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The levels of norharman are high enough after smoking to affect monoamineoxidase B in platelets

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Abstract

Epidemiological studies suggest that smoking reduces the risk for Parkinson's disease. It has been hypothesized that inhibition of monoamineoxidase contributes to this action. The present study examined the contribution of the beta-carbolines norharman, an inhibitor of monoamineoxidase B, and harman, an inhibitor of monoamineoxidase A, which are present in high concentrations in tobacco smoke to the protective action. Nineteen active smokers and five nonsmokers smoked one and two cigarettes. The levels of norharman and harman increased in plasma from smokers and nonsmokers. Ex vivo saturation kinetic experiments revealed that the baseline affinity constant of monoamineoxidase in platelets from smokers was higher than that of nonsmokers in contrast to the maximum turnover rate, which did not differ. Acute smoking affected the monoamineoxidase in nonsmokers only. It is discussed that inhibition of both isoforms of monoamineoxidase is necessary for the neuroprotection and that both norharman and harman play an important role.

Keywords: Norharman; Harman; Neuroprotection; Monoamineoxidase; Parkinson's disease

1. Introduction

Smoking is associated with severe health problems affecting lung, pancreas, kidney, and the cardiovascular system. The effects on the brain are more complex. Smoking improves working memory, long-term memory, attention, and increases arousal (Sahakian et al., 1989; Jones et al., 1992; Rusted and Warburton, 1992). Neuroprotective properties were concluded from observations demonstrating a reduced prevalence of smokers among patients with Parkinson's disease compared with the general population (Baron, 1986; Morens et al., 1995; Checkoway and Nelson, 1999; Gorell et al., 1999; Werneck and Alvarenga, 1999). These findings suggested protection of dopaminergic neurones in the brain by constituents of tobacco smoke.

The neuroprotective action of tobacco smoke cannot be generalized to all types of neurones because such an association was not consistently demonstrated in Alzheimer's disease, the other major neurodegenerative disease. Smokers had a doubling of the risk for dementia and Alzheimer's disease. The risk factor further increased to 4.6 in smokers without the APOE $\varepsilon 4$ allele compared to never smokers (Ott et al., 1998). Another study confirmed those findings with a somewhat lower relative risk of 1.9–2.1 for active smokers (Merchant et al., 1999). Although this issue remains controversial, Fratiglioni and Wang (2000) concluded in a meta-analysis that smoking seems to play a causative role in dementia and cognitive impairment, possibly due to the known risk effect for vascular diseases. On the other hand, a negative association of Parkinson's disease with smoking has been consistently found.

The search for neuroprotective constituents in smoke was unsuccessful yet. Positron emission tomography studies have shown a 40% decrease in binding capacity of the tracer substance of monoamineoxidase B (EC 1.4.3.4) in the brains of active smokers relative to nonsmokers or former smokers (Fowler et al., 1996). Monoamineoxidase B catalyzes the oxidative deamination of dopamine and produces a large amount of hydrogen peroxide, which can further react to form the hydroxyl radical HO* in the presence of divalent iron (Fenton reaction, Halliwell, 1992; Reiter, 1995; Cadenas and Davies, 2000). The reaction contributes to an

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increase in the steady-state concentration of reactive oxygen species within both the mitochondrial matrix and the cytosol. The reactive oxygen species cause damage to mitochondrial components and initiate degradative processes by reacting with lipids, DNA and susceptible amino acids in proteins. Thus, the reactive oxygen species are possibly implicated in the pathogenesis of Parkinson's disease (Fahn and Cohen, 1992; Berman and Hastings, 1999).

These findings suggested that inhibition of monoamineoxidase B would reduce the rate of progression of Parkinson's disease. Selegiline (L-deprenyl) inhibits monoamineoxidase B irreversibly and was among the first compounds to be evaluated in a double blind prospective clinical study (The Parkinson Study Group, 1989). Neuropathological studies on nigral degeneration have shown that selegiline reduces the severity of neuronal loss in the lateral tier of the substantia nigra pars compacta (Rinne, 1991). The neurorescuing actions of selegiline are clearly demonstrated by the protection it affords to dopaminergic neurones after administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine at a time period when most of it is metabolized to the neurotoxic 1-methyl-4-phenylpyridinium ion (Riederer et al., 2000). In contrast to these regular observations, a large clinical trial found excess mortality in patients treated with a combination of levodopa and selegiline (Ben-Shlomo et al., 1998).

