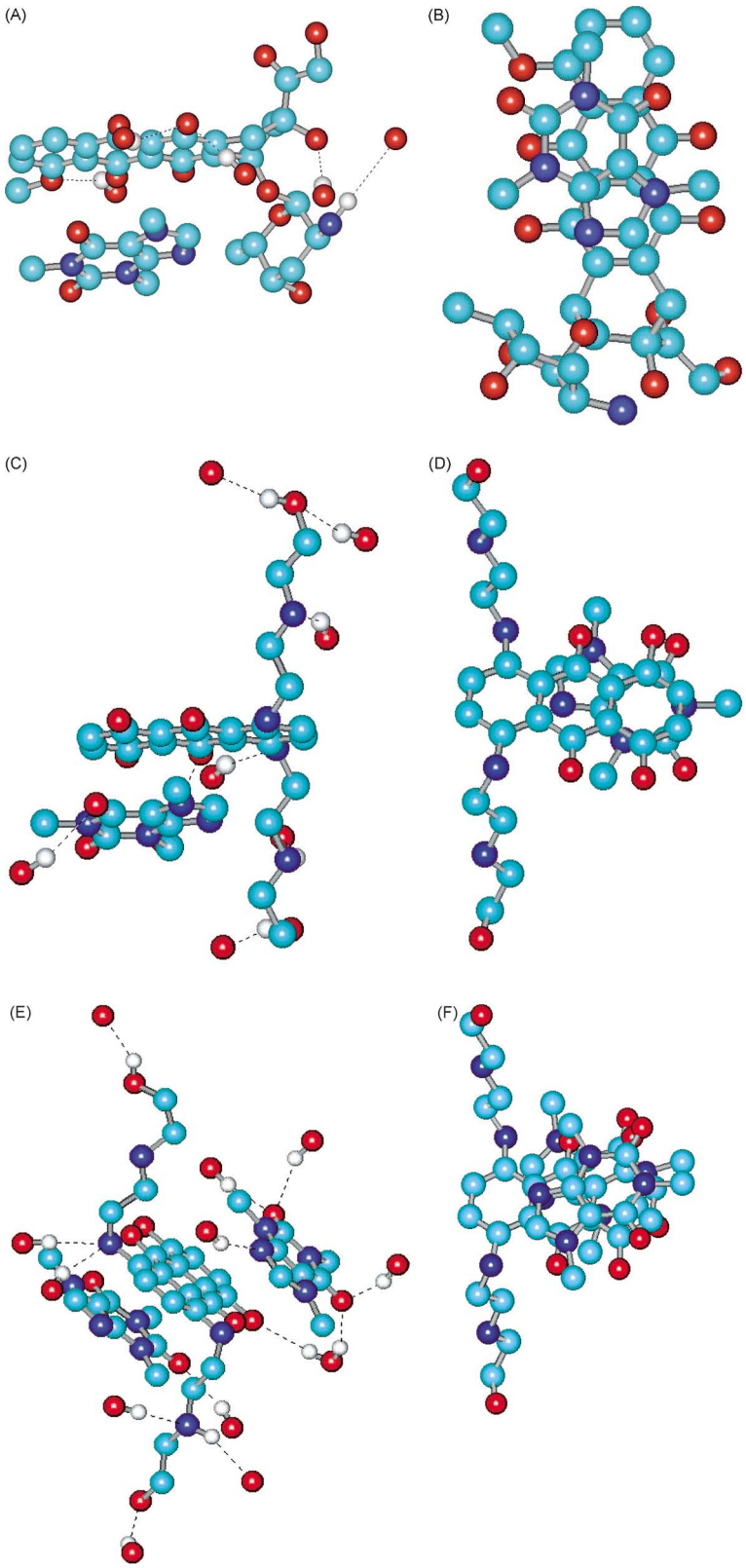

Fig. 8. Molecular modeling geometry optimization of the $DOXH^+$–CAF complex in the presence of 133 water molecules (colored version). All water molecules, with exception of this which form hydrogen bond (doted lines) have been removed (A: side view, B: from the top view) The energy of the complex formation corrected for the energy of formation of the components and the water shell molecules not attached by hydrogen bonding $\Delta E = -7.4$ kcal/mol, the winding angle between the axes of aromatic anthracene system and the xanthine rings $\alpha = 30°$. (C, D) The structure of 1:1 $MITH^+$–CAF complex calculated in the presence of 77 water molecules; $\Delta E = -8.7$ kcal/mol, $\alpha = 26°$. (E, F) The structure of 1:2 $MITH^+$–$(CAF)_2$ calculated in the presence of 141 water molecules; $\Delta E = -29.9$ kcal/mol, $\alpha_1 = 20°$, $\alpha_2 = 30°$. For the clarity of the picture, the double bonds and hydrogen atoms in the parts B, D and E have not been shown. Hydrogen atom: small grey, carbon: cyan, nitrogen: deep blue, and oxygen atom: red spheres.

4. Discussion

Recently accumulated statistical data indicate the protective effect of caffeine consumption against several types of cancer diseases. There are also reports about protective effect of caffeine and other xanthines against tumors induced by PAHs. One of the explanations of this phenomenon is based on biological activation of such carcinogens by cytochromes that are also known for metabolism of caffeine. In the accompanying paper (Part I, [2]) we provide evidence (flow cytometry and the cell cycle analysis) that the CAF on two DNA alkylating agents, which do not require the biological activation, depend on their ability to form stacking (π–π) complexes. In this study, we use physicochemical techniques (computer aided light absorption and microcalorimetry), and molecular modeling to examine previously published qualitative data. This published both by our and other groups data, indicates that CAF is able to modify the cytotoxic and/or cytostatic action of the two well known antitumor drugs DOX and MIT. To obtain the quantitative results from the experimental data we used the statistical-thermodynamical model