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Impaired mutagenic activities of $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium) and MPP^+ (1-methyl-4-phenylpyridinium) due to their interactions with methylxanthines

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Abstract—MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a neurotoxin causing symptoms that resemble those observed in patients suffering from Parkinson's disease. However, in animal or human organisms, MPTP is converted to $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium) and further to MPP^+ (1-methyl-4-phenylpyridinium); the latter compound is the actual neurotoxin. In this report, we demonstrate that $MPDP^+$ and MPP^+ can form stacking complexes with methylxanthines (caffeine and penthoxifylline), which leads to significant impairment of the biological activity of these toxins (as measured by their mutagenicity).

1. Introduction

Parkinson's disease is a neurodegenerative disorder of still unclear etiology, though there are data indicating contribution of both genetic and nongenetic causes.[1–3] The most characteristic symptoms of this disease are motor deficits, including bradykinesia, rigidity, and resting tremor. These symptoms are results of the massive striatal dopamine deficiency, which arises from degeneration of dopaminergic neurons in the substantia nigra and the loss of striatal dopaminergic fibers.

Among nongenetic causes of Parkinson's disease, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) appears to be one of the best studied chemicals. It is a neurotoxin, which induces in humans and animals a syndrome virtually identical to Parkinson's disease. This occurs due to MPTP-dependent selective damage of dopaminergic neurons, predominantly those originating in the substantia nigra pars compacta. Therefore, MPTP is extensively used in studies on animal models of this disorder.[4]

Various studies indicated a protective effect of coffee intake or of moderate doses of caffeine (1,2,3-trimethylxanthine) on risk of Parkinson's disease in various human populations.[5–7] Indeed, it was demonstrated that caffeine attenuates MPTP-induced loss of striatal dopamine and dopamine transporter binding sites,[8,9] and improves the memory deficits observed in rats treated with this neurotoxin.[10] Since caffeine is an antagonist of the adenosine A_{2A} receptor,[11] and the effects of caffeine on MPTP-treated animals were mimicked by several A_{2A} antagonists and by genetic inactivation of the A_{2A} receptor,[8,12,13] it was initially suggested that caffeine attenuates MPTP toxicity solely by the A_{2A} receptor blockade.[8,14] However, recent studies demonstrated that caffeine can form stacking (π–π) complexes with MPTP.[15] Moreover, induction of mutations in a microbiological mutagenicity assay (a biological activity

Keywords: Parkinson's disease; Stacking complexes; Mutagenicity; $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium); MPP^+ (1-methyl-4-phenylpyridinium); Methylxanthines.
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of MPTP which is completely independent of the A_{2A} receptor blockade), was found to be significantly reduced by caffeine.[15] Therefore, it was suggested that caffeine may attenuate neurotoxicity of MPTP and may reveal its protective effects on the risk of Parkinson's disease not only by blocking the A_{2A} receptor but also by sequestering neurotoxin molecules in mixed complexes, especially in stomach.[15]

The problem with the model presented above is that a derivative of MPTP, rather than this compound itself, is an actual neurotoxin. Namely, after intake by a human or animal, MPTP is metabolically converted to $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium) and further to MPP^+ (1-methyl-4-phenylpyridinium), a derivative which causes all the neuronal disturbances described above.[16–18] Therefore, we asked whether caffeine and other methylxanthines can form stacking complexes with $MPDP^+$ and MPP^+, and reduce their biological activity.

2. Results

2.1. Microcalorimetric studies on direct interactions between methylxanthines and $MPDP^+$ and MPP^+

Chemical structures of the main compounds studied in this work: caffeine (1,2,3-trimethylxanthine, CAF), pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)xanthine, PTX), $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium), and MPP^+ (1-methyl-4-phenylpyridinium) are presented in Figure 1.

To study direct interactions between $MPDP^+$, MPP^+ and chosen methylxanthines (caffeine and pentoxifylline), we employed the method of titration microcalorimetry, proved previously to be useful in this kind of studies.[15] Ten portions of $MPDP^+$ (concentration range 0.01–0.09 mM) or MPP^+ (concentration range 0.01–0.09 mM) were added to solutions of methylxanthines: caffeine at initial concentration of 98.8 mM or pentoxifylline at initial concentration of 100.4 mM. Control measurements were also performed, to estimate heats of dilution of each compound in the mixtures ($MPDP^+$, MPP^+, and methylxanthines with buffer titrations) and heat exchange between syringe and cell (buffer with buffer titration) (data not shown).

