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Caffeine, pentoxifylline and theophylline form stacking complexes with IQ-type heterocyclic aromatic amines

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ABSTRACT

Methylxanthines (MTX), in particular caffeine (CAF), are known as the most widely consumed alkaloids worldwide. Many accumulated statistical data indicate the protective effect of CAF intake against several types of cancer. One of the possible explanations of this phenomenon is direct non-covalent interaction between CAF and aromatic mutagen/carcinogen molecules through stacking (π–π) complexes formation. Here we demonstrate that CAF and other MTX, pentoxifylline (PTX) and theophylline (TH), form stacking complexes with carcinogenic imidazoquinoline-type (IQ-type) food-borne heterocyclic aromatic amines (HCAs). We estimated neighborhood association constants (K_{AC} of the order of magnitude of 10^2 M^{-1}) in neutral and acidic environment and enthalpy changes (ΔH values between −15.1 and −39.8 kJ/mol) for these interactions using UV–Vis spectroscopy, calculations based on thermodynamical model of mixed aggregation and titration microcalorimetry. Moreover, using Ames test with *Salmonella typhimurium* TA98 strain and recently developed mutagenicity assay based on bioluminescence of *Vibrio harveyi* A16 strain, we demonstrated a statistically significant reduction in HCAs mutagenic activity in the presence of MTX.

1. Introduction

Caffeine (CAF), a methylxanthine, which is a major component of several popular beverages (coffee, tea, energy drinks), is known as the most widely consumed alkaloid worldwide. Statistical data from the USA revealed that the average intake of CAF can reach 193 mg/day per person, mainly consumed as the constituent of coffee among adults and soft drinks among children and teenagers [1]. Among various effects of the consumption of caffeinated beverages, a relationship between cancer occurrence and CAF intake appears to be a matter of great interest. Many epidemiological data concerning this issue are contradictory, however, some reports revealed protective effect of coffee consumption against breast, liver and kidney cancer [2]. Direct stacking interaction between CAF and polycyclic aromatic mutagens and/or carcinogens is mentioned as one of the possible explanations of this protective action of CAF. Such sequestration of mutagen/carcinogen molecules in stacking complexes with CAF may decrease their biologically active concentration and, in consequence, lower their mutagenic/carcinogenic effects. It has been demonstrated previously that CAF not only forms stacking aggregates with model aromatic mutagens: quinacrine mustard (QM), ICR170 and ICR191, but also decreases their mutagenicity and minimizes cell cycle perturbation [3,4]. Another methylxanthine derivative, pentoxifylline (PTX), which is mainly administered to humans as a drug [5], forms stacking aggregates with QM, ICR170 and ICR191 and diminishes their mutagenic activity as well [4]. CAF and PTX are also proven to form stacking hetero-complexes with neurotoxins, which may be delivered to humans as food and beverage contaminants [6,7]. Additionally, CAF, PTX, and another methylxanthine–theophylline (TH) form stacking aggregates with anticancer drugs, reducing in this way their biological activity [8].

Heterocyclic aromatic amines (HCAs) are created during meat and fish processing at temperatures above 150 °C [9]. Apart from food, HCAs are found in small concentrations in rain water, sewage, exhaust fumes, cigarette smoke [10], river water [11] and among air pollutants [12]. Wakabayashi and colleagues estimated the average daily intake of HCAs at between 0.4 and 16 μg per person [13]. HCAs exhibit strong mutagenic activity, which is significantly increased after their metabolic activation by cytochrome P450 in liver [14]. What is more, they have been demonstrated to be carcinogenic in laboratory animals [15]. The relationship between frequent meat consumption and breast [16], colorectal [17], pancreatic [18], and urothelial cancer [19] suggests that HCAs may play a significant role in human carcinogenesis.

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As HCAs are present in commonly consumed food, it seems extremely difficult to eliminate them completely from regular human diet. Instead, Dashwood proposes several ways to diminish their mutagenic and carcinogenic activity, such as the reduction of meat consumption, modification of cooking methods, inhibition of HCAs formation and biological activation, and activity of interceptor molecules [20].

Chlorophyllin (CHL), which is one of the best-studied interceptor molecules, is proven to reduce effectively the activity of a broad range of aromatic mutagens and carcinogens, such as polycyclic aromatic hydrocarbons, aflatoxin B_1 and HCAs [21]. It has been shown that CHL, through stacking complexes formation with HCAs, diminishes their mutagenic activity [22]. What is more, the administration of CHL causes the decline in HCA–DNA adducts formation in liver tissue, due to the reduction of carcinogen absorption in the digestive tract [23].

There are some reports indicating that CAF can reduce the biological activity of HCAs as well [24,25]. The most frequently proposed explanation for this phenomenon is the influence of CAF on the metabolic activation of HCAs. However, little is known about the possibility of CAF to interact directly with HCAs. Both CAF and HCAs possess aromatic structure, therefore they are capable of stacking complexes formation. Consequently, such sequestration of HCAs molecules in complexes with CAF might possibly play a role in modulation of HCAs biological activity, as it has been previously described for CHL.

In this paper we demonstrate the formation of stacking complexes between five imidazoquinoline-type (IQ-type) HCAs and three methylxanthines (MTX). We determined neighborhood association constants and enthalpies of hetero-complexes formation using UV/Vis spectroscopy, calculations based on thermodynamical model of mixed aggregation and titration microcalorimetry. Moreover, we used Ames test and newly developed bioluminescence mutagenicity assay in order to check the mutagenic potency of CAF–HCA mixtures.

2. Materials and methods

2.1. Materials

Methylxanthines (MTX): caffeine (CAF; 1,2,3-trimethylxanthine), pentoxifylline (PTX; 3,7-dimethyl-1-(5-oxohexyl)xanthine) and theophylline (TH; 1,3-dimethylxanthine) were purchased from Sigma–Aldrich (St. Louis, USA). Heterocyclic aromatic amines (HCAs): IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline), IQx (2-amino-3-methylimidazo[4,5-*f*]quinoxaline), MeIQ (2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline), MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline) and 7,8-DiMeIQx (2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline) were purchased from Toronto Research Chemicals (Toronto, Canada). Structures of above mentioned chemicals are presented in Fig. 1. CAF, PTX and TH stock solutions were prepared by dissolving their weight amounts in appropriate buffer in concentrations about 10^{-1} M for CAF and PTX and 10^{-2} M for TH. HCAs stock solutions were prepared by dissolving their weight amounts in distilled water (HCAs concentrations in mM range) and stored in darkness at 4 °C. For microcalorimetric titrations HCAs solutions were prepared by dilution of proper volume of water stock solution in appropriate buffer. The accurate concentrations of HCAs solutions were assayed colorimetrically using earlier determined molar absorption coefficients, presented in Table 1. HP buffer (containing 5 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), pH 7.1) and citrate buffer (containing 0.12 M NaOH, 0.2 M citric acid, pH 3.1) were filtered through a 0.2 μm pore Millex Millipore filters and degassed.