of mixed aggregation, to find the ''neighborhood'' association constants K_{AC} of the CAF–drug interaction ($128 \pm 10\ M^{-1}$ and $356 \pm 21\ M^{-1}$ for DOX–CAF and MIT–CAF complex formation, respectively). In addition, the favorable enthalpy change of CAF–MIT ($\Delta H = -11.3$ kcal/mol) was measured by microcalorimetry titration. The molecular modeling (semi-empirical and force field method) allowed us to obtain the geometry of these complexes, which also indicated the favorable energy (ΔE) of complex formation the protonated drug's molecules in aqueous environment (−7.4 and −8.7 kcal/mol for DOX–CAF·$5H_2O$ and MIT–CAF·$8H_2O$ complex, respectively). The molecular modeling calculation indicates the existence of CAF–drug complex in which the MIT molecules are intercalated between two CAF molecules ($\Delta E = -29.9$ kcal/mol). These results indicate that the attenuating effect of caffeine on cytotoxic or mutagenic effects of some polycyclic aromatic mutagens cannot be the result of metabolic activation in the cells but simply is the physicochemical process of the sequestering of aromatic molecules (e.g. carcinogens or mutagens) by formation of the stacking complexes. It should be mentioned, however, that the geometry of DOX–CAF and MIT–CAF complexes presented in this report (Fig. 8A–D) differs from the structures described before. The molecular modeling calculation described in this report has not taken under consideration that both DOX and MIT molecules are protonated and without including the water molecules in the energy computation. The need of taking these factors into consideration in molecular modeling of the aqueous systems seems to be important in the view of the well-known fact that hydrophobic (in this case π–π) interactions are competitive to hydrophilic one. In addition, we have shown before that releasing of water molecules bound to substrates during complex formation can increase the enthalpy of the system [28] and the accompanying paper [2]. The energy of complex formation calculated by us (in the aqueous environment) is less negative (−7.4 and −8.7 kcal/mol for DOX–CAF·$5H_2O$ and MIT–CAF·$8H_2O$ complex), than those calculated *in vacuo* (−15.089 and −11.997 kcal/mol, respectively) [6].