Approximate values of heat of interaction between methylxanthines and $MPDP^+$ or MPP^+ were corrected by subtraction of the sum of heats of dilution of adequate compounds. The obtained results (exemplified in Fig. 2) were extrapolated to values of [$MPDP^+$ or MPP^+]/[methylxanthine] → 0. Calculated values of heats

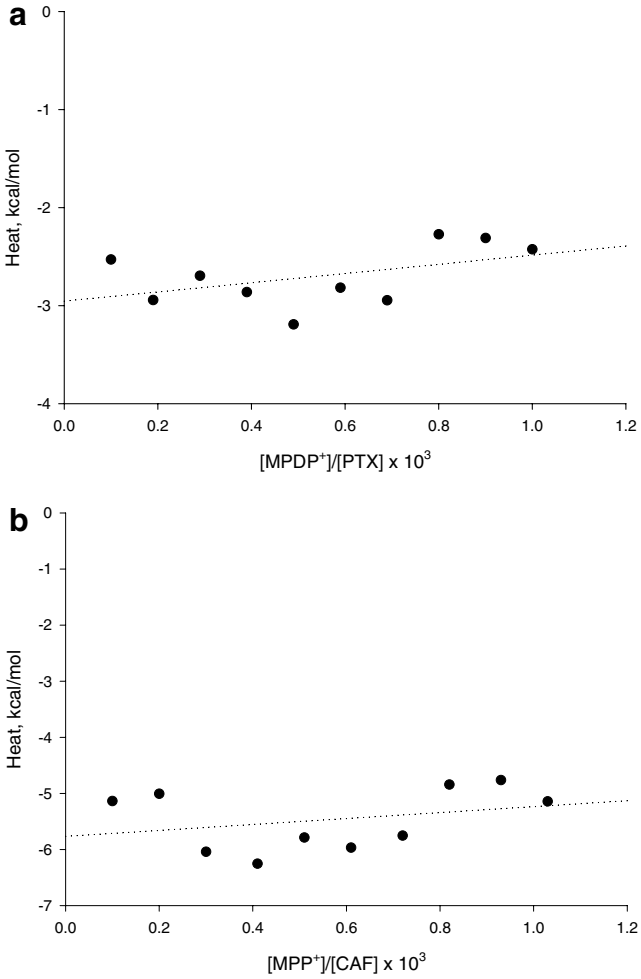

Figure 2. Heat of interaction between $MPDP^+$ and pentoxifylline (a), and between MPP^+ and caffeine (b) estimated by microcalorimetric titration. For calculation of the heat of interaction (see results presented in Table 1), the heat of every reaction was corrected for the heat of dilution of components (not shown) and estimated by extrapolation to [$MPDP^+$ or MPP^+]/[methylxanthine] → 0.

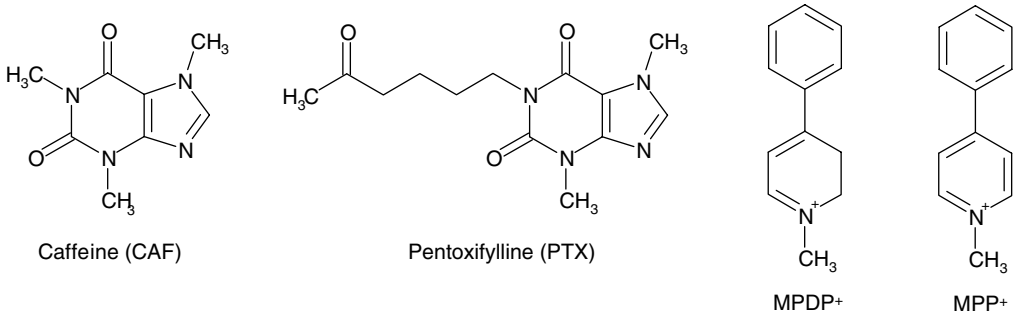

Figure 1. Chemical structures of caffeine (1,2,3-trimethylxanthine, CAF), pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)xanthine, PTX), $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium), and MPP^+ (1-methyl-4-phenylpyridinium).

