

Chapter 6 – Article 2

The interactions of caffeine with monoamine oxidase

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Abstract

Aims: Caffeine has been used as scaffold for the design of inhibitors of monoamine oxidase (MAO) A and B and substitution at the C8 position with a variety of moieties yields structures with high MAO inhibition potencies. Although the MAO inhibitory properties of numerous caffeine derivatives have been characterized, the possibility that caffeine inhibits the MAOs has not been investigated in detail. Based on the therapeutic applications and potential adverse effects of MAO inhibition, this study examines the interactions of caffeine with the MAOs.

Main methods: Employing the recombinant human enzymes, the potencies by which caffeine inhibits the in vitro catalytic activities of the MAOs were recorded and expressed as the IC_{50} and K_i values. The reversibility of inhibition was determined by measuring the recovery of enzyme activity after dialysis of enzyme-caffeine mixtures.

Key findings: Caffeine acts as a MAO inhibitor with K_i values of 0.70 mM and 3.83 mM for the inhibition of MAO-A and MAO-B, respectively. The results show that caffeine binds reversibly and competitively to both MAO enzymes.

Significance: Although structural modifications of caffeine lead to compounds with highly potent MAO inhibitory potencies, caffeine is a weak inhibitor of MAO-A and MAO-B. At doses achieved by normal human consumption, the MAO inhibitory potencies of caffeine are unlikely to be of pharmacological relevance in humans. The MAO inhibitory effects of caffeine should however be taken into consideration when using this drug in in vitro and tissue culture experiments where higher doses and concentrations of caffeine are often used.

Keywords: caffeine; monoamine oxidase; inhibition; competitive; reversible

Introduction

Caffeine (**1**) is the most widely consumed psychoactive substance in the world (Fig. 1). Almost all caffeine ingestion occurs via the diet, most of it from coffee and tea (Fredholm et al. 1999). The acute and, particularly, chronic exposure to caffeine appear to have only minor harmful effects on human health, and as a result, no restrictions on the use of caffeine is imposed by governmental regulatory agencies. While caffeine possesses diverse pharmacological actions, its stimulatory effects in the central nervous system are best known (Fredholm et al. 1999). The biochemical mechanisms by which caffeine exerts its pharmacological actions are not well understood. Laboratory evidence suggests that, at dietary doses, caffeine most likely antagonizes adenosine receptors, particularly the A₁ and A_{2A} receptor subtypes. These antagonistic effects at adenosine receptors may account for most of caffeine's peripheral and central effects. The reported K_d values (receptor–ligand dissociation constants) for the antagonism of human A₁ and A_{2A} receptors are 12 and 2.4 μM, respectively (Fredholm et al. 1994). Considering that ingesting a single cup of coffee may give peak plasma concentrations of 1–10 μM in humans, interactions of caffeine with especially the A_{2A} receptor seems likely (Fredholm et al. 1999). Behavioural data, however, suggests that caffeine may possess other biochemical mechanisms of action (Garrett and Holtzman 1995). It has been shown that caffeine may cause the direct release of calcium (probably by acting on ryanodine receptors) (McPherson et al. 1991), and inhibit 5'-nucleotidase and alkaline phosphatase (Fredholm et al. 1978; Fredholm and Lindgren 1983). These effects, however, only occur at millimolar concentrations, and are thus unlikely to be of significance at dietary doses of caffeine. Caffeine is also reported to inhibit cyclic nucleotide phosphodiesterases although with relatively low potency (Smellie et al. 1979). Based on the widespread consumption of caffeine and the possibility that caffeine may possess multiple biochemical mechanisms of action, the identification of these mechanisms is urgently required.