Regardless of the inconsistent results with the monoamineoxidase B inhibitor selegiline, the question remains which compound(s) in the tobacco smoke is/are responsible for the consistently observed inhibition of monoamineoxidase B (Oreland et al., 1981; Berlin et al., 1995). Nicotine, its metabolite cotinine, thiocyanate, and hydrazine are no inhibitors of monoamineoxidase B (Yong and Perry, 1986). Yu and Boulton (1987) determined an inhibition constant value $K_i = 4 \times 10^{-2}$ M for nicotine. Oreland et al. (1981) determined a concentration of 5×10^{-4} M nicotine inhibiting the enzyme activity by 20%. This concentration is about 2000 times higher than that which can be expected in the blood of heavy smokers.

An inhibitor of monoamineoxidase, which is present in tobacco smoke in remarkably high concentrations, is norharman (synonymous: beta-carboline; in addition, beta-carboline is the group name for a family of natural and synthetic compounds). An amount of 12.6 μg Norharman/g tobacco is present in the bright cigarette smoke (Poindexter and Carpenter, 1962). Norharman inhibits preferentially monoamineoxidase B ($K_i = 730$ nM, brain tissue, rats, May et al., 1991). It occurs naturally in human blood plasma (Rommelspacher et al., 1991). The highest natural concentration was found in substantia nigra from humans (16 nmol/kg tissue, Matsubara et al., 1993). The levels increased in plasma from acutely smoking subjects (Breyer-Pfaff et al., 1996). Norharman readily crosses the blood–brain barrier and is accumulated in the brain (partition factor ~ 3, Fekkes and Bode, 1993). Harman, the methylated derivative of norharman, was measured in addition because it is present in tobacco smoke (3.6 μg/g tobacco measured in the bright cigarette smoke; Poindexter and Carpenter, 1962) and inhibits monoamineoxidase A ($K_i = 220$ nM, May et al., 1991).

The aim of the present study was to confirm the increase of the normal levels of norharman and harman by acutely smoking and to extend these investigations to platelets where monoamineoxidase B is expressed. Furthermore, the basic pharmacokinetic characteristics of norharman and harman were assessed in both compartments. Ex vivo experiments were performed to examine whether the determined levels of norharman in platelets from active smokers are sufficient to affect monoamineoxidase activity. The monoamineoxidase B in platelets serves as a model for the monoamineoxidase B in the brain (Pletscher, 1978; Stahl, 1985; DaPrada et al., 1988). The findings will be discussed with respect to the neuroprotective role of norharman and harman in Parkinson's disease.

2. Materials and methods

2.1. Subjects

The group consisted of male subjects of active smokers ($n = 19$) and nonsmokers ($n = 5$) averaging 26.05 ± 2.9 years (mean ± S.D.; range 21–31 years) and 29.4 ± 4.5 years (range 26–37 years), respectively. Smokers consumed at least 12 cigarettes/day. Subjects were excluded from the study who reported that the average consumption differed in the last 6 months. The subjects had no history of substance abuse or dependence besides tobacco and no first grade relatives known to be drug dependent. The probands did not report psychiatric and somatic diseases. The study had been approved by the ethics committee of the Rudolf Virchow-Klinikum of the Free University of Berlin. All subjects gave their written consent after full understanding of the study.

2.2. Study design

The day preceding the collection of blood no tryptamine-rich food was allowed, e.g. wine, beer, cheese, and herring, because tryptamine is regarded as the precursor of norharman and harman (Susilo and Rommelspacher, 1988; Rommelspacher et al., 1991). The last cigarette was smoked on the evening before so that at least 10 h passed by without smoking before the collection of blood samples. The experiment started at 9 a.m. in the outpatient department of our clinic.