Table 1. Values of heats of interactions between $MPDP^+$, MPP^+ and chosen methylxanthines calculated on the basis of microcalorimetric studies

Mixed compounds	Calculated heat of interaction, ΔH[a] (kcal mol^{-1})
$MPDP^+$ and caffeine	−8.2 ± 0.4
$MPDP^+$ and pentoxifylline	−2.9 ± 0.1
MPP^+ and caffeine	−5.8 ± 0.2
MPP^+ and pentoxifylline	−4.4 ± 0.2

[a] Calculated heats of interactions between various compounds ± SE are presented. Examples of experimental results are shown in Figure 2.

of interactions between $MPDP^+$, MPP^+ and chosen methylxanthines, shown in Table 1, are typical for staking interactions.

2.2. The absorption spectra and calculation of the association constant

Another technique we aimed to use in our study on interactions between $MPDP^+$, MPP^+ and methylxanthines was light absorption spectrometry. However, it was not possible to employ this method to estimate direct interactions between MPP^+ and caffeine or pentoxifylline, because spectra of these compounds overlap in the UV wavelength range (data not shown).

Absorption spectra of the mixtures of $MPDP^+$ with caffeine are presented in Figure 3. An increase in caffeine concentration in the mixture caused lowering of the maximum peak (hypochromic effect) and its insignificant red shift (bathochromic effect). These spectra changes indicate two predominant components in the mixture: a free form of $MPDP^+$ and its bound form in a complex with caffeine. The spectrum of the $MPDP^+$–caffeine complex was calculated by the extrapolation for each wavelength of mixture spectra to $C_{TA}/C_{TC} \rightarrow 0$, where C_{TA} and C_{TC} are total concentrations of $MPDP^+$ and caffeine in the mixtures, respectively. Then, the spectra of mixtures were decomposed into weighted sum of components, as described previously.[19,20] Based on the thermodynamical model of mixed aggregation,[20] concentrations of all components in the mixture and the neighborhood association constant (K_{AC}) were calculated. The comparison of the experimental and calculated data is presented in Figure 4.

A similar procedure was carried out for testing interactions between $MPDP^+$ and pentoxifylline (data not shown). Calculated neighborhood association constants (K_{AC}) were 138.7 ± 8.6 M^{-1} and 59.7 ± 3.9 M^{-1} (values ± SE) for $MPDP^+$ with caffeine and $MPDP^+$ with pentoxifylline, respectively.

Light absorption spectra changes and calculated neighborhood association constants (K_{AC}) for $MPDP^+$ with caffeine and pentoxifylline again (similarly to results presented in Section 2.1.) suggest formation of stacking complexes between these methylxanthines and $MPDP^+$. The obtained values are quite similar to those reported

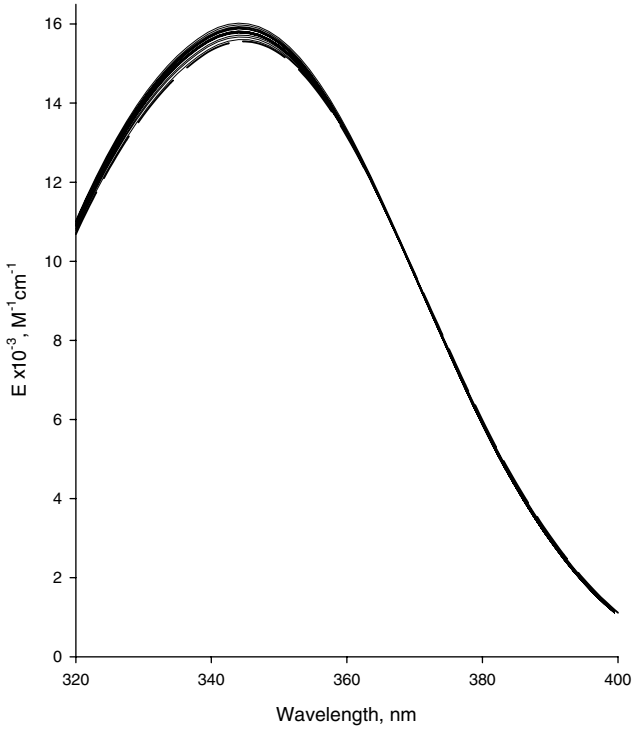

Figure 3. Spectrophotometric analysis of titration of $MPDP^+$ (initial concentration of 0.65 mM) with caffeine (stock solution concentration 92.2 mM). The upper thin line represents the spectrum of free $MPDP^+$, other thin lines below represent the spectra of the mixture of $MPDP^+$ with caffeine (concentration range in cuvette 0.7–16.8 mM). The dashed thick line (on the bottom) represents the spectrum of the complex $MPDP^+$–caffeine obtained numerically as described previously.[20]