2.2. Light absorption spectroscopy

The 2 mL aliquots containing mutagen were placed in a quartz cuvette (1 cm light path) and titrated with 5–150 μL of methylxanthine solution. The absorption spectra of each mixture were

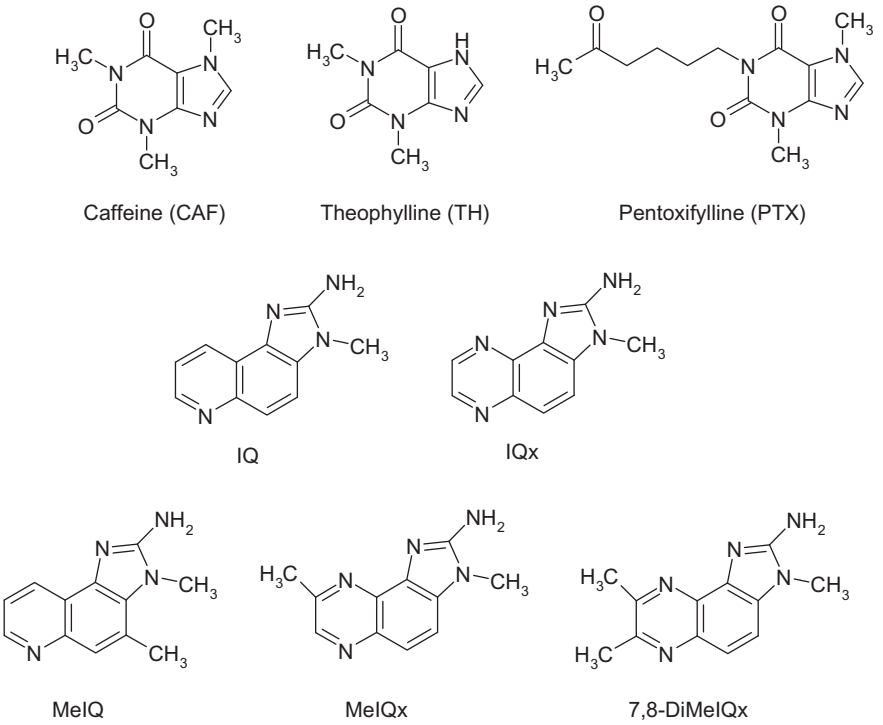

Fig. 1. Chemical structures of examined methylxanthines and IQ-type heterocyclic aromatic amines.

Table 1
Molar absorption coefficients (ε_M) of selected IQ-type heterocyclic aromatic amines in HP buffer, pH 7.1 at 20 ± 0.1 °C.

Compound	Wavelength, nm	ε_M, M^{-1} cm^{-1}
IQ	339.0	2947
IQx	339.5	3231
MeIQx	341.0	3645
7,8-DiMeIQx	338.0	5598
MeIQ	327.0	2885

measured using Beckman's DU 650 spectrophotometer at 0.5 nm intervals and stored in a digital form. All spectroscopic experiments were performed in HP and citrate buffer at 20 °C (±0.1 °C) and are expressed in the form of molar absorption coefficient (E_λ, M^{-1} cm^{-1}).

2.3. Calculations of the association constants

Calculations of the association constants of HCAs with MTX complex formation were performed with statistical thermodynamics of mixed aggregation based on Zdunek et al. model, which has been described and used previously [3,4,6–8,26,27]. Notations and definitions described previously by Weller et al. [28] were used. All calculations in this paper were performed using Sigma Plot 11 (Systat Software, Inc.), Excel (Microsoft) and Mathcad 14 (Parametric Technology Corporation) software.

2.4. Microcalorimetry

The microcalorimetric titrations were performed at 25 ± 0.1 °C in HP buffer, using Nano-Isothermal Titration Calorimeter III model 5300 (Calorimetry Sciences Corporation). 10 μL portions of appropriate mutagen (concentrations: IQ – 1.33 mM, IQx – 1.1 mM, MeIQ – 1.03 mM, MeIQx – 1.48 mM, 7,8-DiMeIQx – 1.17 mM) were added to 950 μL of the titrated solution containing methylxanthine (initial concentrations: CAF – 15.83 mM for titration with IQ, IQx and 7,8-DiMeIQx, CAF – 15.43 mM for titration with MeIQ and MeIQx, PTX – 15.24 mM, TH – 15.15 mM) and the heat of the process was measured as a function of time (μW s^{-1}). The results of titrations were corrected for the heat of components' dilution, and finally expressed as heat per mole of added mutagen (kJ mol^{-1}).

2.5. Ames mutagenicity assay

The *Salmonella* mutagenicity test [29] was performed with *Salmonella typhimurium* strain TA98 without metabolic activation. We used procedure described by Mortelmans and Zeiger [30] with modifications. A mixture containing 100 μl of the overnight culture of *S. typhimurium* TA98 (corresponding to 1×10^8 colony forming units), 50 μl of 3% NaCl and 100 μl of a test chemical dilution (or 100 μl of water as the negative control) was incubated for 4 h in darkness at 37 °C and 220 rpm. Afterwards the mixture was centrifuged, bacterial pellet was washed with 0.6% NaCl, resuspended in 300 μl of 0.6% NaCl containing 0.1 μmol of histidine and 0.1 μmol of biotin and spread on GM plate. Revertant colonies were counted after cultivation for 48 h at 37 °C in darkness. All experiments were performed in triplicate. Bacterial toxicity was determined by observation of the auxotrophic background (background lawn). All analyzed mixtures were proven to be non-toxic towards *S. typhimurium* TA98, as no alterations in the background lawn were observed.