As one can see from Fig. 6 and the data published before [6,7,25,27,28,36,40] that, because of relatively low values of association constants, the effective binding of the DNA-damaging agents required high (mM) CAFs concentration. The question, therefore, arises if this phenomenon has any relevance to the protective effect of CAF consumptions against the risk of cancer development reported in several population statistic studies. One of the facts that support this hypothesis is that none of such effects was observed in the action of inorganic, aliphatic or physical agent (e.g. UV or X radiation). It was observed exclusively in the case of DNA-damaging agents having the flat, aromatic moieties, which are able to form of stacking (π–π) complexes with xanthines. In most cases such high concentration of ''intercepting'' agents has been used either in cell ([2] and the reference cited therein) or bacteria [16] cell's culture experiments, or when the tumor inducing agent was administrated together with high concentration of xanthines (e.g. in the same place of the entry of the agent). The classical example of such study is tumor induction by the PAH hydrocarbons (from tobacco smoke condensate), when applied on the skin of animal together with CAF [41], or feeding rats with the mixture of DMBA and CAF [42,43]. It is quite unlikely that the consumption of CAF containing drinks by humans can produce mM concentration of CAF in the blood stream. Such concentration, however, can be easily reached in the upper stomach by consumption of one cup of coffee (60–200 mg). At this concentration the CAF can ''intercept'' some carcinogens present in food thus reducing their mutagenic effects (e.g. [17,44]). One can speculate, for example, that this interception may reduce the absorption of mutagen in the upper digestive track. As mentioned above some, but not all, carcinogens required biological activation (e.g. by cytochromes P450). The possible explanation of antagonistic effect of CAF and this activation is the fact that CAF is also metabolized by this enzyme, and may compete with carcinogens for the cytochromes [4]. Another explanation, which we believe is most likely, is that in the complex (especially this presented in Fig. 8E and F) the aromatic DNA-damaging agents is protected by CAF molecules against the action of enzyme.

As explained before, CAF concentration in the human blood, which is high enough to intercept the mutagen molecule, cannot be achieved while drinking reasonable amount of CAF containing drinks. However, the one very interesting studies were reported (Denda *et al.* [45]) in which the effect of the pancreatic tumorigenesis induced by 4-hydroxyaminoquinoline-1-oxide (4-HAQO) in rats

were examined. In this experiment, the 4-HAQO and CAF (the later at the maximum tolerated dose, i.e. up to 120 mg/kg body weight) were injected i.v. or s.c.). Such high CAF concentration in the blood stream will be harmful for human and cannot be reached by drinking of CAF-contained beverages (an equivalent of consuming of more than 100 cups of coffee) but is likely that in extremely high concentration of CAF injected i.v. [45] intercepts the mutagen and forms the stacking complex in blood. This can support the hypothesis that the interception of mutagens can occur most likely in the upper digestive track. Denda *et al.* [45] also reported that the CAF has dose-dependent effect in decreasing of the total number of nodules developed. Interestingly, these authors observed that the low dose (30 mg/kg body weight) CAF has an opposite effect and actually potentates the tumorigenesis of 4-HAQO. The later effect can be explained by the suppression of DNA repair mechanism by CAF, observed frequently in the case of inorganic, aliphatic of physical mutagenic agents. The answer to the question whether the CAFs effect is of protective, or the mutagenic enhancing nature, could only be established by broad population statistical study. The data of such study published so far indicate that the former effect is predominant (see references cited in our accompanied paper (Part I), and [28]). There is, however, no indication, both in the literature, or in our experiments, that xanthines such as CAF can reverse the damage to nucleic acids when this damage to DNA has already been done. We have finished studies of CAF, and other xanthine–pentoxifylline, complex formation with some other aromatic compound (e.g. ellipticine, ethidium, propidium and fluoresceine) that will be published soon as consecutive parts of this series.

Note added in proof

Following submission of this paper several papers have been printed which are not contradictory to our findings and may be of interest to cytologists (e.g., E. Bender *et al.*, Cytometry 2001;43:38–45 and B. Ardelt *et al.*, Int J Oncol 2001;18:849–53), or scientists interested in stacking complex formation (D.B. Davies *et al.*, Molec Phys 1999;97: 439–51, Molec Phys 2000;98:1961–71, J Chem Perkin Trans 2001;2:61–67 and Eur Biophys J 2001;30: 354–66).

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