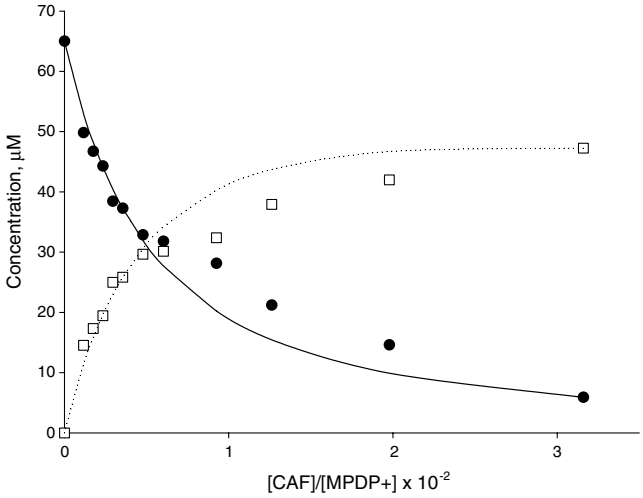

Figure 4. The comparison of the experimental results and calculated data of numerical analysis of $MPDP^+$–caffeine interactions in mixtures. Circles represent the concentration of a free form of $MPDP^+$ and squares—the concentration of $MPDP^+$ bound to caffeine and forming a complex (experimental data). Solid line represents the concentration of a free form of $MPDP^+$, and dotted line—the concentration of $MPDP^+$ bound to caffeine and forming a complex, calculated using the mixed aggregation model,[20] based on the estimated neighborhood association constant K_{AC} = 138.7 ± 8.6 (SE) M^{-1}.

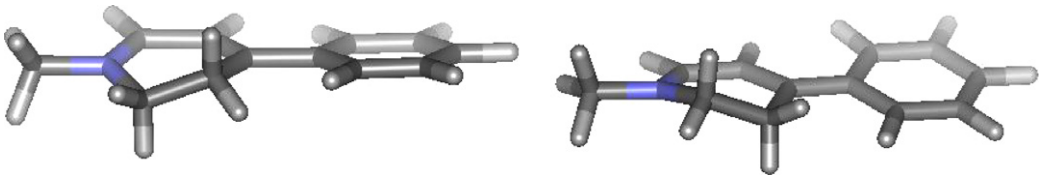

Figure 5. Two possible $MPDP^+$ conformations, determined by molecular modeling. Colors: oxygen—red; nitrogen—blue, carbon—dark gray, hydrogen—light gray.

for this kind of interactions between several aromatic compounds and methylxanthines.[21,22]

2.3. Molecular modeling

Since results of microcalorimetric and spectroscopic studies suggested that $MPDP^+$ and MPP^+ may form stacking complexes with caffeine and pentoxifylline, we performed molecular modeling studies to test this possibility in silico. Our analysis revealed that conformation changes of a $MPDP^+$ molecule may occur in the 2,3-dihydropyridine ring due to possible rotation of the bound between two carbons: C2 and C3 (sp3 hybridization) (Fig. 5). Torsion angle (N1–C2–C3–C4) in the 2,3-dihydropyridine ring can adopt values in the range from −37° to 37°. These angle changes also affect relative positions of the two rings (phenyl and 2,3-dihydropyridine rings), which are demonstrated in Figure 5. MPP^+ adopts one possible conformation where two aromatic rings are almost parallel (data not shown, but see Fig. 6).

The most stable, possible stacking complexes between $MPDP^+$ or MPP^+ and methylxanthines (caffeine and pentoxifylline) are presented in Figure 6. The distance between rings, in all presented conformations is equal to about 3.7 Å. The complex formation energies have been estimated at about -4.7 kcal mol^{-1} and -6.2 kcal mol^{-1} for $MPDP^+$–caffeine and $MPDP^+$–pentoxifylline, respectively. Similar values of complex formation energy were calculated for MPP^+–caffeine (-4.9 kcal mol^{-1}) and MPP^+–pentoxifylline (-5.7 kcal mol^{-1}) interactions.