Caffeine has been used as scaffold for the design of inhibitors of the monoamine oxidase (MAO) A and B enzymes (Vlok et al. 2006; Strydom et al. 2010). It has been documented that substitution of caffeine at the C8 position with a variety of moieties

yields structures with high MAO inhibition potencies. Examples of such structures are (E)-8-(3-chlorostyryl)caffeine (**2**) (Vlok et al. 2006) and 8-benzyloxycaffeine (**3**) (Strydom et al. 2010). These compounds are potent inhibitors of the MAOs with IC_{50} values in the nanomolar to micromolar ranges. (E)-8-(3-Chlorostyryl)caffeine inhibits MAO-B with an IC_{50} value of 0.128 μ M (Vlok et al. 2006) while 8-benzyloxycaffeine inhibits both MAO-A and MAO-B with IC_{50} values of 1.24 μ M and 1.77 μ M, respectively (Strydom et al. 2010). Although the MAO inhibitory properties of numerous caffeine derivatives have been characterized, the possibility that caffeine inhibits the MAOs has not previously been investigated in detail. This study therefore examines the interactions of caffeine with human MAO-A and MAO-B.

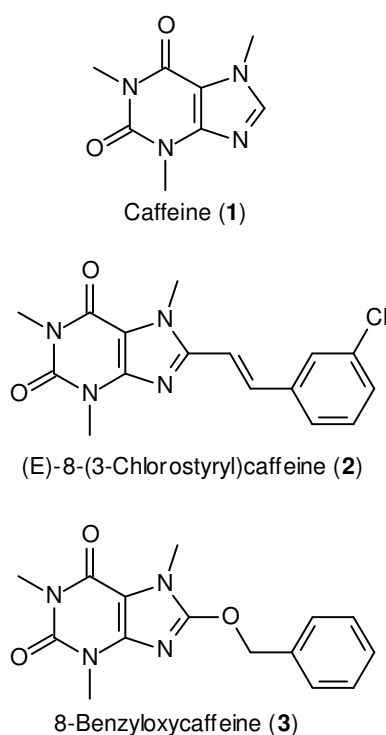


Fig. 1. The structures of caffeine (**1**), (E)-8-(3-chlorostyryl)caffeine (**2**) and 8-benzyloxycaffeine (**3**).

The possibility that caffeine may inhibit MAO-A and MAO-B is of significance since these enzymes are of pharmacological and toxicological importance. MAO-A selective inhibitors are used in the treatment of depressive illness since they enhance the central

levels of serotonin, norepinephrine and dopamine (Yamada and Yasuhara 2004). MAO-B selective inhibitors may elevate dopamine levels in the basal ganglia of the brain and are thus used in the treatment of Parkinson's disease (Fernandez and Chen 2007). In Parkinson's disease therapy, MAO-B inhibitors are frequently combined with levodopa, the metabolic precursor of dopamine, since MAO-B inhibitors may enhance the levels of dopamine derived from levodopa (Finberg et al. 1998). The most notable toxicological effect of MAO inhibition is the potentiation of tyramine-induced pressor responses. When MAO-A inhibitors are combined with tyramine, which is present in certain foods, a potentially fatal hypertensive reaction may occur (Da Prada et al. 1988). Tyramine is normally metabolized by MAO-A in the intestines, which limits its entry into the systemic circulation. The inhibition of intestinal MAO-A results in excessive amounts of tyramine reaching the circulation, and since tyramine induces the release of norepinephrine from peripheral neurons, this may lead to a hypertensive crisis (Da Prada et al. 1988). Another notable toxicological effect of MAO-A inhibition is serotonin toxicity. Serotonin toxicity is a potentially fatal syndrome which develops when 5-hydroxytryptaminergic agents, such as selective serotonin reuptake inhibitors (SSRIs) or serotonin-releasing agents, and MAO-A inhibitors are combined (Ramsay et al. 2007; Stanford et al. 2010). Because MAO-A inhibitors reduces the MAO-A-catalyzed degradation of serotonin, such combinations may lead to excessive extracellular serotonin concentrations in the central nervous system.

Materials and methods

Microsomes from insect cells containing recombinant human MAO-A or MAO-B (5 mg/mL), kynuramine dihydrobromide and caffeine were obtained from Sigma–Aldrich®. 8-Benzyloxycaffeine was synthesized according to the reported method (Strydom et al. 2010). Fluorescence spectrophotometry was conducted with a Varian® Cary Eclipse fluorescence spectrophotometer.