2.6. Vibrio harveyi mutagenicity test

The *V. harveyi* mutagenicity test was performed according to Podgorska and Wegrzyn [31] with *V. harveyi* A16 strain (a dim *luxE* mutant), which was obtained from Dr. Beata Podgórska from the Department of Microbiology, University of Gdańsk, Poland. Bacteria were cultivated in the liquid BOSS medium [32] containing 1% bacto-peptone, 0.3% beef extract, 0.1% glycerol and 3% NaCl (pH 7.3) at 30 °C and 200 rpm. Dilutions of tested chemicals (alone or in appropriate mixtures) were added to exponentially growing cultures and the cultivation was continued for 3 h. Then, 250 μL of each culture was placed on the 96-well plate (Nunc 96 Well Optical Bottom White Plate; Thermo Fischer Scientific, Roskilde, Denmark) and its absorbance at 575 nm and luminescence was measured using Wallac 1420 Victor^2 plate spectrometer (PerkinElmer). The luminescence of each culture was corrected for its absorbance and the results were presented as relative light units (RLU).

2.7. Statistical analysis

The results of mutagenicity tests were evaluated statistically with Statistica 8.0 (StatSoft) software. We used one-way variance analysis (ANOVA), which was followed by the post hoc RIR–Tukey's test. Significance level was established at $\alpha = 0.05$.

3. Results

3.1. Spectroscopic measurements and calculations

The absorption spectra of IQ titrated with MTX (CAF, PTX and TH) are presented in Fig. 2. In order to reflect changes only in a structure of IQ, all the spectra were measured in the range of wavelengths above 300 nm, where light absorption of MTX is insignificantly low. IQ and other examined HCAs do not dimerize in the range of concentrations employed in spectroscopic titrations, which was checked in separate experiments (data not shown). Hypochromic and bathochromic (red shift) effects indicate that, upon the addition of MTX to IQ solution, a new absorbing component, which is IQ complexed with MTX, appears in the mixture. By extrapolation of molar extinction coefficient, for each wavelength, to $C_{TA}/C_{TC} \rightarrow 0$ (where C_{TA} and C_{TC} are the total concentrations of IQ and appropriate MTX, respectively), the spectrum of IQ–MTX complex was calculated.

The spectra of mixtures containing IQ and MTX were decomposed into a weighted sum of components by non-linear regression analysis, as previously described [33]. The example of such two-component analysis for IQ–CAF mixture is presented in Fig. 3. The spectrum of mixture was decomposed into spectra of IQ free form and IQ complexed with CAF. Based on such analysis conducted for all IQ–CAF mixtures, we were able to estimate the concentrations of free IQ (C_A^*) and IQ complexed with CAF (X_{BA}^*) for various [IQ]/[CAF] ratios.

Because of the complexity of analyzed system (indefinite self-association of CAF), we used thermodynamical model of mixed aggregation, previously described by Zdunek et al. [26] to calculate "neighborhood" association constant (K_{AC}) of IQ–CAF complex formation and concentrations of all possible "neighborhoods" in the mixture. Based on the mass conservation law, we calculated molecular concentration of aggregated CAF (X_{CC}) and molecular concentration of IQ complexed with CAF (X_{BA}) (results given in Table 2). The comparison of the measured and calculated values, presented in Fig. 4, indicates a good correlation between these two sets of data.

Using procedure described above, we analyzed possible interactions between remaining IQ-type HCAs and MTX in neutral (pH 7.1) and in acidic (pH 3.1) environment. We demonstrated that all examined HCAs formed stacking aggregates with CAF, PTX and TH in different pH conditions. Calculated "neighborhood"

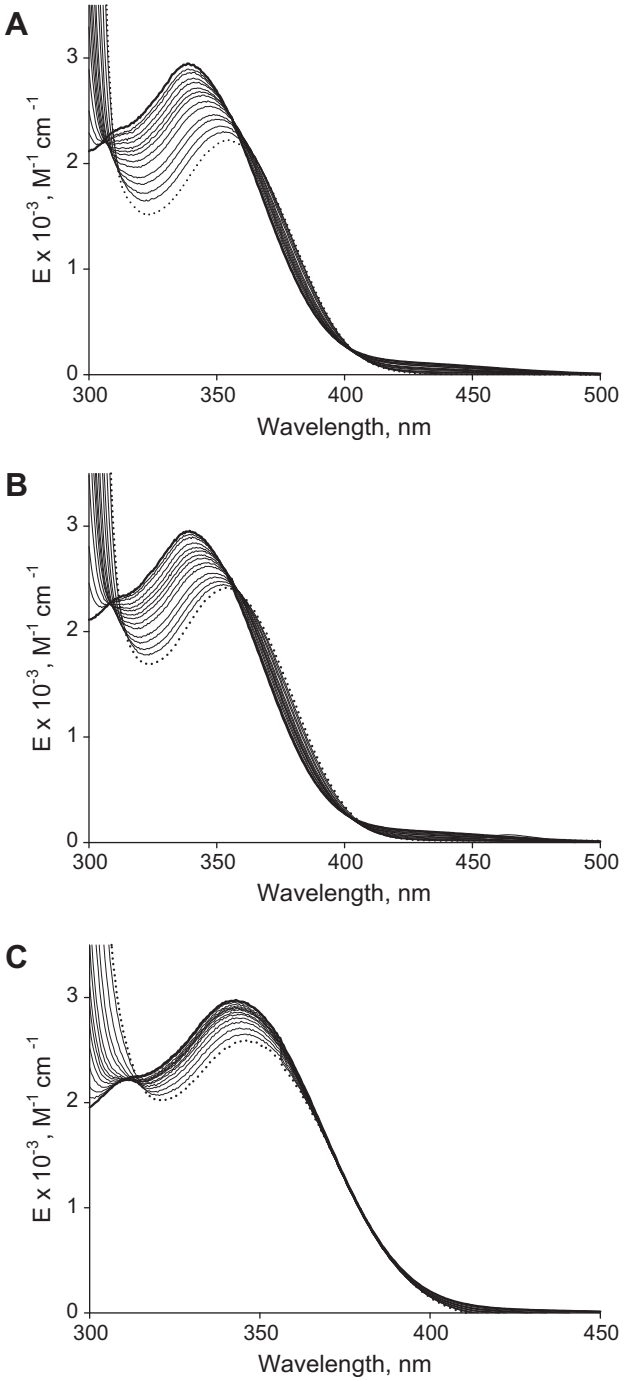

Fig. 2. Spectrophotometric titrations of IQ with methylxanthines: IQ (initial concentration 232 μM) with caffeine (concentration ranging from 0.26 mM to 19.43 mM) (panel A), IQ (initial concentration 228 μM) with pentoxifylline (concentration ranging from 0.31 mM to 23.09 mM) (panel B) and IQ (initial concentration 51.2 μM) with theophylline (concentration ranging from 49 μM to 3.61 mM) (panel C). Thick solid line on the top represents the spectrum of free IQ. Thin lines represent the spectra of IQ and MTX mixtures. Dotted line represents calculated spectrum of IQ–MTX complex.

association constants (K_{AC}) of HCAs–MTX interaction are presented in Table 3.