2.4. Mutagenicity of $MPDP^+$ and MPP^+

We asked whether formation of stacking complexes between methylxanthines and $MPDP^+$ or MPP^+ can influence biological activities of these toxins. Since it was proposed previously that caffeine may attenuate toxicity of MPTP and its derivatives by acting as an A_{2A} receptor blocker,[8,14] to estimate effects of direct interactions between MPTP derivatives ($MPDP^+$ and MPP^+) and methylxanthines on their biological activities we aimed to use an assay which is totally independent of the A_{2A} receptor (otherwise results would always be unclear due to the influence of the effects of methylxanthines on the A_{2A} receptor).

Apart from neurotoxicity, considerable mutagenic activities of MPTP, $MPDP^+$, and MPP^+, determined using the Ames test, were demonstrated previously.[23] However, other authors reported opposite results, suggesting that MPTP and MPP^+ are not mutagenic (mutagenicity was measured also in the Ames test).[24] Nevertheless,

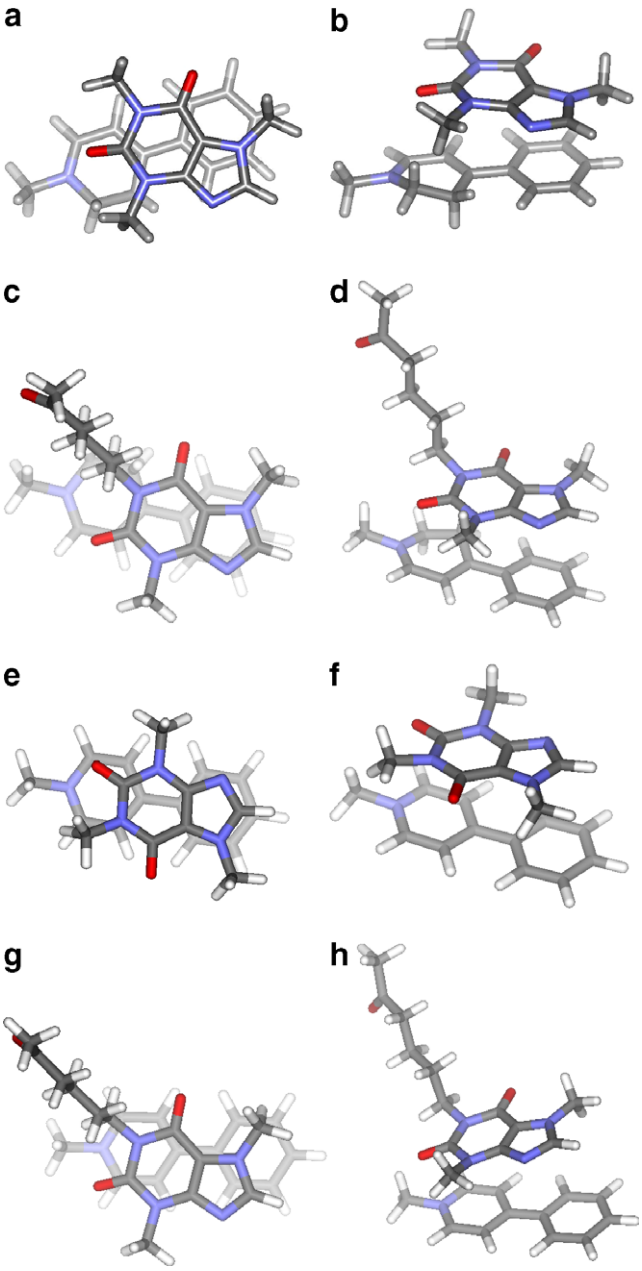

Figure 6. The most probable lowest energy stacking complex between $MPDP^+$ and caffeine (a,b), $MPDP^+$ and pentoxifylline (c,d), MPP^+ and caffeine (e,f), and MPP^+ and pentoxifylline (g,h), from the top view (a,c,e,g) and the side view (b,d,f,h), obtained by molecular modeling. Colors: oxygen—red; nitrogen—blue, carbon—dark gray, hydrogen—light gray.