Blood for baseline values was collected from an arm vein (timepoint A, −2 min). Then (start of the first smoking session), a filterless cigarette was smoked as fast as possible, whereby the probands were asked to inhale the smoke as deep as possible. The maximum timeperiod allowed was 3 min. Five minutes after the start of the first smoking session, the second blood sample was collected (timepoint B). Ten minutes later, the third sample was drawn (timepoint C, 15

min). Sixty minutes after the start of the first smoking session (timepoint D; start of the second smoking session), two cigarettes of the same brand as in the first smoking session were smoked within 8 min followed 5 min later by the forth sampling (timepoint E, 13 min after the start of the second smoking session). Seventeen minutes later (timepoint F, 30 min after the start of the second smoking session), the fifth collection was performed. The final collection occurred 120 min after the start of the second smoking session (timepoint G; for details, see Figs. 1–4).

A total of 60 ml blood was collected dropwise into tubes containing 1.5% EDTA in 0.7% NaCl (3 ml for 60 ml blood). The tubes were immediately placed on ice and subsequently centrifuged at 4 °C and 200 × *g* for 15 min without break. The supernatant (platelet-rich plasma) was carefully removed. The platelet concentration in the platelet-rich plasma was counted in an aliquot sample by using a coulter counter. The other portion of the sample was centrifuged for 10 min at 27,000 × g_{av} and 4 °C (Hereus Varifuge 3.OR, Germany). The resulting supernatant (plasma) was decanted, the volume determined, and stored at − 80 °C. The resulting pellet containing the platelets was stored at − 80 °C until use. The timeperiod between freezing and work up of the plasma and platelet samples was not longer than 3 weeks.

2.3. Measurement of norharman and harman in plasma

After thawing, plasma samples were centrifuged at 33,000 × g_{av} and 4 °C for 10 min (Sorvall, RC5C, Du Pont, Wilmington, DE). A 4 ml aliquot of the supernatant was put on a cartridge (phenyl, Baker, Phillipsburg, NJ), which had been washed with 2.5 ml of 10 mM sodium phosphate buffer, pH 7.0, followed by 5 ml distilled methanol (Merck, Darmstadt, Germany) and adjusted to pH 8 by 5 ml of 10 mM sodium phosphate buffer. After the sample had run through the cartridge, a washing step with 2.5 ml buffer followed. Beta-carbolines were eluted from the cartridge by 5 ml distilled methanol, which was subsequently evaporated to dryness (Rotavapor, Büchi, Switzerland). The residue was dissolved in 400 μl of 70% methanol.

The determination of norharman and harman was performed using a system that consisted of a Hewlett Packard chromatograph (high-performance liquid chromatography; type 1090) with a fluorescence detector (type 1046A), a Chem Station, (HP 79994A; Palo Alto, CA), and a 250 × 4.6 mm reversed phase column (VDS optilab, Berlin, Germany, packing material: Inertsil C_8, 5 μm) and a precolumn (1.5 cm) containing the same material. Three aliquots of the sample (140 μl each) of the same subject were eluted under three different conditions: Method 1: flow 1 ml/min, 55% methanol/45% ammonium acetate buffer, 10 mM, pH 8, octane sulfonic acid, 2.5 mM, gradient elution, with 51.7% methanol at 11 min. Method 2: flow 1.5 ml/min, 10% acetonitrile 90%, 90% ammonium acetate buffer, isocratic flow for 4 min, then within 30 s to 43% acetonitrile followed by isocratic elution. Method 3: flow 1.5 ml/min, 38.9% acetonitrile (90%) and ammonium acetate buffer, 50 mM, pH 9, containing 2.5 mM octane sulfonic acid (isocratic conditions). The respective retention times for norharman and harman were: (1) 8.6 and 10.1 min, (2) 10.4 and 11.0 min, and (3) 7.0 and 8.0 min.

For both beta-carbolines, the limit of detection was about 5 pg/4 ml aliquot sample (three times baseline) and the recovery was 90–95%. Each run consisted additionally of several plasma samples spiked with internal standards of authentic harman and norharman to assess the recovery of both compounds. Separate reports provide details of the method concerned here (Rommelspacher et al., 1991; Spies et al., 1995, 1996).