3.2. Thermal effects of HCAs–MTX interactions

In order to determine enthalpies of HCAs–MTX complex formation, we analyzed direct interactions between IQ-type HCAs and

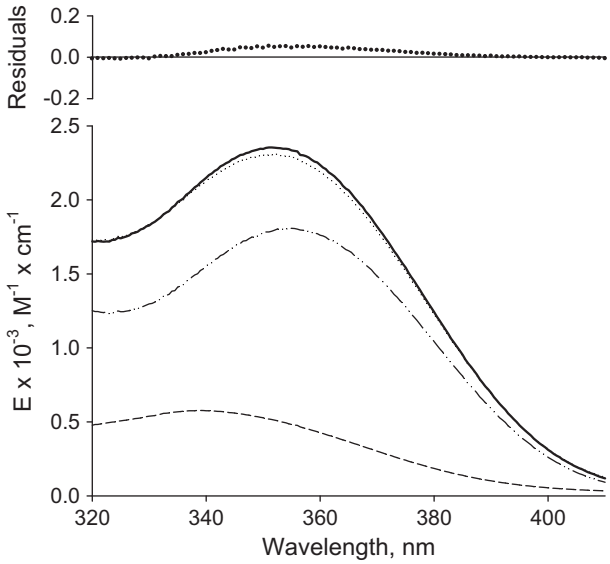

Fig. 3. The example of two-parameter analysis of IQ–caffeine mixture (for composition see Table 2, sample #11). Component weighted spectra represent the spectra of components multiplied by their molar fraction in the mixture. (A) Residuals of the measured spectrum and weighted sum of components (free IQ and IQ complexed with caffeine) spectra. (B) Spectrum of IQ–caffeine mixture (solid line), weighted sum of components spectrum (dotted line), component weighted spectra of free IQ (dashed line) and IQ complexed with caffeine (dashed-dotted line).

MTX using titration microcalorimetry. We performed four independent experiments to exclude the heat of every examined component' dilution for each analysis. The results of titration of IQ with CAF, buffer with buffer, buffer with IQ and CAF with buffer are presented in Fig. 5. These curves represent the changes in power required to maintain a zero temperature difference between the microcalorimeter sample and reference cells after each titration and are presented as a function of time.

Thermal effects of IQ–CAF interaction were estimated taking into consideration the thermal effects of every component' dilution (Fig. 6). These effects are exothermic, in contrast to endothermic effects of components' dilution.

Finally, the approximate enthalpy change (ΔH) of IQ–CAF complex formation was estimated by the extrapolation of experimental points to infinite dilution of IQ (Fig. 7) and is equal to −32.4 kJ/mol of injected titrant. Using similar procedure we estimated enthalpy changes of complex formation between remaining IQ-type HCAs and MTX (results presented in Table 4).

3.3. Mutagenicity of IQ–MTX mixture

We used *S. typhimurium* TA98 assay (Ames test) to check whether stacking aggregates formation between IQ and MTX can influence biological activity of this mutagen. Results of this assay for IQ–CAF mixtures are presented in Fig. 8. We demonstrated that mutagenic activity of IQ is significantly inhibited in a dose-dependent manner upon the addition of CAF (Fig. 8A). We did not observe mutagenic activity of CAF alone against *S. typhimurium* TA98 cells in all tested concentrations. Based on total concentrations of IQ and CAF in the mixture, and neighborhood association constant (K_{AC} = 86.8 M^{-1}) we were able to estimate molar fractions of free form of IQ and IQ complexed with CAF. Relationship between the mutagenicity of IQ and its free molar fractions in the IQ–CAF mixtures is presented in Fig. 8B.

Moreover, we observed that TH and PTX reduced the mutagenic activity of IQ as well. The number of revertants decreased from

Table 2
Titration of IQ with caffeine in HP buffer, pH 7.1 at 20 ± 0.1 °C.

Sample #	C_{TC}, mM	C_{TA}, μM	C_C, mM	C_{CC}, mM	C_{AC}, μM	C^*_A, μM	C_A, μM	X^*_{BA}, μM	X_{BA}, μM	X_{CC}, mM	K_{AC}, M^{-1}
0	0.00	231.80	0.00	0.00	0.00	231.80	231.80	0.00	0.00	0.00	–
1	0.26	231.20	0.25	0.00	10.09	219.30	221.30	11.96	9.98	0.00	105.20
2	0.52	230.70	0.50	0.00	19.62	210.90	211.50	19.73	19.20	0.00	89.54
3	1.04	229.60	0.98	0.01	37.13	195.10	193.90	34.45	35.63	0.02	83.64
4	1.56	228.50	1.46	0.03	52.83	182.70	178.70	45.77	49.77	0.05	78.52
5	2.06	227.40	1.91	0.04	66.96	167.40	165.30	59.97	62.03	0.09	83.22
6	2.57	226.30	2.36	0.07	79.74	156.20	153.60	70.06	72.72	0.13	82.66
7	3.07	225.20	2.79	0.10	91.33	144.40	143.20	80.84	82.07	0.18	85.07
8	4.05	223.10	3.62	0.17	111.50	125.60	125.60	97.55	97.55	0.32	86.92
9	5.02	221.10	4.41	0.25	128.30	108.80	111.40	112.30	109.70	0.47	90.50
10	7.36	216.10	6.20	0.52	159.90	80.01	85.72	136.10	130.30	0.97	95.21
11	9.59	211.30	7.80	0.85	181.40	62.87	68.81	148.40	142.50	1.59	96.31
12	13.78	202.30	10.50	1.64	206.90	39.67	48.32	162.70	154.00	3.03	104.58

Abbreviations: C_{TC}: total CAF concentration; C_{TA}: total IQ concentration; C_C: concentration of CAF monomer; C_{CC}: concentration of neighborhoods CAF–CAF complexes; C_{AC}: concentration of neighborhoods IQ–CAF complexes; C^*_A: concentration of IQ monomer (measured spectrophotometrically); C_A: concentration of IQ monomer; X^*_{BA}: concentration of IQ complexed with CAF (measured spectrophotometrically); X_{BA}: concentration of IQ complexed with CAF; X_{CC}: concentration of CAF–CAF complexes; K_{AC}: neighborhood association constant IQ–CAF; mean K_{AC} = 86.9 ± 2 (SE).