IC₅₀ determinations

The IC₅₀ values for the inhibition of the recombinant human MAOs were determined according to a previously described protocol (Petzer et al. 2012). All enzyme reactions

were conducted in potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl) to a final volume of 500 μ L. The reactions contained six different concentrations of caffeine (0.1–30 mM) or the reference MAO inhibitor 8-benzyloxycaffeine (0.003–100 μ M), and the MAO-A/B mixed substrate kynuramine (45 μ M for MAO-A and 30 μ M for MAO-B). Stock solutions of the test inhibitors were prepared in DMSO and were added to the reactions to yield a final concentration of 4% DMSO. Similar reactions were also carried out in the absence of inhibitor. The reactions were initiated with the addition of MAO-A or MAO-B (0.0075 mg protein/ml) and incubated for 20 min at 37 °C. After termination of the reactions with the addition of 400 μ L NaOH (2 N), 1000 μ L water was added, and the concentrations of 4-hydroxyquinoline were subsequently measured by fluorescence spectrophotometry (λ_{em} = 310; λ_{ex} = 400 nm) ([Novarolli et al. 2005](#)). 4-Hydroxyquinoline is the ultimate MAO-catalyzed oxidation product of kynuramine and, unlike kynuramine, is fluorescent in alkaline medium. For the quantitation of 4-hydroxyquinoline, linear calibration curves (4-hydroxyquinoline: 0.047–1.5 μ M) were constructed. The MAO catalytic rates were calculated and fitted to the one site competition model incorporated into the Prism[®] software package (GraphPad). The IC₅₀ values were determined in triplicate from the resulting sigmoidal concentration–inhibition curves and are expressed as mean $\pm$ standard deviation (SD).

Dialysis

The reversibility of the MAO inhibition by caffeine was examined by dialysis ([Harfenist et al. 1996](#)). Slide-A-Lyzer[®] dialysis cassettes (Thermo Scientific[®]) with a molecular weight cut-off of 10 000 and a sample volume capacity of 0.5–3 ml were used for these studies. The MAO enzymes (0.03 mg/ml) were combined with caffeine, at concentrations equal to fourfold the IC₅₀ values for the inhibition of MAO-A and MAO-B, respectively. These mixtures (final volume of 0.8 ml) were prepared in potassium phosphate buffer (100 mM, pH 7.4, 5% sucrose) and contained 4% DMSO as co-solvent. The mixtures were preincubated for 15 min at 37 °C. As controls, MAO-A and MAO-B were similarly preincubated in the presence of the irreversible inhibitors, pargyline and (R)-deprenyl, respectively, as well as in the absence of inhibitor. The concentrations of pargyline [IC₅₀(MAO-A) = 13 μ M] ([Strydom et al. 2012](#)) and (R)-deprenyl [IC₅₀(MAO-B) = 0.079

μM] ([Petzer et al. 2012](#)) employed were equal to fourfold their reported IC_{50} values for the inhibition of the respective enzymes. The enzyme–inhibitor mixtures were subsequently dialyzed at 4 °C in 80 mL of dialysis buffer (100 mM potassium phosphate, pH 7.4, 5% sucrose). The dialysis buffer was replaced with fresh buffer at 3 h and 7 h after the start of dialysis. At 24 h after dialysis was started the reactions were diluted twofold with the addition of kynuramine (dissolved in potassium phosphate buffer, 100 mM, pH 7.4, made isotonic with KCl). The final inhibitor concentrations in these reactions were equal to twofold their IC_{50} values and the final concentration of kynuramine was 50 μM . The residual MAO activities were subsequently measured by fluorescence spectrophotometry as described above. For comparison, undialyzed mixtures of the MAOs and caffeine were maintained at 4 °C over the same time period. All reactions were carried out in triplicate and the residual enzyme catalytic rates were expressed as mean $\pm$ SD.