2.4. Measurement of norharman and harman in platelets

The pellet containing the platelets was thawed and diluted in 4 ml of a mixture of ice-cold acetonitrile, distilled water, and 6 M hydrochloride (ratio: 40:37:3) containing 0.1 mM EDTA. The homogenate was divided into four aliquots of 4 ml each (~ 400 mg tissue/aliquot). Standard solutions of 0.2 ng norharman and harman were added to one aliquot and 0.5 ng norharman and harman to a second aliquot, respectively. The samples were centrifuged 10 min at 27,460 × g_{av} and 4 °C, in a Sorvall centrifuge (RC5C). The supernatant was decanted and the pellet washed once with 4 ml of the mixture mentioned above. Both resulting supernatants were combined and 300 μl of 5 M potassium hydroxide was added. Thereafter, the samples were lyophilized (~ 40 h). The dry residue was dissolved in 4 ml sodium phosphate buffer, pH 7.0 (cartridge buffer). The supernatant was applied to a cartridge (phenyl, Baker). The cartridge had been equilibrated with 10 mM sodium phosphate buffer, pH 7.0, followed by distilled methanol and again 5 ml of the sodium phosphate buffer. After a wash step with the cartridge buffer, the beta-carbolines were eluted by 5 ml methanol. The cartridges were utilized three times maximum. The eluate was dryed by a rotavapor (Büchi, Switzerland). The dry residue was diluted in 400 μl of 50% methanol. A 120 μl aliquot was applied to the high-performance liquid chromatography column as described for plasma. Only silanized glassware was used for all experiments.

2.5. Determination of monoamineoxidase activity in platelets

A total of 30 ml of venous blood was drawn as described for beta-carbolines and centrifuged for 15 min at 4 °C (200 × *g*). The supernatant containing the platelet-rich plasma was centrifuged for 10 min at 2000 × *g*. The resulting pellet was homogenized in a mixture of 10 ml of 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.4) and 5 mM EDTA, with the aid of a glass/teflon Potter and centrifuged for 10 min at 40,000 × g_{av}. The washing procedure was repeated once.

The final pellet was homogenized in 4 ml of 0.2 M potassium phosphate buffer and used for the monoamineoxidase assay.

The fluorometric assay was performed according to a method described by Kraml (1965) with minor modifications (600 μl final volume, 500 μl of 50 mM potassium phosphate buffer and EDTA, 50 μl homogenate (~ 15 μg protein)). Under standard conditions, six concentrations of kynuramine in duplicate (5–160 μM for Lineweaver–Burk analysis) were used. A single concentration of kynuramine was used in displacement studies (20 μM).

After preincutation for 30 min at 37 °C, the reaction was started by adding of 50 μl substrate. Thirty minutes later, the incubation was stopped by adding 400 μl 10% trichloroacetic acid and centrifugation at 10,000 × g_{av} for 6 min.

A total of 800 μl of the resultant supernatant was stirred with 2 ml of 1 M sodium hydroxide. Then, the fluorescence of the reaction product 4-hydroxyquinoline was measured in a fluorescence spectrometer (excitation = 315 nm; emission = 380 nm). The results were calculated in nmol of 4-hydroxyquinoline formed/min/mg protein with the aid of internal 4-hydroxyquinoline (product) standard and after protein determination with bovine serum albumin as standard (Bradford, 1976).

2.6. Pharmacokinetic data analysis

For the determination of the pharmacokinetic parameters of norharman and harman in plasma and platelets of smokers the following dimensions were used: time (min), plasma concentration (pg/ml), platelet concentration (ng/10^9 platelets), and dose (arbitrary). The two consecutive smoking sessions (= treatments) with a dosing period of 3 and 8 min, respectively, administered to the subjects were one cigarette at time 0, and two cigarettes at time 60 min after first dosing. Bioanalytical results reported as below limit of quantification were transformed to "0" before t_{max} and "missing" after t_{max}.

Noncompartmental analysis (Gabrielsson and Weiner, 2000) was performed using the software WinNonlin™, version 3.1 or 3.2, Pharsight, Mountain View, CA. For evaluation, model 202 (constant infusion) was used. The results of the bioanalytical determination have been used with a weighting factor of y^{-1} in order to account for the magnitude of the measured concentrations. This weighing scheme assigns relatively larger weights to lower concentrations.