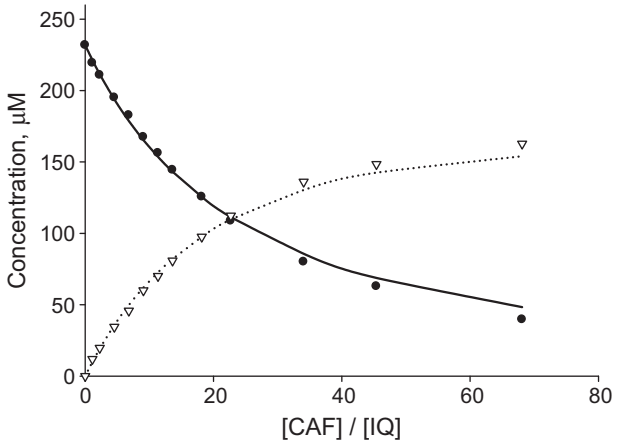

Fig. 4. The comparison of the results of two-parameter analysis of IQ–caffeine mixture. Lines represent concentrations of IQ molecules calculated using statistical–thermodynamical model of mixed aggregation (listed in Table 2), basing on the estimated association constant (K_{AC}) equal to 86.9 ± 2 (SE) M^{-1}. Solid line represents the concentration of free IQ. Dotted line represents the concentration of IQ complexed with caffeine. Symbols represent concentrations of IQ molecules calculated using two-parameter analysis of the spectra (an example of such analysis in given in Fig. 3). Circles represent the concentration of free IQ. Triangles represent the concentration of IQ complexed with caffeine.

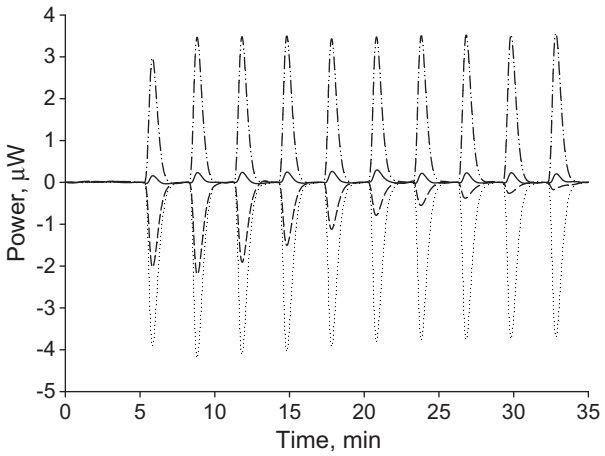

Fig. 5. Microcalorimetric titration of caffeine with IQ (dashed-dotted line), buffer with buffer (solid line), buffer with IQ (dashed line) and caffeine with buffer (dotted line) presented as changes in power required to maintain the system at constant temperature after each titration.

106.0 ± 8.2 for IQ alone (10 μg/plate) to 79.7 ± 8.7 and 41.3 ± 1.5, when IQ was used in mixture with TH (0.33 mg/plate) and PTX (1 mg/plate), respectively. Neither TH nor PTX exerted mutagenic effect against *S. typhimurium* TA98 cells in examined concentrations.

3.4. Mutagenicity of MeIQx–MTX mixtures

We did not observe any mutagenic effect of MeIQx (10 and 20 μg/plate, which corresponds to concentrations 200 and 400 μM) in *S. typhimurium* TA98 assay.

In turn, we detected mutagenic activity of MeIQx in recently developed modified *V. harveyi* mutagenicity test, which seems to be more sensitive than Ames test. Results of this test, based on

Table 3
Neighborhood association constants (K_{AC}) estimated for interaction of selected IQ-type HCAs with methylxanthines in HP (pH 7.1) and citrate (pH 3.1) buffer at 20 ± 0.1 °C.

	K_{AC} ± SE, M^{-1}				
	IQ	IQx	MeIQx	7,8-DiMeIQx	MeIQ
HP buffer, pH 7.1 ± 0.1					
Caffeine	86.9 ± 2.4	77.0 ± 2.6	60.8 ± 1.6	95.1 ± 1.5	94.4 ± 1.5
Pentoxifylline	84.9 ± 2.1	70.9 ± 2.6	60.4 ± 2.4	96.1 ± 4.0	101.1 ± 1.8
Theophylline	258.2 ± 7.6	154.7 ± 6.8	192.3 ± 12.7	108.1 ± 5.1	129.1 ± 9.8
Citrate buffer, pH 3.1 ± 0.1					
Caffeine	33.7 ± 1.3	45.7 ± 2.3	49.6 ± 2.6	55.8 ± 2.6	24.1 ± 1.3
Pentoxifylline	29.6 ± 1.8	38.5 ± 1.7	43.0 ± 2.5	49.3 ± 2.3	23.9 ± 4.3
Theophylline	97.7 ± 5.7	113.1 ± 4.2	97.0 ± 3.0	114.2 ± 3.5	61.9 ± 7.9

SE – standard error.

