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Biochemical Pharmacology 63 (2002) 625–634

Biochemical Pharmacology

The modulation of the DNA-damaging effect of polycyclic aromatic agents by xanthines Part I. Reduction of cytostatic effects of quinacrine mustard by caffeine☆

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Received 30 January 2001; accepted 15 October 2001

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 is based on biological activation of such carcinogens by cytochromes that are also known for metabolism of caffeine. However, there is also numerous data indicating reverse effect on cytotoxicity of anticancer drugs that inhibit the action of topoisomerase I (e.g. Camptothecin or Topotecan) and topoisomerase II inhibitors (e.g. Doxorubicin, Mitoxantrone or mAMSA). In this work we tested the hypothesis that the caffeine protective effect is the result of sequestering of aromatic mutagens by formation of stacking (π–π) complexes. As the models for the study we have chosen two well-known mutagens, that do not require metabolical activation: quinacrine mustard(QM, aromatic, heterocyclic nitrogen mustard) and mechlorethamine (NM2, aliphatic nitrogen mustard). The flow cytometry study of these agents' action on the cell cycle of HL-60 cells indicated that caffeine prevents the cytotoxic action of QM, but not that of NM2. The formations of stacking complexes of QM with caffeine were confirmed by light absorption, calorimetric measurements and by molecular modeling calculation. Using the statistical thermodynamics calculations we calculated the ''neighborhood'' association constant ($K_{AC} = 59 \pm 2\ M^{-1}$) and enthalpy change ($\Delta H^{0'} = -116\ cal\ mol^{-1}$); the favorable entropy change of complex formation ($\Delta S^{0'} = 7.72\ cal\ mol^{-1}\ K^{-1}$, due to release of several water molecules, associated with components in the process of complex formation). The Gibbs' free energy change of QM–CAF formation is $\Delta G^{0'} = -2.41\ kcal\ mol^{-1}$. We were unable to detect any interaction between NM2 and caffeine either by spectroscopic or calorimetric measurement. In order to establish, whether the intercalation of QM plays any role in cytotoxic effect we tested, as a control, non-alkylatiatig, but also intercalating QM derivative—quinacrine (Q). The later had no cytostatic effect on HL-60 cell even at there order of higher concentration than QM or NM2 but, similar to QM forms (which we demonstrated) stacking complexes with caffeine ($K_{AC} = 75 \pm 3\ M^{-1}$). These results strongly indicate, that the attenuating effect of caffeine on cytotoxic or mutagenic effects of some mutagens, is not the results of metabolic processes in the cells, but simply the physicochemical process of sequestering of aromatic molecules (potential carcinogens or mutagens) by formation of stacking complexes with them. 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 concentration of mM level). There is, however, no indication either in the literature or in our experiments that xanthines can reverse the damage to nucleic acids when the damage to DNA has already occurred.

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

☆ Presented in part at 3rd International Students' Scientific Conference, Gdansk [1].

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Abbreviations: CAF, caffeine; Q, quinacrine; QM, quinacrine mustard; NM2, mechlorethamine; DAPI, 2,4′-diamidino-6-phenylindole dichloride; dsDNA, double stranded DNA; 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; C_{CC} C_{AA} and C_{AC}, the ''nearest neighbors'' concentrations; X, ''molecular'' concentration; Z, denote partition function equal to the summation of statistical weight of all possible oligomers.

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00904-2

1. Introduction

Caffeine (CAF) has a myriad of effects on living organisms. In oncology, most studies have focused on the propensity of CAF to potentate the cytological effects of variety of DNA damaging agents, such as an ionizing radiation, alkylating compounds, *cis*-platinium and cyclophosphamide, hydroxyurea and others [2–7]. The mechanism by which CAF enhances the toxicity of these agents is believed to involve suppression of DNA repair and/or DNA synthesis [8–10]. Because the CAF is probably the most commonly used alkaloid worldwide, these findings intensified the cancer epidemiological study on the adverse effects, the consumption of the CAF containing beverages, i.e. coffee, tea and cocoa. The recent reviews of medical literature on this subject suggest [11] that coffee does not have appreciable effect on a number of diseases but the CAF has not been shown to be teratogenic [12].

Surprisingly, there were certain published studies which indicate that CAF consumption was inversely associated with the cancer risk of a digestive tract [13–15], breast [16,17], pancreas [18] or thyroid [19]. A biologic mechanism for such risk reduction has not been established [20]. There is very interesting study by Rothwell which describes a dose-related inhibition of chemical carcinogenesis (cigarette smoke condensate) in mouse skin by CAF [21]. The effect of CAF on cyclic AMP synthesis was considered as a possible explanation of this phenomenon.