Table 2. Mutagenicity of $MPDP^+$ and MPP^+ assessed using the *V. harveyi* mutagenicity assay

Compound	Concentration (μM)	Number of induced neomycin-resistant mutants[a]
None	0	0
$MPDP^+$	0.1	109 ± 8
	0.3	278 ± 14
	0.6	357 ± 71
	1.2	528 ± 46
	2.4	535 ± 72
MPP^+	0.3	15 ± 5
	0.6	39 ± 6
	1.2	52 ± 10
	2.4	76 ± 8
	3.6	176 ± 43

[a] Number of induced mutants was calculated by subtraction of the number of neomycin-resistant colonies found in the absence of an additional compound (equal to 48 ± 8) from the number of neomycin-resistant colonies observed in experiments with particular compound added. The presented results are mean values from three or more measurements ± SD.

Table 3. Effects of methylxanthines on mutagenicity of $MPDP^+$ and MPP^+

Methylxanthine[a] and its concentration	Number of induced neomycin-resistant mutants in the presence of[b]		
	No toxin	$MPDP^+$ (1.2 μM)	MPP^+ (3.6 μM)
None	0	528 ± 46	176 ± 43
CAF (10 μM)	12 ± 10	293 ± 114	103 ± 36
CAF (100 μM)	21 ± 15	70 ± 7	50 ± 17
CAF (500 μM)	9 ± 5	51 ± 10	31 ± 11
CAF (1000 μM)	10 ± 7	47 ± 4	7 ± 5
PTX (10 μM)	3 ± 3	278 ± 55	116 ± 17
PTX (100 μM)	5 ± 7	143 ± 42	78 ± 15
PTX (500 μM)	2 ± 8	77 ± 39	52 ± 25
PTX (1000 μM)	−1 ± 8	51 ± 14	24 ± 10
TH (10 μM)	3 ± 2	176 ± 27	159 ± 35
TH (100 μM)	0 ± 8	114 ± 32	64 ± 5
TH (500 μM)	6 ± 5	77 ± 21	42 ± 17
TH (1000 μM)	5 ± 5	54 ± 5	15 ± 6

[a] Abbreviations: CAF, caffeine; PTX, pentoxifylline; TH, theophylline.
[b] Number of induced mutants was calculated by subtraction of the number of neomycin-resistant colonies found in the absence of additional compound (equal to 48 ± 8) from the number of neomycin-resistant colonies observed in experiments with particular compound(s) added. The results showed are mean values from three or more measurements ± SD.

recent studies, in which another microbiological mutagenicity assay (the *Vibrio harveyi* mutagenicity test) was employed, indicated mutagenic activities of MPTP.[25] Thus, we asked whether it is possible to observe mutagenicity of $MPDP^+$, and MPP^+ using this novel assay.

Various concentrations of $MPDP^+$ and MPP^+ were tested in the *V. harveyi* mutagenicity assay after addition of these compounds to bacterial cultures. We have detected unambiguous mutagenic activities of both tested compounds, though higher mutagenicity of $MPDP^+$ than that of MPP^+ was evident (Table 2).

2.5. Inhibition of mutagenic activities of $MPDP^+$ and MPP^+ by methylxanthines

Detection of a biological activity (mutagenicity) of $MPDP^+$ and MPP^+ that can be studied in the A_{2A} receptor-independent assay (*V. harveyi* mutagenicity assay) allowed us to test whether interactions between these compounds and methylxanthines influence their activities. Caffeine, pentoxifylline, and theophylline (which is one of the most common methylxanthines, but whose relatively low solubility excluded it from the microcalorimetric and spectroscopic studies) were employed in these experiments.

None of the tested methylxanthines caused appearance of a significantly changed (relative to control experiments without any additional compounds) number of mutants in the *V. harveyi* mutagenicity assay at concentrations of 1 mM or lower (Table 3). However, addition of caffeine, pentoxifylline or theophylline into solutions of $MPDP^+$ or MPP^+ decreased their mutagenic activities in a dose-dependent manner (Table 3). Therefore, we conclude that methylxanthines can decrease mutagenicity of $MPDP^+$ and MPP^+, though large molar excess (at least several fold, but optimally a few hundred fold) of a methylxanthine is necessary to obtain an unambiguous effect.