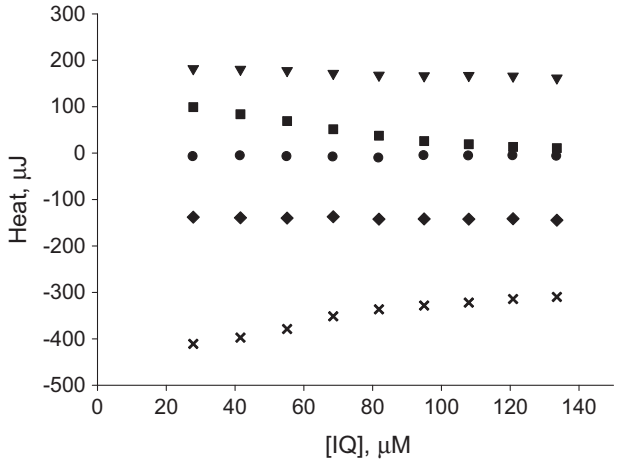

Fig. 6. Thermal effects of microcalorimetric titration of caffeine with buffer (triangles), buffer with IQ (squares), buffer with buffer (circles) and caffeine with IQ (diamonds). Crosses represent the heat of IQ–caffeine complex formation, calculated as the differences between the heat of titration of caffeine with IQ and sum of heats of titration of buffer with buffer, caffeine with buffer and buffer with IQ.

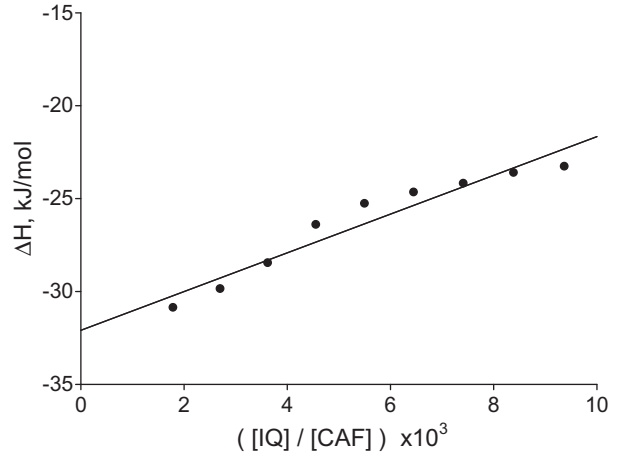

Fig. 7. The heat of interaction of caffeine (CAF) with IQ (corrected for heats of interaction of buffer with buffer, caffeine with buffer and buffer with IQ, see Fig. 6) calculated as kJ/mol of injected IQ. The enthalpy change (ΔH) of IQ–caffeine interaction was calculated by the linear regression of experimental points to infinite dilution of IQ ([IQ]/[CAF] → 0) and is equal to −32.4 kJ/mol.

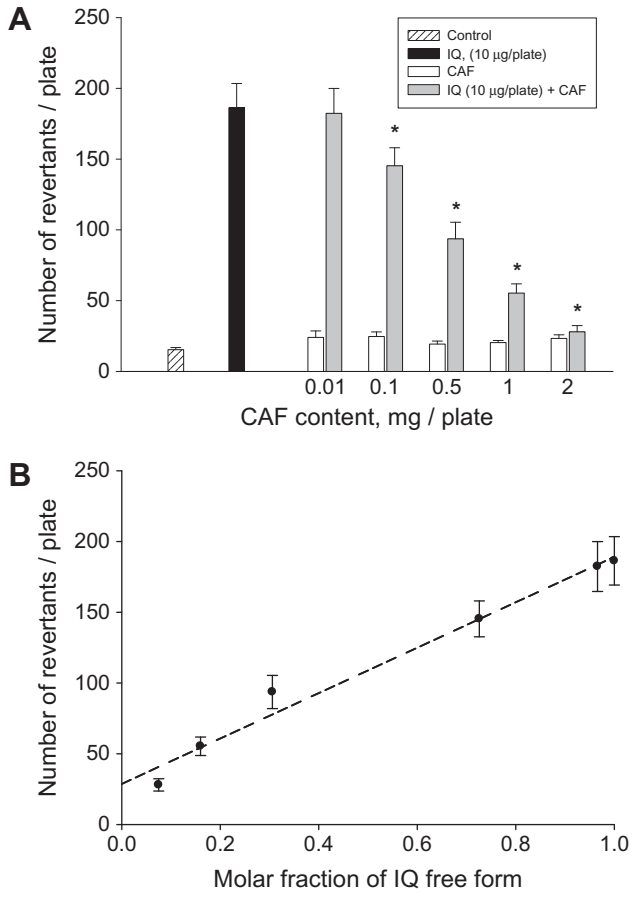

Fig. 8. Mutagenic activity of IQ, caffeine (CAF) and IQ–CAF mixtures in *S. typhimurium* TA98 test (Panel A) and relationship between mutagenic activity of IQ and its free molar fraction in the IQ–CAF mixtures (Panel B). (A) Overnight cultures of *S. typhimurium* TA98 were treated with either 10 μg IQ, 0.01–2 mg CAF or 10 μg of IQ and indicated concentrations of CAF and incubated for 4 h. Afterwards bacteria were spread on plates and revertants were counted after cultivation for 48 h. Results are reported as means ± SD; * – values significantly different from IQ alone ($p < \alpha$, $\alpha = 0.05$). (B) Molar fractions of IQ free form in IQ–CAF mixtures were calculated basing on neighborhood association constant of IQ–CAF complex formation ($K_{AC} = 86.9\ M^{-1}$, see Table 3). Number of revertants per plate for each IQ–CAF mixture were fitted to molar fractions of IQ free form by linear regression ($r^2 = 0.98$).

bioluminescence ability of *V. harveyi* A16 strain (a dim *luxE* mutant), are expressed as relative light units (RLU) bars. Mutagenic activity of MeIQx was examined at 0.1 μM concentration. This concentration of mutagen was non-toxic to bacteria, but exhibited strong mutagenic activity, as determined in preliminary experiments. We used *V. harveyi* mutagenicity test to check the influence of MTX on the mutagenic activity of MeIQx. Mutagenic activity of MeIQx, CAF and MeIQx–CAF mixtures is presented in Fig. 9A. Surprisingly, CAF proved to be notably mutagenic at examined concentrations (above half of MeIQx mutagenic effects for CAF concentration range 0.1–100 μM and slightly above MeIQx mutagenic effects for 1 mM CAF) against *V. harveyi* A16 strain. Thus, one can expect increased mutagenic activity of MeIQx in the presence of CAF, as a consequence of a possible synergistic action of these compounds against bacterial cells. However, our results demonstrated no increase in mutagenic activity of MeIQx–CAF mixture in comparison to mutagenic activity of CAF. In contrast, considerable decrease in mutagenic activity of the MeIQx–CAF mixture with respect to the activity of mutagen alone was evident, especially for the mixture containing 100 μM CAF (Fig. 9A).