Caffeine consumption can also significantly reduce mammary carcinoma multiplicity induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) in rats [22]. Recently published study on a benzo[*a*]pyrene (BP) induced lung tumor in mice confirmed that CAF is among cancer preventive agents [23]. Both DMBA and BP are procarcinogens and require biological activation by cytochromes P450 [24,25]. These enzymes also metabolize CAF and it was proposed that protective effect of CAF is a result of competitive interaction with cytochromes [26,27]. However, similar protective effect of CAF has been observed for several polycyclic aromatic compounds that do not require cytochromes for their biological activity. Intercalating anticancer drugs Doxorubicin, Mitoxantrone and mAMSA [28–32], which are believed to be the topoisomerase II blockers, and non-intercalators, topoisomerase I inhibitors, Camptothecin and Topotecan [33] belong to a group of such agents. There is a possibility these phenomena may be a result of CAF having an effect on topoisomerases action, or inhibition of RNA synthesis [32]. However, we believe that formation of stacking (π–π) complexes [34] between CAF and polycyclic aromatic drugs is responsible for the reduction toxicity of these drugs. Our studies confirmed the formation of such complexes with several anticancer drugs [31,33] and the fluorescence DNA ligands DAPI and ethidium bromide [35]. We also proposed statistical thermodymical models that describe the formation of these complexes [36]. According to our hypothesis, the mixed aggregation with the CAF reduces free drug concentration in solution, which is then reflected as diminished pharmacological activity. To confirm this hypothesis we have chosen two well-known mutagenic agents as a subject of this study: QM and NM2. Both these compounds are derivatives of nitrogen mustard (Fig. 1) and in an aqueous solution spontaneously form reactive arizidinium ions that can alkylate nucleic acids and proteins [37,38]. The reason for the selection of QM and NM2 for our studies is that they do not require any enzymatic activation. QM, however, can bind to DNA by intercalation [39] while NM2, as a aliphatic compound, can not.

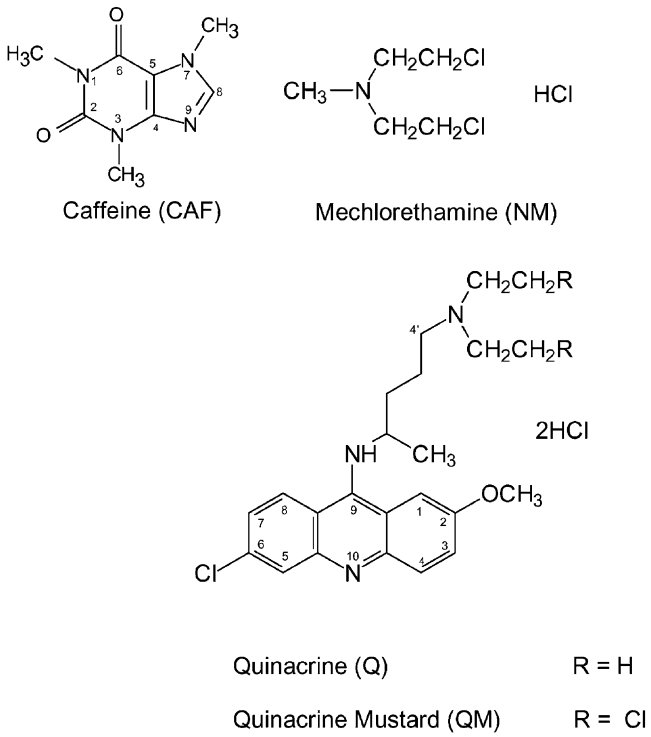

Fig. 1. Chemical structures of CAF, NM2, Q and QM.

To test whether this process has any effect on cell cytotoxity we included the quinacrine (Q, Fig. 1) to our study; this compound intercalates, but does not alkylate nucleic acids.

2. Materials and methods

2.1. Materials

Caffeine (1,2,3-trimethylxanthine, Fig. 1) and quinacrine mustard (3-chloro-7-methoxy-9-(1-methyl-4-2,2′-dichlorodiethyloethylamino-butylamino)acridine, Fig. 1), mechlorethamine (*N*-methyl-2,2′-dichlorodiethylamine, Fig. 1) and Hepes were purchased from Sigma Chemical Co. and quinacrine (3-chloro-7-methoxy-9-(1-methyl-4-diethylethylaminebutylamino)acridine, Fig. 1) was from

Aldrich Chemical Co. Stock solutions were prepared by dissolving their weight amounts in distilled water, and then the stock solutions were diluted in the appropriate buffer (filtered through a 0.45 μm pore Millet Millipore filter).

2.2. Cell cultures

Human promyelocytic leukemia cells (line HL-60) were maintained in suspension culture at 37° in T-75 flasks containing RPMI (Gibco RL) supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc.), 100 unit/mL penicillin, 100 mg/mL streptomycin (Gibco RL), and 2 mM L-glutamine (Gibco RL). The cells were split every other day, and the cell densities in the culture did not exceed 5×10^5 cells/mL.

2.3. Drug treatment of HL-60 cells

Exponentially growing HL-60 cells were exposed for 4 hr to QM (0.2 μM), NM2 (0.2 μM) or Q (25 μM) with or without CAF (final concentration 5 mM). In addition in the control experiment cells were exposed to CAF (5 mM) only. The cells were then washed in HBSS and fixed in 50% ethanol in coldroom (4°).