3. Discussion

Administration of MPTP to humans or animals causes symptoms closely resembling those of Parkinson's disease,[4] which can be alleviated by caffeine and its derivatives.[5–13] This protective action of caffeine was proposed to be due to a blockage of the adenosine A_{2A} receptor,[8,14] however, another mechanism is also possible as direct interactions between this methylxanthine and MPTP have been demonstrated recently.[15] Since MPTP is metabolized to $MPDP^+$ and then to MPP^+, which is an actual neurotoxin, we investigated interactions between these MPTP derivatives and methylxanthines.

Microcalorimetric measurements and molecular modeling revealed that formation of stacking complexes between methylxanthines and metabolic derivatives of MPTP ($MPDP^+$ and MPP^+) is likely. Although light absorption spectrometric studies with $MPDP^+$ showed minor spectral shifts, and one might consider instability of this compound, in our opinion the most probable interpretation of these results is formation of stacking complexes between tested methylxanthines and $MPDP^+$, which supports the conclusion made on the basis of microcalorimetry and molecular modeling. Thus, we asked whether interactions of $MPDP^+$ and MPP^+ with methylxanthines influence biological activities of these neurotoxins. To avoid any overlap with the A_{2A}

receptor-dependent activity of caffeine, we measured mutagenicity of $MPDP^+$ and MPP^+ in the presence and absence of methylxanthines using a microbiological assay (the *V. harveyi* mutagenicity test).

Our results corroborate the previously published conclusion that MTPT and its derivatives are mutagens,[23] but they are in contrast to suggestions of other authors who could not detect mutagenic activities of MPTP and MPP^+ using the Ames test.[24] We suspect that this discrepancy is due to the fact that the authors who reported a lack of mutagenicity of these compounds used their relatively high concentrations (about 200 μM).[24] At such concentrations of MPTP, a small number of detected mutants were observed in both microbiological mutagenicity assays (Ames test and *V. harveyi* mutagenicity assay),[15,23] which was supposed to arise from induction of multiple mutations in cells, causing lethal effects or a drastic decrease in bacterial growth rate, and thus, preventing formation of bacterial colonies.[15]

Using the *V. harveyi* mutagenicity assay, we have demonstrated that methylxanthines impair mutagenic activities of $MPDP^+$ and MPP^+. Therefore, we conclude that formation of stacking complexes between methylxanthines and either $MPDP^+$ or MPP^+ results in their biological inactivation due to capturing of these compounds in large complexes.

These findings may have implications for mechanisms of caffeine-mediated protective effects on the risk of Parkinson's disease, described previously.[5–7] These mechanisms can involve both blocking of the A_{2A} receptor (as proposed previously)[8,14] and inactivation of the neurotoxins by formation of stacking complexes with them. Since neurotoxins belong to the most important nongenetic causes of Parkinson's disease,[2] and compounds structurally similar to MPTP, $MPDP^+$, and MPP^+ may be quite common in a polluted environment, the protective effects of caffeine and other methylxanthines can be important in prevention of this disorder.

As shown in Table 3, 50% biological inactivation of $MPTP^+$ by methylxanthines requires about 10 times molar excess of caffeine, pentoxifylline or theophylline, but 90% inactivation occurs when this excess is higher than 100 times. Quite similar results were obtained by us for MPP^+ and were reported previously for MPTP.[15] Since biological inactivation of MPTP and its derivatives is very effective only at a high molar excess of methylxanthines, it appears that this mechanism may be important in stomach (where caffeine can be found at high concentrations, even millimolar in the case of high coffee consumption) rather than in blood or brain (where it would be perhaps impossible to achieve such concentrations of methylxanthines without serious side effects). Therefore, although toxins occur at relatively low concentrations even in polluted environment, we suggest that methylxanthines might have mainly a role in protection of uptake of neurotoxins from a digestive tract after their oral administration (large stacking complexes should be excreted). In this light, it is worth mentioning that caffeine- and theophylline-mediated inhibition of uptake of MPP^+ by human intestinal cells has been reported recently (though those authors neither investigated nor suggested direct interactions between methylxanthines and MPP^+).[26,27] However, if a toxin has already been transported to the brain, then the A_{2A} receptor blocking mechanism, rather than sequestration of this toxin by methylxanthines, may prevent or alleviate symptoms of the Parkinson's disease.

4. Experimental

4.1. Chemicals

$MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium), MPP^+ (1-methyl-4-phenylpyridinium), caffeine (1,2,3-trimethylxanthine, CAF), pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)xanthine, PTX), and theophylline (1,3-dimethylxanthine, THF) were purchased from Sigma–Aldrich.