Table 4
Enthalpies (ΔH) of complex formation between selected IQ-type HCAs and methylxanthines calculated on the basis of microcalorimetry studies.

Enthalpy ± SE, kJ/mol					
	IQ	IQx	MeIQx	7,8-DiMeIQx	MeIQ
Caffeine	−32.4 ± 0.6	−18.7 ± 0.2	−22.1 ± 0.5	−26.9 ± 0.4	−29.6 ± 2.3
Pentoxifylline	−34.1 ± 1.3	−20.8 ± 1.0	−21.2 ± 0.7	−29.5 ± 1.0	−39.8 ± 0.9
Theophylline	−37.4 ± 0.8	−15.1 ± 0.1	−15.7 ± 0.4	−20.8 ± 0.2	−34.6 ± 1.0

SE – standard error.

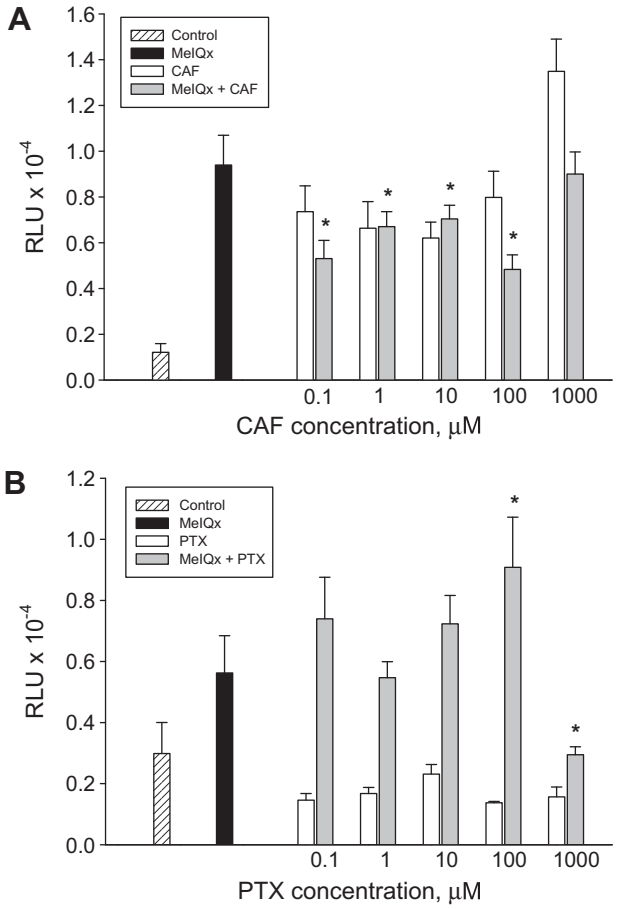

Fig. 9. Mutagenic activity of MeIQx, caffeine (CAF), MeIQx–CAF mixture (Panel A), pentoxiphylline (PTX) and MeIQx–PTX mixture (Panel B) in *Vibrio harveyi* A16 strain mutagenicity assay. Exponentially growing cultures of *V. harveyi* A16 strain were treated with either 0.1 μM MeIQx, 0.1–1000 μM CAF (PTX), or 0.1 μM MeIQx and indicated concentrations of CAF (PTX) and incubated for 3 h. Afterwards, luminescence of each culture was measured and expressed as relative light units (RLU) per A_{575} of bacterial culture; * – values significantly different from MeIQx alone ($p < \alpha$, $\alpha = 0.05$).

We used similar procedure for MeIQx–PTX mixtures (Fig. 9B). In contrast to CAF, we did not observe mutagenic effects of PTX. In addition, a significant decrease in mutagenic activity of MeIQx was observed only for mixture containing 1 mM PTX.

We also tested mutagenicity of MeIQx–TH mixtures in *V. harveyi* assay (data not shown). We did not observe significant decrease in MeIQx mutagenic activity upon the addition of TH (TH concentration range 0.1–100 μM), however, TH alone proved to be mutagenic at 100 μM concentration.

4. Discussion

It has been previously reported that CAF and other MTX can form stacking complexes with several aromatic compounds: anticancer drugs [8,27], fluorescence dyes [26,33–36], mutagens [3,4], neurotoxins [6,7], and others [37,38]. The authors of papers cited above assumed that hetero-association of MTX with these compounds may diminish their biological activity.

Spectroscopic and calculation results presented in this paper indicate the presence of direct stacking interactions between all examined IQ-type HCAs and MTX in several solutions (see Table 3). For all tested compounds, increasing concentration of HCA–MTX stacking aggregates (observed during the titration of HCAs with MTX) is associated with the decline in the concentration of free active form of mutagen molecules (C_A) in the mixture (see Table 2 and Fig. 6). Calculated "neighborhood" association constants (K_{AC}) values are in good agreement with K_{AC} values determined previously for several aromatics – CAF (PTX or TH) systems [3,4,7,8,26,27,33,34]. What is more, K_{AC} values of HCAs–MTX interactions correspond to hetero-association constants calculated basing on different calculation models [39–42].

K_{AC} values for HCAs–TH complexes formation, presented in Table 3, are higher than K_{AC} values calculated for HCAs–CAF and HCAs–PTX complexes at both neutral and acidic pH (except for 7,8-DiMeIQx at neutral pH, for which K_{AC} values are comparable). Similar relationship was observed for interactions between anticancer drug doxorubicin (DOX) and MTX [8]. Calculated K_{AC} values were equal to 128.3, 173.0 and 364.5 M^{-1} for DOX–CAF, DOX–PTX and DOX–TH complex formation, respectively. However, K_{AC} values of mixed complex formation for other anticancer drugs, daunomycin (DAU) and mitoxantrone (MIT) with these MTX did not demonstrate similar relationship [8]. Andrejuk and colleagues also showed that hetero-association constants for interaction of several aromatic molecules are higher for TH than CAF [42]. They interpreted this difference mainly as a consequence of the absence of a 7-Me group in TH molecule, which is present in CAF [42].