2.4. Flow cytometry

HL-60 cells were rehydrated from the ethanol fixative by centrifugation and resuspension in 1 mL of HBSS. Then they were stained as previously described [40] using 1.0 μg/mL 2,4′-diamidino-6-phenylindole dichloride (DAPI)·[41] in a buffer containing 10 mM PSA (Calbiochem), 100 mM NaCl, 2 mM $MgCl_2$, and 0.1% Triton X-100 (Sigma), pH 6.8, at 0–4°. The blue fluorescence of DAPI bound to DNA was measured with IPC-22 flow cytometer (Ortho Diagnostic). The data were stored on a computer and analyzed for DNA cell cycle distribution.

2.5. Spectrophotometry

Light absorption spectra were measured using Beckman's DU 650 spectrophotometer connected with Polystat's thermostat constant circulator (25 ± 0.1°). The 2 mL of the buffer (20 mM Hepes, 150 mM NaCl, pH 6.8) containing QM or Q, the initial concentration of which were calculated by measuring their absorption at the isosbestic points ($E_{343} = 5.78 \times 10^3$ and $E_{423} = 9.77 \times 10^3\ M^{-1}\ cm^{-1}$, respectively) were placed in a quartz cuvette (1 cm light path) and titrated with 10–50 μL of CAF (concentration ~ 0.1 M) dissolved in the buffer. The spectra were measured at 1 nm interval and stored on a computer. The spectra were then corrected for the absorption of the buffer and CAF, and expressed in the form of molar absorption coefficience (E_λ, $M^{-1}\ cm^{-1}$). In the case of NM2 the opposite titration procedure (CAF titrated with NM2) was applied.

2.6. Calculations of the association constants of QM or Q with CAF

Calculations of the association constants of QM or Q with CAF were accomplished using statistical thermodynamics of mixed aggregation [36,42]. In this short description of the model, we follow the notation and definition used by Weller *et al.* [42]. The systems contain two types of molecules: A (i.e. QM or Q), and C (CAF) which can form different self- and mixed-aggregates of a type

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

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

Most intercalators form dimers in solution, even in micromolar concentrations, depending on the value of their dimerization constants (K_D). QM and Q, however, have very low K_D equal $45 \pm 3\ M^{-1}$ calculated based on the light absorption measurement, as described before [42] and ~800 [39], respectively, and therefore, they can be omitted in thermodynamic calculation (i.e. j can be reduced to 0 or 1). Relative concentrations of the components are calculated using the partition function Z of the system, which is obtained by adding the statistical weights of all the states accessible to all types of oligomers [36,42–44]. 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 [36,42–44]:

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

Terms C_A and C_C denote the concentration of isolated (i.e. free or "molecular") A and isolated C molecules in solution. K_{CC} and K_{AC} denote the nearest neighbors equilibrium constants of association of C with C and A with C, respectively. The total "molecular" concentrations of A (C_{TA}) and C (C_{TC}), can be expressed in form of equations [44]:

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

and

$$C_{TC} = C_C \frac{\partial Z}{\partial C_C} = C_C \left[\frac{1 + K_{AC} C_A}{1 - C_C (K_{CC} + K_{AC}^2 C_A)} \right]^2. \quad (3)$$

These two equations enable one to calculate the unknown concentration C_C and association constant K_{AC}, knowing K_{CC} ([43], and the references cited therein), and C_A, C_{TA}, C_{TC} obtained from spectroscopy measurements. The solution of the Eqs. (2) and (3) has to be calculated by numerical method. Using the partition function Z, and calculation method in a way similar to that used to obtain Eqs. (2) and (3), it is possible [42,44] to find the concentrations of neighborhoods CC, and AC:

$$C_{CC} = K_{CC} \frac{\partial Z}{\partial K_{CC}} = K_{CC} \left[\frac{C_C (1 + K_{AC} C_A)}{1 - C_C (K_{CC} + K_{AC}^2 C_A)} \right]^2, \quad (4)$$

and

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

It should be remembered that C_{CC}, and C_{AC} are not concentrations of CC, and AC, respectively. They count all neighborhoods in all possible oligomers. For example, in the oligomer ACCCACC, there are, three CC, and three AC neighborhoods. However, if the free component concentrations of the system (e.g. C_A and C_C) are known, it is possible to calculate the ''molecular'' concentrations of components, e.g. in the molecular concentrations of bound A form mass conservation law: $C_{BA} = C_{TA} - C_A$. It is also possible to calculate the molecular concentration of the monomer of CAF in the mixture C_C from Eqs. (2)–(5).

2.7. Microcalorimetry

The microcalorimetric titrations were performed at $30 \pm 0.1^\circ$ in the buffer (50 mM Hepes, 0.15 M NaCl, pH 6.8) using Omega Titration Calorimeter (Microcal Inc.), [45]. Next, portions of the titrant (CAF, 135 mM), 10 μL each, were added to 1.3 mL of the titrated solution and the heat process was measured as a function of time (μcal s^{-1}). The result of titration was corrected for heat of components dilution, and expressed as the heat per injection, as previously described [33,44].

2.8. Molecular modeling was performed using HyperChem, Hypercube Inc. software

We used semi-empirical method (*PM3*) that let one to calculate electronic properties, optimized geometries and total energy. We used geometry optimization method to find a minimum *E* energy (stable) configuration for the molecular system.