Construction of Lineweaver–Burk plots

Sets, each consisting of six Lineweaver–Burk plots ($1/V$ vs. $1/[S]$), were constructed to examine the modes of MAO-A and MAO-B inhibition by caffeine. The first plot was constructed in the absence of caffeine and the remaining five plots were constructed in the presence of different concentrations of caffeine. The caffeine concentrations selected for the studies with MAO-A ranged from 0.19 to 0.95 mM, while for the studies with MAO-B the concentrations of caffeine ranged from 1.25 to 6.25 mM. Kynuramine at eight different concentrations (15–250 μM) served as substrate and the concentrations of recombinant human MAO-A and MAO-B employed were 0.015 mg/ml. The rates of the MAO-catalyzed formation of 4-hydroxyquinoline were measured by fluorescence spectrophotometry as described above. Linear regression analysis was performed using Prism[®] 5 ([Manley-King et al. 2011](#)). K_i values were estimated from the x-axis intercept ($-K_i$) of a replot of the slopes of the Lineweaver–Burk plots versus inhibitor concentration.

Results

IC_{50} values for the inhibition of the MAOs by caffeine

As mentioned in the materials and methods, recombinant human MAO-A and MAO-B were used to investigate the inhibitory properties of caffeine. Enzyme activity measurements were carried out by measuring the extent to which kynuramine, a MAO-A/B mixed substrate, is oxidized by the MAOs to ultimately yield 4-hydroxyquinoline. 4-Hydroxyquinoline concentrations were conveniently measured by fluorescence spectrophotometry ($\lambda_{\text{ex}} = 310 \text{ nm}$; $\lambda_{\text{em}} = 400 \text{ nm}$) since both caffeine and the kynuramine are non-fluorescent under the measurement conditions (Novaroli et al. 2005).

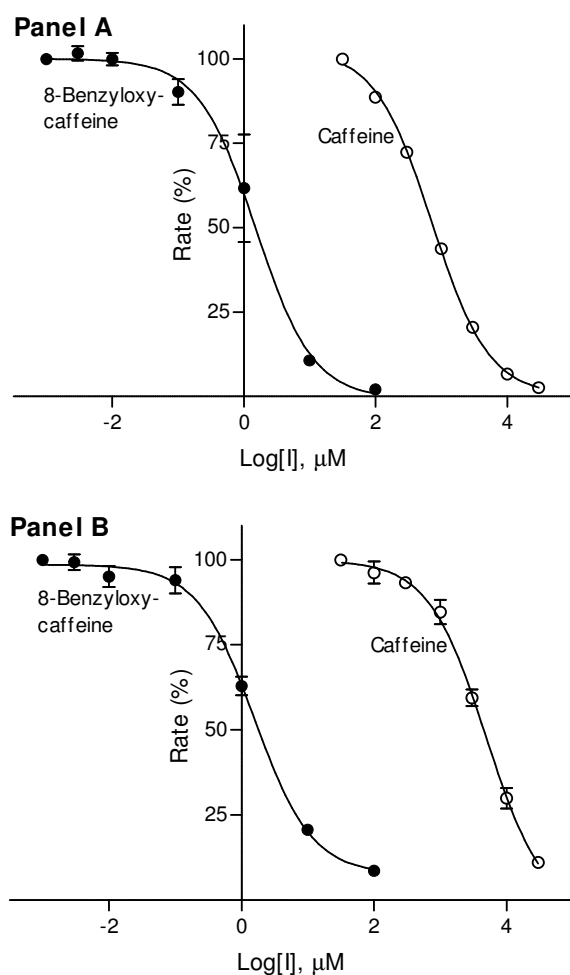


Fig. 2. The sigmoidal concentration-inhibition curves for the inhibition of human MAO-A (panel A) and MAO-B (panel B) by caffeine (**1**) and 8-benzoyloxycaffeine (**3**).