4.2. Microcalorimetry

The microcalorimetric titrations were performed at 30 ± 0.1 °C in the buffer composed of 5 mM Hepes and 150 mM NaCl, pH 7.1, using Omega Titration Calorimeter (Microcal Inc.). Ten microlitre portions of $MPDP^+$ or MPP^+ (stock solution concentrations were 1.32 mM and 1.34 mM, respectively) were added to 1.3 ml of the titrated solution (containing caffeine at initial concentration of 98.8 mM or pentoxifylline at initial concentration of 100.4 mM) and the heat process was measured as a function of time (μcal s^{-1}). The results of titration were corrected for heat of components' dilution, and expressed as the heat per mole of $MPDP^+$ or MPP^+, as described previously.[20]

4.3. Light absorption spectrometry

Light absorption spectra were measured using Beckman's DU 650 spectrophotometer connected to Polystat's thermostat constant circulator (25 ± 0.1 °C). Two millilitres of a $MPDP^+$ solution (initial concentration of 0.65 mM and 0.67 mM for caffeine and pentoxifylline titration, respectively) in a buffer (5 mM Hepes, 150 mM NaCl, pH 7.1) was placed in a quartz cuvette (1 cm light path) and titrated with 10–150 μl of 92.2 mM caffeine or 133.0 mM pentoxifylline (dissolved in water). The spectra were measured in the range of $MPDP^+$ absorption (above 320 nm), at 0.5 nm interval, and stored on a computer disk. The spectra were expressed in terms of the molar absorption coefficient (E_λ, M^{-1} cm^{-1}). The spectra used for calculations were smoothed using ''smooth 2D data'' algorithm (Smoother Loess, Polynomial Degree 2) implemented in SigmaPlot 2000 ver.6 software (SPSS Inc.).

4.4. Calculations of the association constants

Calculations of the association constants of $MPDP^+$ with caffeine and pentoxifylline were accomplished using statistical thermodynamics of mixed aggregation.[19,20,28] Previously described notations and definitions were employed.[29]

4.5. Molecular modeling

All the calculations were performed using HyperChem, Hypercube Inc. software. We used the semi-empirical PM3 method with steepest descent algorithm for geometry optimization and minimization of total energy of the system. Molecules of caffeine, pentoxifylline, $MPDP^+$ and MPP^+, and their several possible configurations in complexes, were built and optimized (terminal condition—RMS gradient lower than 0.2 for molecules alone or 0.3 for complexes). On the basis of these calculations, optimal geometries of the complexes have been evaluated. The energy of $MPDP^+$-methylxanthine and MPP^+-methylxanthine complex formation was estimated by subtraction of the energies of formation of caffeine, pentoxifylline, $MPDP^+$ or MPP^+ (all molecules in the same conformation as in the complex) from total energy of the complex.

4.6. The *Vibrio harveyi* mutagenicity assay

The *V. harveyi* mutagenicity assay is based on the use of a series of genetically modified strains of this bacterium.[30,31] *V. harveyi* is a bacterium naturally sensitive to neomycin, but mutants resistant to this antibiotic can be isolated. The frequency of the appearance of such mutants increases in the presence of mutagens in a dose–response manner.[30,32] To assess mutagenicity of $MPDP^+$ and MPP^+ in the presence or absence of methylxanthines, a previously described procedure,[30,32] with subsequent modifications,[33] was employed. Briefly, *V. harveyi* BB7XM strain (chosen in preliminary experiments from the series of genetically modified tester strains as the most sensitive strain to mutagenicity of $MPDP^+$ and MPP^+) was cultivated at 30 °C in the BOSS medium.[34] To exponentially growing cultures, indicated amounts of $MPDP^+$ and MPP^+ and/or methylxanthines were added and cultivation was continued for 3 h. Then, 5×10^6 cells were spread onto BOSS agar plates containing neomycin (0.1 mg ml^{-1}). Following 48-h incubation at 30 °C, neomycin-resistant colonies were counted. To estimate number of spontaneous neomycin-resistant mutants, analogous experiments were performed in which neither $MPDP^+$ nor MPP^+ nor methylxanthines were added. Number of such mutants was then subtracted from number of mutants in cultures treated with MPTP and/or caffeine.

Acknowledgments

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