These calculations adjust atomic coordinates in steps to find a configuration in which net forces on each atom are reduced to zero; this is usually a local minimum on the potential surface. To take into consideration hydrophobic and hydrophilic forces, we ''put'' our molecular system into periodic box containing the large number of water molecules; these periodic boundary conditions simulate a continuous system with a constant density of molecules.

3. Results

3.1. Cell cycle distribution

Effect of CAF on QM- and NM2-induced perturbation of HL-60 cell cycle. CAF alone, at concentration of up to 5 mM, has relatively (as compared to the action of DNA active drugs) little effect on the cell cycle distribution when analyzed 4 hr after CAF addition [33]. Data presented in Fig. 2A and B and Table 1 confirmed this observation. Exposure of HL-60 cells to equimolar concentration (0.2 μM) of QM and NM2 resulted in arrest of these cells in $G_2 + M$-phase of the cycle, characterized by accumulation of cells in this compartment by 50 and 54%, respectively. Addition of 5 mM CAF at the same time as NM2 slightly changes cell cycle distribution (Fig. 2C vs. D, Table 1) whereas almost completely reduces the $G_2 + M$ block exerts by QM alone (Fig. 2E vs. F). The control experiments with the addition of Q, up to 20 μM concentration, had no measurable effect on the cell cycle of HL-60 cells (data not shown). The absence of cell cycle effect by Q (even at concentration higher by three orders of magnitude than QM and NM2) indicates, that the $G_2 + M$ block produced by these drugs are the result of the presence of nitrogen mustard moiety in the latest drugs, and not their ability to intercalate to dsDNA (both QM and Q are intercalators while NM2 is not). These data indicates that cell cycle effects are connected with the presence of nitrogen mustard group in the drug molecules and, most importantly, that CAF can effectively reduces them in the case aromatic agent only.

Table 1
Effect of caffeine on the cycle perturbation of HL-60 cells induced by NM2 and QM

NM2 (μM)	QM (μM)	CAF (mM)	Cell cycle distribution (%)		
			G_1	S	$G_2 + M$
–	–	–	48	32	20
–	–	5	52	17	31
0.2	–	–	20	26	54
0.2	–	5	18	36	46
–	0.2	–	36	14	50
–	0.2	5	39	33	28

3.2. Light absorption spectroscopy

Light absorption spectroscopy was used to measure light absorption spectra of NM2, QM and Q in solutions alone and in the presence of CAF. The spectrum of CAF in the equimolar amount of NM2 was the same as spectrum of CAF alone, which indicates that CAF does not interact with NM2 (not shown). The absorption spectra of QM and Q titrated with CAF are presented in Fig. 3. Bathochromic and hypochromic effects are clearly visible for these spectra. These are characteristic for aromatic chromophore (π–π) interactions (well-known effect of bases stacking in nucleic acids or the chromophore intercalation). The presence of an apparent isosbestic point at 347 nm in the spectra indicates that two components are predominant in the mixture, because CAF light absorption above 350 nm is very weak, and the spectra were corrected for this (see Section 2), it is obvious that they represent QM,