The sigmoidal concentration–inhibition curves for the inhibition of MAO-A and MAO-B by caffeine are given in Fig. 2. The results show that caffeine inhibits human MAO-A with an IC_{50} value of 0.761 ± 0.040 mM. As positive control, the reference inhibitor, 8-benzyloxycaffeine, inhibits MAO-A with an IC_{50} value of 1.01 ± 0.064 μ M, a value which is similar to that previously reported (1.24 μ M) (Strydom et al. 2010). Caffeine also inhibits human MAO-B, although with lower potency compared to its activity towards MAO-A. The results document that caffeine inhibits MAO-B with an IC_{50} value of 5.08 ± 1.09 mM, a potency approximately 6.7-fold lower than that recorded for the inhibition of MAO-A. As positive control, the reference inhibitor, 8-benzyloxycaffeine, inhibits MAO-B with an IC_{50} value of 1.61 ± 0.095 μ M. As expected this value is similar to the literature value (1.77 μ M) (Strydom et al. 2010).

Reversibility of inhibition

In order to evaluate the reversibility of MAO-A and MAO-B inhibition by caffeine, the enzymes were combined with caffeine and the resulting mixtures were dialyzed. The degrees of recovery of MAO catalytic activity were subsequently measured (Harfenist et al. 1996). For a reversible interaction between caffeine and the MAOs, enzyme activity is expected to recover following dialysis, while for an irreversible interaction, enzyme catalytic activities cannot be recovered with dialysis. For the purpose of this study MAO-A and MAO-B were combined with caffeine, at a concentration equal to $4 \times IC_{50}$. These mixtures were preincubated for a period of 15 min and subsequently dialyzed for 24 h. The results are given in Fig. 3 and show that, after 24 h dialysis of the enzyme–caffeine mixtures, the activities of MAO-A and MAO-B are almost completely recovered to levels of 97% and 96%, respectively, of the control values (recorded in the absence of inhibitor). In contrast, the residual MAO-A and MAO-B activities of undialyzed enzyme–caffeine mixtures are 37% and 39%, respectively, of the control values. The recovery of MAO activity with dialysis is consistent with reversible interactions between the MAO enzymes and caffeine. After similar preincubation of MAO-A and MAO-B with the irreversible inhibitors, pargyline and (R)-deprenyl, respectively, and dialysis of the resulting mixtures, the residual enzyme activities are recovered to levels of only 4.4% and 2.8% of the control values. The observation that the MAO activities are not

recovered after dialysis is consistent with irreversible interactions of pargyline and (R)-deprenyl with MAO-A and MAO-B, respectively.