The relevant pharmacokinetic parameters were determined as follows: C_{max} as observed maximum concentration of beta-carbolines in plasma or platelets, corresponding to t_{max}, t_{max} as blood sampling time of maximum observed concentration of beta-carbolines taking the entire curve into consideration, but relating it to the last smoking session, λ_z as first order rate constant associated with the apparent terminal (log–linear) portion of the curve after the second smoking (z), determined from the slope of the regression of log concentration vs. time. $T_{1/2,z}$ as the apparent terminal half-life was then calculated by $t_{1/2,z} = \ln 2/\lambda_z$.

Due to the investigational nature of this study and the small sample size per matrix and analyte, all parameters were compared only descriptively.

Prior to the second smoking session (time-point D), no blood sample was drawn from smokers. In order to obtain the order of this concentration, an extrapolation of the last concentration measured after the first smoking session to time-point D was performed using the individual estimated apparent terminal elimination rate constant. In order to indicate this approach, a dashed line was used to follow the time course in Figs. 1 and 2.

2.7. Statistics

Differences between the two groups of probands were calculated by means of the Statistical Package for the Social Sciences (version 9.0). The assessment of changes over time was performed by the Friedman test. In case of significant differences, the Wilcoxon test was used. Differences between smokers and nonsmokers were calculated by the Mann–Whitney U-test. Correlation coefficients were determined by using the Spearman test. P-values are not adjusted for the number of correlations tested. A $P \leq 0.05$ was considered significant. The results in the text are presented as mean ± S.D., in the plots as mean ± S.E.M.

3. Results

3.1. Norharman in plasma

The concentration of norharman changed over time in the groups of smokers (smokers: $\chi^2 = 73.77$, $df = 6$, $n = 19$, $P < 0.001$; nonsmokers: $\chi^2 = 9$, $df = 6$, $P = 0.168$; Fig. 1). In nonsmokers, the value did not reach significance possibly due to the small number of subjects and their inability to inhale the smoke deeply.

The baseline mean value of the smokers was 19.2 ± 19.4 pg/ml (mean ± S.D., Fig. 1, timepoint A). Five minutes after the start of the first smoking session, the concentration increased significantly (116 ± 50.8 pg/ml; $P < 0.001$). Ten minutes later, the concentration was still above baseline (94.6 ± 48.5 pg/ml; $P < 0.001$). Five minutes after the second smoking session, the concentration was 179 ± 86.6 pg/ml and 30 min after the start of the second smoking session, the concentration was 120 ± 54.1 pg/ml. The decrease was significant ($P < 0.001$, comparison of timepoints E and F of Fig. 1). One-hundred twenty minutes after the start of the second smoking session the concentration reached almost baseline (35 ± 20.1 pg/ml; $P = 0.113$ compared to baseline value). The correlation coefficient for two dependent variables reached statistical significance between timepoints C and E: r = 0.54, $P = 0.016$ and E and F: $r = 0.89$, $P < 0.001$.

The control group consisted of five nonsmokers who smoked filterless cigarettes of the same brand as the smokers. We asked the nonsmokers to inhale the smoke as

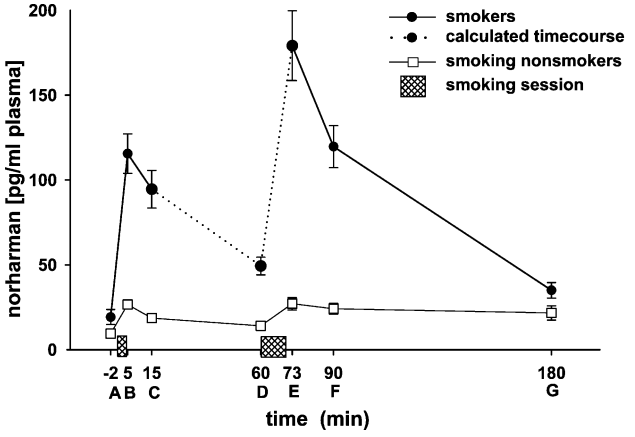

Fig. 1. Timecourse of the levels of norharman (beta-carboline) in plasma from smokers and nonsmokers after smoking one (timepoint 0 min; B) and two (timepoint 60 min; D) filterless cigarettes respectively. In smokers, the levels at timepoint D were not determined but calculated from the pharmacokinetic data found in the present study. The values are the means± S.E.M. from 19 (smokers) and 5 (nonsmokers) male subjects. The smokers were abstinent from smoking for at least 10 h before the first blood sample was drawn (timepoint A, baseline-value).