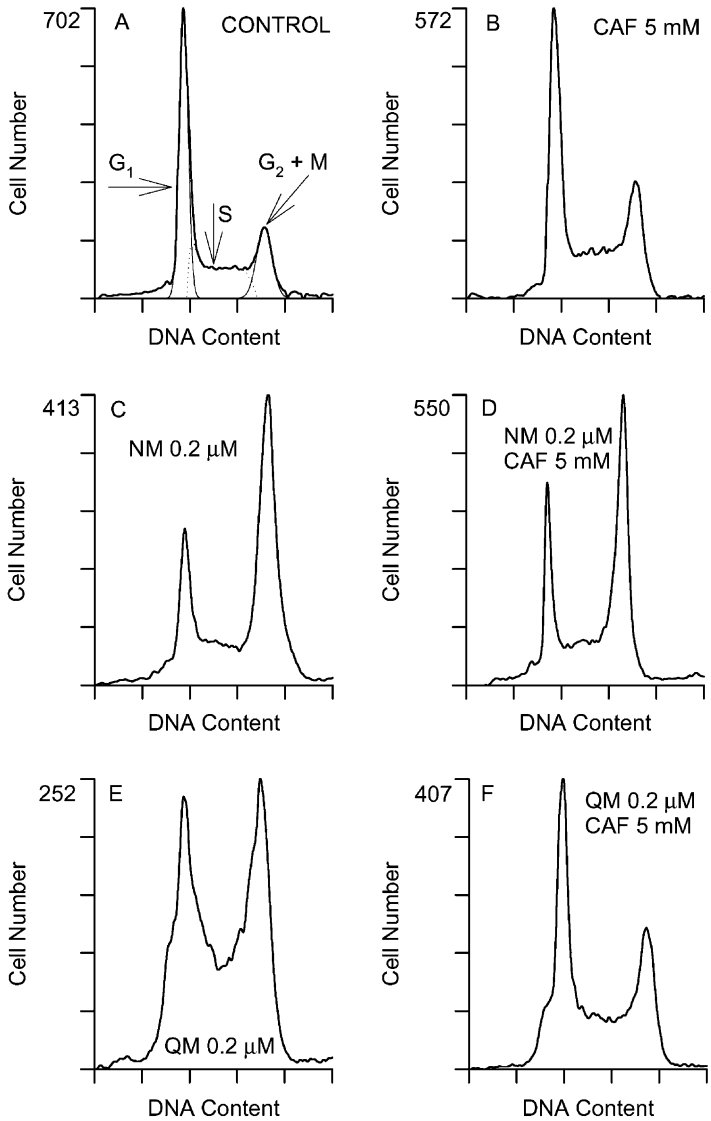

Fig. 2. Frequency distribution histograms representing fluorescence of HL-60 cells treated with QM, NM2 and CAF, stained with DAPI. Cell cycle distributions are summarized in Table 1.

and Q monomer and QM and Q monomer associated with CAF molecules. The dimerization constants of QM and Q are very low (45 ± 3 and $\sim 800\ M^{-1}$, respectively), and spectra of their dimers (or the dimers complexes with CAF) cannot be detected at range of chromophore concentrations (μM). Therefore, the extrapolation of spectra to infinite dilution, e.g. $(C_{TA}/C_{TC}) \rightarrow 0$, [36] the spectra of QM–CAF and Q–CAF complexes can be calculated. By expressing the spectra in molar absorption coefficient E_λ, the spectra of chromophore–CAF mixtures, can be decomposed into a weighed sum of components by non-linear least squares regression analysis as demonstrated in Fig. 4A. For this calculation, we used SigmaPlot program based on the Marquardt–Levenberg algorithm. The decomposition allowed us to calculate molar fraction (Θ) of free and bound chromophores monomer in the mixture. The correlation coefficients of spectra decomposition by this procedure were close to $r^2 = 0.99$ in all cases. The results of this calculation for QM are shown in Table 2. The same procedure was used to estimate the association of Q with CAF. This result already described indicates the interaction of only aromatic mutagens with CAF.

3.3. Calculation of QM–CAF and Q–CAF "neighborhood" association constant (K_{AC})

Using data obtained by spectrophotometric titration, CAF self-association constant $K_{CC} = 11.3\ M^{-1}$ (as reported by Fritzsche *et al.* [43]), the total concentrations of mutagens (C_{TA}) and CAF (C_{TC}), and the measured free mutagen concentrations (C_A), as already described, we were able to calculate concentrations of all other components of the system. Using thermodynamical model of mixed association [36], described in Section 2, we calculated all concentrations of neighborhoods in the complexes

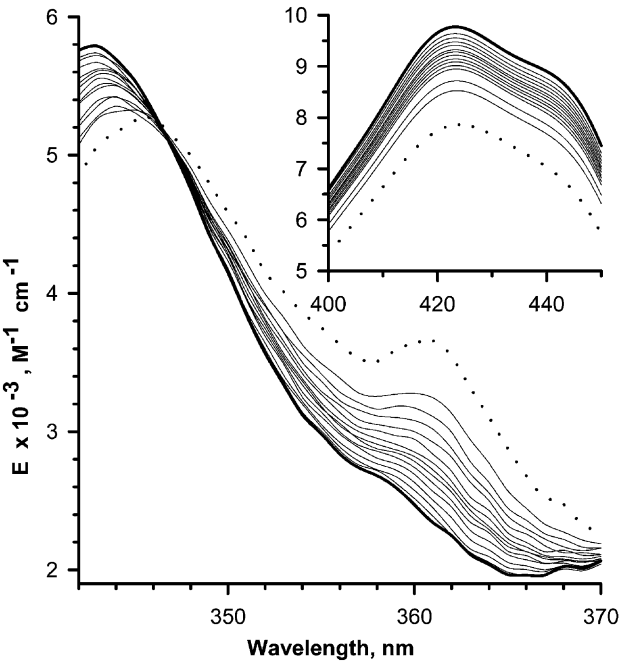

Fig. 3. Light absorption spectrum of QM alone (—), and QM titrated with CAF (—). The concentrations of components are given in Table 1. The dotted line (· · · ·) represents extrapolated ([QM]/[CAF]) → 0, [36] spectrum of QM–CAF complex. Insert: light absorption spectra of Q (initial concentration 21 μM (—)) titrated with CAF (0.25–9.2 mM, thin line). The dotted line (· · · ·) represents extrapolated to ([Q]/[CAF]) → 0 [36] spectrum of Q–CAF complex.