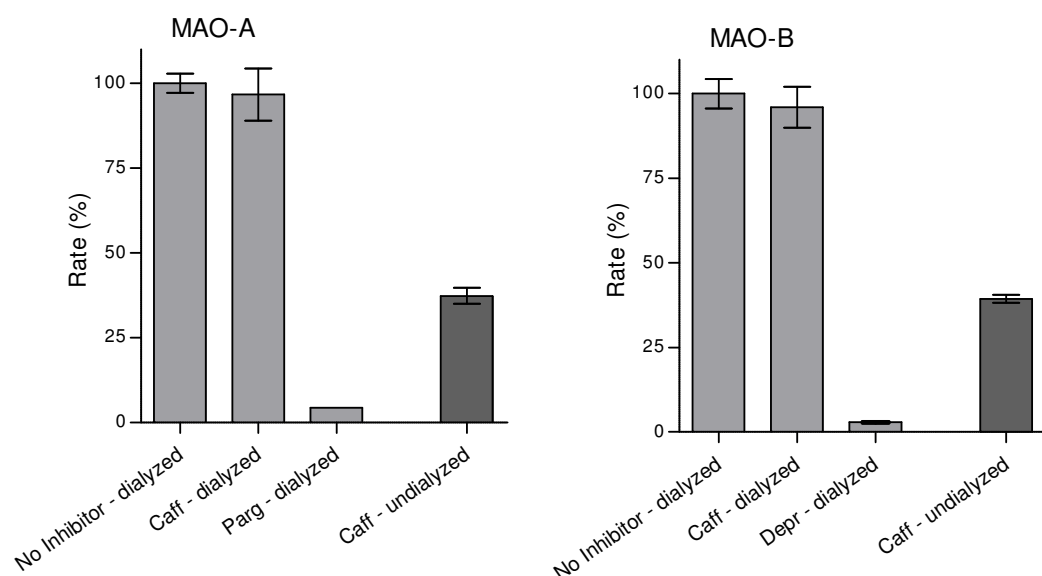


Fig. 3. Reversibility of inhibition of human MAO-A (left) and MAO-B (right) by caffeine. MAO-A and MAO-B were preincubated for a period of 15 min with caffeine, at a concentration of $4 \times IC_{50}$. The mixtures were dialyzed for 24 h and the residual MAO activities were measured (Caff–dialyzed). For comparison, the MAOs were similarly preincubated in the absence (No inhibitor–dialyzed) and presence of the irreversible inhibitors, pargyline (Parg–dialyzed) and (R)-deprenyl (Depr–dialyzed), respectively, and dialyzed. The residual MAO activities of undialyzed mixtures of MAO-A and MAO-B with caffeine (Caff–undialyzed) are also shown.

Mode of inhibition

As shown above, caffeine interacts reversibly with MAO-A and MAO-B. It is likely that the modes of inhibition of both isozymes are competitive since caffeine derivatives such as 8-benzyloxycaffeine has been shown to inhibit the MAOs competitively ([Strydom et al. 2010](#)).

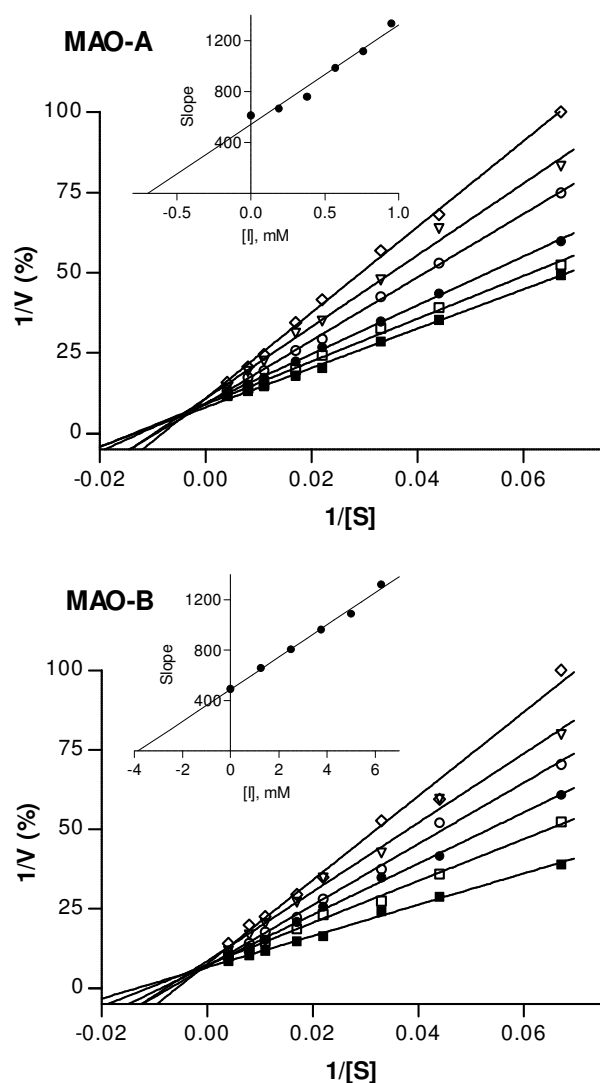


Fig. 4. Lineweaver-Burk plots of human MAO-A (top) and MAO-B (bottom) activities in the absence (filled squares) and presence of various concentrations of caffeine. The insets are the plots of the slopes of the double-reciprocal plots versus inhibitor concentration. For the studies with MAO-A the concentrations of caffeine employed were 0.19–0.95 mM, while for the studies with MAO-B the concentrations of caffeine employed were 1.25–6.25 mM.