In order to check how the differences at pH between various parts of gastrointestinal tract (where food-borne HCAs and MTX may encounter) can influence HCA–MTX complex formation, we performed spectroscopic experiments between IQ-type HCAs and MTX in both neutral and acidic environment. Our results demonstrate HCAs–MTX stacking complexes formation in acidic as well as in neutral environment, however, K_{AC} values obtained in acidic solution are significantly lower except 7,8-DiMeIQx–TH (see Table 3). Protonation, especially of HCAs and partially of MTX, in acidic environment may be responsible for this phenomenon.

Microcalorimetric titration results confirmed mixed stacking complexes formation between HCAs and MTX with enthalpy changes (ΔH) between −15.1 and −39.8 kJ/mol (Table 4). Similar results were obtained earlier for interaction of CAF with fluorescent dye DAPI ($\Delta H = -46.47$ kJ/mol [26]) and mitoxantrone ($\Delta H = -47.31$ kJ/mol [27]), as well as for CAF and PTX with metabolically converted 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) and 1-methyl-4-phenylpyridinium (MPP^+) with ΔH values equal to −34.33 kJ/mol, −24.28 kJ/mol, −12.14 kJ/mol and −18.42 kJ/mol for $MPDP^+$–CAF, MPP^+–CAF, $MPDP^+$–PTX and MPP^+–PTX interactions, respectively [7]. Corresponding ΔH values for interactions between MTX and several aromatic compounds were obtained with NMR spectroscopy. Enthalpies of hetero-complex formation for CAF ranged from −20.4 for CAF–acridine orange interaction to −27.2 kJ/mol for CAF–actinomycin D interaction [40], whereas enthalpies of hetero-complex formation for TH ranged from −15.0 to −34.3 kJ/mol for TH–novantrone and TH–proflavine hetero-complexes formation, respectively [42].

It has been previously demonstrated that CAF exerts protective effects against mutagenic activity of anticancer drugs (DAU, DOX, MIT) [8], neurotoxins (MPTP, $MPDP^+$, MPP^+) [6,7] and model mutagens (quinacrine mustard, ICR191, ICR170) [4]. These protective effects were explained by direct interaction of CAF with aromatic compounds. Here we show, with the use of *S. typhimurium* TA98 assay, that CAF reduces the mutagenic activity of IQ in a dose-dependent manner (Fig. 8A). PTX and TH also diminish mutagenic activity of IQ against *S. typhimurium* TA98, which was demonstrated by decreased number of revertants for IQ–PTX and IQ–TH mixtures in comparison to number of revertants induced by IQ alone. Moreover, we noticed a strong linear relationship ($r^2 = 0.98$) between the mutagenicity of IQ and molar fraction of IQ free form in the appropriate mixture with CAF (Fig. 8B), which indicates that direct interactions between IQ and CAF may play a

vital role in observed phenomenon. Such sequestration of IQ molecules in complexes with CAF or other MTX may lead to the decline in the concentration of free mutagen, reducing in this way its availability for bacterial cells.

In addition, we intended to perform similar experiments against *S. typhimurium* TA98 with MeIQx and MTX mixtures. However, in contrast to IQ, no considerable mutagenic effects were observed even at relatively high concentrations of MeIQx. Therefore, we used recently developed *V. harveyi* A16 bioluminescence mutagenicity assay, in which 0.1 μM MeIQx was proven to be mutagenic (Fig. 9). We demonstrated considerable lowering of MeIQx mutagenic activity in the presence of CAF and surprisingly, relatively high level of CAF mutagenicity against *V. harveyi* A16 cells (Fig. 9A). It has been shown previously that CAF may be mutagenic to bacteria, and, in higher concentrations, to mammalian cells, but *in vivo*, probably due to the activity of detoxifying enzymes, CAF is devoid of mutagenic effects [43]. Additionally, mutagenic activity of CAF at low concentrations in bacterial mutagenicity assays is often difficult to observe and ambiguous to interpret. Thus, it seems that the usage of the bioluminescence mutagenicity assay enabled us to observe even weak mutagenic effects of CAF, probably due to higher sensitivity of this method in comparison to the Ames test (see Fig. 8A) or methods based on *V. harveyi* BB7 or BB7XM strain [31]. In contrast to CAF, PTX did not exert mutagenic activity in *V. harveyi* A16 test. Moreover, statistically significant decrease in mutagenic activity of MeIQx was observed only in the presence of 1 mM PTX (Fig. 9B). We estimated (based on calculated K_{AC} value = 84.9 M^{-1}) that only about 10% of MeIQx molecules form hetero-complexes with 1 mM PTX in the mixture. We speculate that concentration of MeIQx molecules available for bacterial cells fluctuates at the level of minimal mutagenic dose and even slight reduction of MeIQx concentration may lead to the protection of bacterial cells against mutagen. Probably for the same reason we did not observe the protective effect of TH against MeIQx mutagenic activity, especially regarding the fact that TH tends to exert weaker protective effect than CAF or PTX, as shown for IQ in the Ames test.

It has been shown earlier, using *S. typhimurium* TA98 strain in the Ames test, that CAF inhibits the mutagenicity of another HCA, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) [24]. The authors suggested that CAF protective action probably results from the competitive inhibition of cytochrome P450 enzymes and/or possible direct non-covalent interactions between PhIP and CAF molecules. It should be indicated that in these experiments PhIP was biologically activated with S9 liver fraction, containing cytochrome P450 enzymes [24]. By contrast, in our experiments IQ and MeIQx were not exogenously activated. For this reason stacking complexes formation seems to be the most possible explanation for the protective effect of MTX against mutagenic activity of IQ and MeIQx.

Many accumulated statistical data indicate the protective effects of CAF against cancer [2]. One of the possible explanations for this phenomenon is direct interaction of CAF with several aromatic compounds exhibiting mutagenic and/or carcinogenic potency. HCAs are mutagens and carcinogens which are mainly consumed as food contaminants. Therefore, it is highly probable that these mutagens can encounter and interact with commonly consumed CAF and other MTX in gastrointestinal tract, where particularly CAF may reach relatively high concentrations (several-fold higher than HCAs concentrations). Demonstrated here mixed HCAs–MTX stacking complexes formation may account for the explanation of CAF and other MTX protective action against HCAs-induced carcinogenesis, but these speculations require further detailed studies.

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