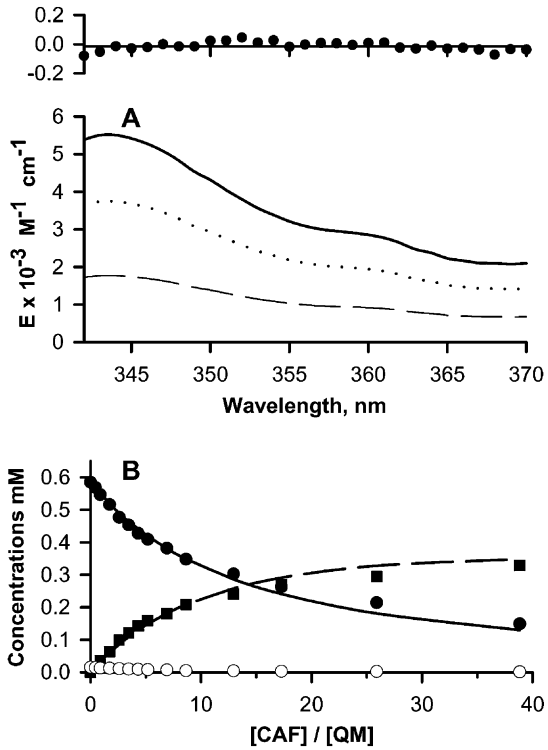

Fig. 4. (A) An example of two parameters numerical analysis decomposition [36] of the light absorption spectrum of QM–CAF mixture (sample no. 6, Table 2). Under the spectrum of the mixture (—), there are the spectra of the individual components, i.e. the complex QM–CAF (· · · ·), and free QM (– – –), weighted to their molar fraction. Residue of the analysis, the difference between the measured and the weighted sum of the components spectra. (B) The comparison of the results of two-parameter analysis of the mixtures QM with CAF using measured data of the concentration QM monomer (●) and QM–CAF complex (■) and concentration of these components ((—) and (– – –), respectively) based on mean K_{AC} (Table 2) calculation. The standard deviation was 0.014 mM in both cases. In addition the "hypothetical" concentration of QM dimer is shown (○) calculated based on concentration of free QM (C_A, and K_D) in the samples. One can see that the fraction of the QM dimer is very low as compared with QM monomer (and most likely in QM dimer–CAF complex), and their presence can not be detected by light spectroscopy concentrations (μM). This, in our opinion, is the justification for using the simplified mathematical model, and two parameter of experimental data analysis.

and the "neighborhood associations" constants K_{AC} (equal 59 and 75 M^{-1} for QM–CAF and Q–CAF interaction, respectively). The results of these calculations for QM–CAF system are shown in Table 2. The comparison of C_A concentrations measured and C_A calculated for QM–CAF and Q–CAF systems are presented in Fig. 4B. The interaction of QM with CAF was also confirmed by measuring the heat of their interaction.

3.4. Microcalorimetry titration

Fig. 5 indicates the negative enthalpy (ΔH) of QM–CAF complex formation. It should be noted that no measurable heat effect was observed when CAF solution was titrated with NM2 (not shown). This result is the next proof that mutagen–CAF complex formation involves interaction of polycyclic aromatic rings of the reactants, and is not the result of interaction of N-nitrogen mustard group presented both in QM and NM2. The possibility of (π–π) complex formation between Q and CAF was also examined by molecular modeling.

3.5. Molecular modeling

Both QM and Q are commercially supplied as dihydrochlorides. They have good solubility in water or in the buffers at pH 5. However, their solubility is drastically reduced in the solution at higher pH. Also, there are significant changes in their light absorption spectra in the solution with pH 5, as compared to pH 7 (not shown). These effects can only be explained by the assumption that, in the neutral pH, QM and Q are predominately in monocationic form. The semi-empirical calculation of *in vacuo* indicated the localization of the most negative potential (−0.072) at atom 4′N QM base. In the periodic box conditions, geometry optimization, the proton forms a covalent bond with length <1 Å with this atom (not shown). We used, mono-protonated (at 4′N) form of the QM for farther molecular modeling of its complex with CAF. The semi-empirical (*PM3* method) geometry optimization of QM–CAF complexes was performed in the periodic box containing 74 water molecules. The energy minimization of the system indicates only two water molecules bound to

Table 2
Titration of QM with CAF in 0.15 M NaCl, 20 mM Hepes, pH 6.8, 25°

Sample	C_{TA} (mM)	C_{TC} (mM)	Θ	C_A (mM)	X_{AA} (μM)	C_{BA} (mM)	C_C (mM)	C_{CC} (mM)	C_{AC} (mM)	K_{AC} (M^{-1})
1	0.585	0.000	0.000	0.585	0.015	0.000	0.000	0.000	0.000	–
2	0.584	0.252	0.025	0.567	0.014	0.015	0.236	0.001	0.015	52.6
3	0.582	0.502	0.061	0.550	0.014	0.035	0.462	0.003	0.036	66.3
4	0.579	1.000	0.108	0.519	0.012	0.063	0.917	0.010	0.064	61.6
5	0.576	1.493	0.172	0.490	0.011	0.099	1.348	0.023	0.080	69.6
6	0.574	1.980	0.211	0.464	0.010	0.121	1.778	0.040	0.128	66.9
7	0.571	2.463	0.250	0.441	0.009	0.143	2.195	0.061	0.153	66.7
8	0.568	2.942	0.280	0.419	0.008	0.159	2.607	0.087	0.172	64.5
9	0.563	3.885	0.321	0.381	0.007	0.180	3.404	0.149	0.198	58.7
10	0.557	4.810	0.375	0.349	0.005	0.209	4.150	0.226	0.233	59.3
11	0.544	7.047	0.443	0.286	0.004	0.241	5.886	0.469	0.276	52.7
12	0.532	9.182	0.506	0.241	0.003	0.269	7.420	0.770	0.316	51.3
13	0.509	13.17	0.580	0.181	0.002	0.295	10.04	1.494	0.358	47.3
14	0.478	18.55	0.688	0.130	0.001	0.329	13.11	2.747	0.430	50.7