To examine the modes of inhibition of MAO-A and MAO-B by caffeine, sets of Lineweaver–Burk plots were constructed. Using eight substrate concentrations (15–250

μM), the MAO catalytic activities were recorded in the absence and presence of five different concentrations of caffeine. The Lineweaver–Burk plots thus constructed are given in Fig. 4. The results show that, for the inhibition of both MAO-A and MAO-B, the Lineweaver–Burk plots are linear and intersect at a single point on the y-axis. This suggests that caffeine most likely interacts competitively with both MAO isozymes. From the replot of the slopes of the Lineweaver–Burk plots versus the inhibitor concentrations, K_i values of 0.70 mM and 3.83 mM are estimated for the inhibition of MAO-A and MAO-B, respectively, by caffeine.

Discussion

Caffeine is considered to be a central nervous system stimulant and is widely consumed by man. Several hypotheses have been formulated to explain the actions of caffeine in the central nervous system. As mentioned in the introduction, three main mechanisms of action have been described to account for caffeine's central effects. These include the antagonism of adenosine receptors, particularly (Nehlig et al. 1992) the A_{2A} receptor subtype, intracellular mobilization of calcium (McPherson et al. 1991) and the inhibition of phosphodiesterases (Smellie et al. 1979). In addition, binding of caffeine to benzodiazepine receptors has been suggested as a possible mechanism of action (Boulenger et al. 1982). Of these mechanisms, the interaction of caffeine with adenosine receptors may explain most of caffeine's central effects (Nehlig et al. 1992). Caffeine is a moderately potent antagonist of adenosine A_1 and A_{2A} receptors, with K_d values in the low micromolar range (Fredholm et al. 1994). Since the peak plasma concentrations reached by caffeine after consumption of a single cup of coffee is within this range, interactions of caffeine with adenosine receptors seems likely, especially with the A_{2A} receptor for which it has a higher binding affinity than for the A_1 receptor (Fredholm et al. 1999). In contrast, caffeine's effects on calcium shifts (McPherson et al. 1991) and phosphodiesterases (Smellie et al. 1979) occur only at millimolar concentrations, well above the circulating plasma concentration of caffeine. The binding affinities of caffeine for benzodiazepine receptor sites also are rather weak (Boulenger et al. 1982). Given this analysis, it is unlikely that the latter mechanisms represent essential mechanisms of action of caffeine in the central nervous system.

The present study shows that caffeine also interacts with the human MAOs. Caffeine acts as a reversible inhibitor with K_i values of 0.70 mM and 3.83 mM for the inhibition of MAO-A and MAO-B, respectively. Although these inhibitory potencies may be considered weak, they are in the same range as those potencies reported for caffeine as an inhibitor of phosphodiesterases ([Smellie et al. 1979](#)) and as a releaser of intracellular calcium ([McPherson et al. 1991](#)). At plasma concentrations achieved after normal coffee consumption, caffeine is, however, unlikely to inhibit the MAOs under physiological conditions and MAO inhibition cannot account for the central nervous system effects of caffeine. The inhibition of the MAOs by caffeine is therefore not of clinical relevance in humans and caffeine is not expected to block the metabolism of monoamine neurotransmitters to lead to the pharmacological and toxicological effects associated with MAO inhibition. The finding that caffeine acts as a reversible MAO-A and MAO-B inhibitor further supports this point of view. Irreversible inhibitors act in a time-dependent manner by which the degree of inhibition increases over time. For example, at a concentration equal to its IC_{50} value, an irreversible inhibitor may suppress enzyme catalytic activity to well below 50%. In fact, given enough exposure time, irreversible inhibitors may completely abolish enzyme activity, even at concentrations well above their IC_{50} values. In contrast, reversible inhibitors causes a maximal inhibition of 50% at a concentration equal to its IC_{50} value, even after prolonged exposure of the enzyme to the inhibitor ([Petzer et al. 2012](#)). Being a reversible inhibitor, caffeine will therefore not inhibit the MAOs to a greater extent than expected from its local concentration at the physiological target. The MAO inhibitory effects of caffeine should, however, be taken into consideration when using this drug in tissue culture and in vitro experiments. In tissue culture experiments, the pharmacological effects of caffeine are frequently examined at high doses (up to 10 mM) where interactions with the MAOs are probable ([Lee et al. 2011](#); [Potta et al. 2012](#); [Tai et al. 2010](#)).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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