Mean $K_{AC} = 59.1 \pm 2\ M^{-1}$. C_{TA} and C_{TC}: total concentration of QM and CAF, respectively; n: molar fraction of the QM in the complex with CAF; C_A: concentration of free QM (experimental); X_{AA}: hypothetical QM dimer concentration calculated based on $K_D = 45 \pm 2\ M^{-1}$ and C_A; C_{BA}: concentration of complexed QM; C_C: concentration of monomer CAF; C_{CC} and C_{AC}: "neighborhood" of CAF–CAF and QM–CAF concentration, respectively; K_{AC}: "neighborhood" association constant QM–CAF.

the complex (one to N9 atom of CAF and one with the hydrogen attached to 4′N atom of QM, see Fig. 6), The energy of complex was then corrected by subtracting the energy formation of QM and CAF in the same water shell, and for the energy of water shell formatting, result in $\Delta E = -10\ cal\ mol^{-1}$. It needs to be mentioned that QM–CAF complex formation resulted in lose of water molecules bound to the substrate molecules. Semi-empirical molecular modeling, by the method already described, indicates that QM and CAF separately, can form up to six hydrogen bonds with water molecules (not shown). In contrast QM–CAF retain only two water molecules. This process of water molecules detachment results in favorable entropy $\Delta S^{0'} = -7.72\ cal\ mol^{-1}\ K^{-1}$. The detail of molecular modeling semi-empirical calculations of aromatic polycyclic complexes will be described elsewhere.

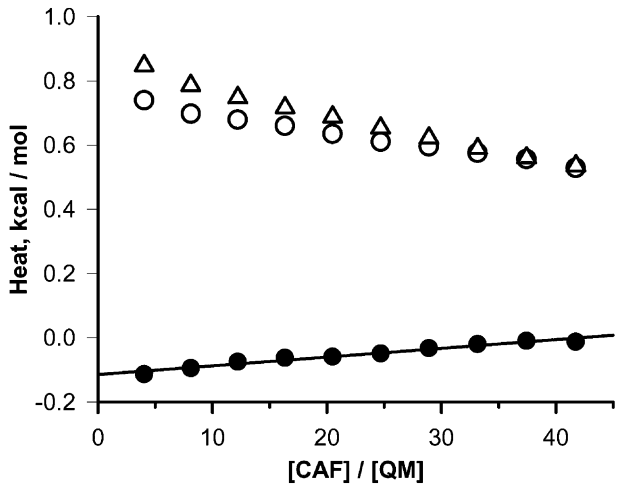

Fig. 5. Microcalorimetric titration of QM 1.3 mL, initial concentration equal 0.109 mM, in 50 mM Hepes, 0.15 M NaCl, pH 6.8, at 30 ± 0.1° with 0.134 M CAF, in the same buffer, at 120 s intervals. The open symbols represent the heat of the component dilution measured in separate experiments, corresponding to heat of dilution during titration of QM with CAF. The result corrected for the heat of dilution of components expressed as ΔH per molar of the titrant (●), as previously described [44]. The value of $\Delta H = -116\ cal\ mol^{-1}$ by the extrapolation to $([CAF]/[QM]) \rightarrow 0$ ($r^2 = 0.97$) was obtained.

4. Discussion

Studies from several laboratories demonstrated that CAF is able to reduce the cytostatic and/or cytotoxic effect of DNA damaging polycyclic aromatic compounds. As a model in this work we have chosen two well-known mutagenic agents as a subject of this study—quinacrine mustard and mechlorethamine. Both these compounds are derivatives of nitrogen mustard (Fig. 1) and in an aqueous solution spontaneously form reactive arizidinium ions that can alkylate nucleic acids and proteins [37,38]. The reason for the selection of QM and NM2 for our studies is that they do not require any enzymatic activation. QM, however, can bind DNA by intercalation [39] while NM2, as aliphatic compound, cannot. To test whether this process has any effect on cell cytotoxity we included the Q (Fig. 1) to our study; this compound intercalates and forms the stacking complexes with CAF, but does not alkylate nucleic acids. The results presented in Sections 3.1 and 3.2 revealed that the nitrogen mustard moiety, and not the ability of QM intercalation into DNA is the important factor in the toxicicity of this agents The present data strongly support the hypothesis that the protective mechanism of CAF is a consequence of sequestration of such agents in stacking complexes with CAF, thus lowering the effective free ligand concentration in the solution, and making the ligand less accessible to the cells. This conclusion is supported by

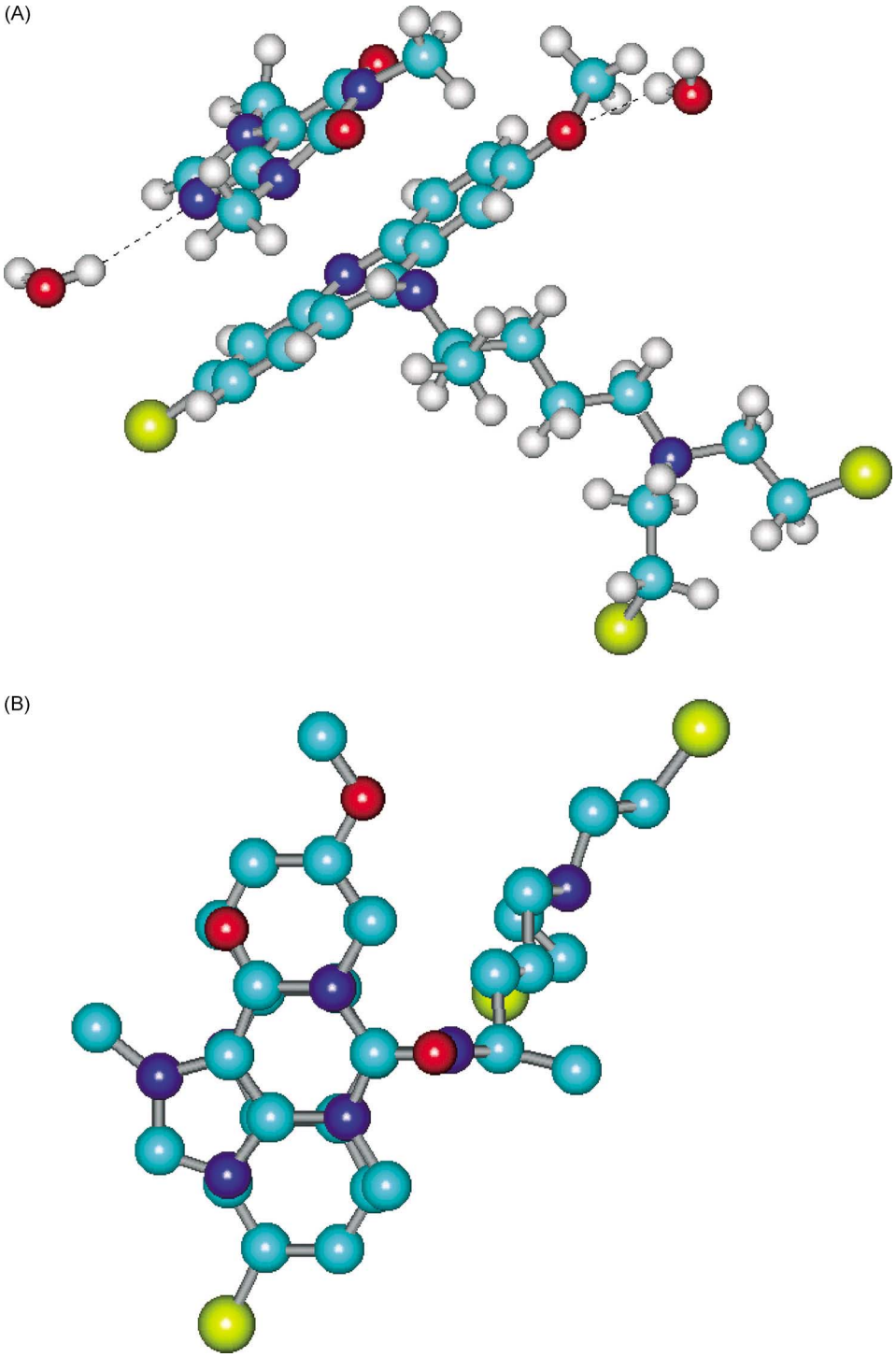

Fig. 6. Semi-empirical geometry optimization of the QM–CAF complex in the periodic box contained 74 water molecules. For the clarity of the pictures all water molecules, except those forming the hydrogen bond (dotted lines) with the complex, as well as the double bonds in molecules were removed. (A) Side view of the complex which indicates almost parallel planes with their average distance of 3.4 Å. (B) Overlaying the QM and CAF molecules in their complex (all hydrogen atoms and multiple bonds were removed, for the clarity of the picture). The tilt between the symmetry axis of the aromatic system of both molecules is about 56°. (Hydrogen atoms: small gray spheres, and chlorine atoms: green, carbon: cyan, nitrogen: deep blue, and oxygen atoms: red spheres).

all experimental techniques implemented in this work, e.g. cell cycle proliferation, light absorption spectroscopy, microcalorymetry and molecular modeling. The caffeine may then be acting as the ''interceptor'' of potential carcinogens (especially in the upper part of digesting track where its concentration can reach the concentration of millimolar level). There is, however, no indication both in the literature and in our experiments, that xanthines can reverse the damage to nucleic acids when this damage to DNA has already occurred.

Acknowledgments

This work was supported by KBN 6 P203 044 06 grant. We are grateful to Piotr Paneth from the Technical University of Lodz, Poland, for his very valuable advice in molecular modeling calculations, and Adam Blaszczak from Institute of Biochemistry and Biophysics Polish Academy of Sciences, Warsaw, Poland for the help in editing this paper.

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