deeply as possible and to smoke as fast a possible. The baseline value was 9.5 ± 5.0 pg/ml (mean ± S.D., timepoint A, Fig. 1, $P=0.3$ compared to baseline of smokers). Five minutes after the start of the first smoking session, the concentration of norharman was 26.6 ± 13.3 pg/ml and 10 min later 18.6 ± 10.0 pg/ml. Immediately before the second smoking session, the concentration was 14.1 ± 6.3 pg/ml. Thirteen minutes and 30 min after the start of the second smoking session, the concentrations were slightly increased (27.1 ± 16.6 and 24.1 ± 15.9 pg/ml, respectively; $P>0.1$).

3.2. Pharmacokinetics of norharman

The terminal part after the second smoking session of each of the 39 graphical representations of the bioanalytical results (plasma: 20, platelets: 19) was used for selection of the data pairs suitable for λ_z determination. For the determination of λ_z in plasma, three data pairs were available; in platelets, only two data pairs. In total, four concentration time profiles were not evaluable due to an increase in the concentration up to 90 min so that there was no elimination phase to be observed.

The results of the pharmacokinetic parameters are summarized with their descriptive statistics in Table 1. For concentrations, the geometric mean is reported. In general, there was a large coefficient of variation. Therefore, the median for all three time or rate pharmacokinetic parameters and the range observed is reported.

In all subjects and both matrices, the mean maximum concentrations of norharman was reached after the second dosing (median 15 min). The increase from the baseline to peak concentrations was approximately eightfold higher in plasma than in platelets. The terminal elimination rate constant revealed a median of 0.0136 and 0.0037 min^{-1} in plasma and platelets, respectively, with a 10-fold larger range in platelets than in plasma. In total, the apparent terminal half-life was determined for 19 plasma concentration–time profiles and 16 platelet concentration–time profiles. A large coefficient of variation (82% for plasma and 188% for platelets) was observed. The median $t_{1/2,z}$ in plasma was 51 min. The apparent terminal half-life in platelets was more than 3 h, thus longer compared to plasma, with four subjects exhibiting a half-life of more than 1000 min, suggesting that there was only very slow elimination occurring.

Table 1
Pharmacokinetic parameters of norharman in plasma and platelets

	n	Median	Minimum	Maximum
Plasma				
C_{max} [pg/ml]	20	159.0[a]	28.8	390.3
t_{max} [min]	20	15	–	–
λ_z [min^{-1}]	19	0.0136	0.0027	0.0258
$t_{1/2,z}$ [min]	19	51.0	26.8	232.3
Platelets				
C_{max} [pg/10^9 platelets]	19	41.1[b]	29.6	82.3
t_{max} [min]	19	15	–	–
λ_z [min^{-1}]	16	0.0037	0.00009	0.0088
$t_{1/2,z}$ [min]	16	190.1	78.4	7368.0

[a,b] Geometric mean.

3.3. Harman in plasma

The concentration of harman changed over time in the groups of both smokers and nonsmokers (smokers: $\chi^2=34.04$, $df=5$, $n=19$, $P<0.001$; nonsmokers: $\chi^2=13{,}8$, $df=6$, $n=5$, $P=0.02$; Fig. 2).

In smokers, the baseline value was 8.7 ± 7.5 pg/ml. Five minutes after the start of the first smoking session, the concentration increased (23.7 ± 11.0, $P<0.001$) and was still elevated at timepoint C (19.1 ± 11.7, $P<0.001$). Thirteen minutes after the start of the second smoking session

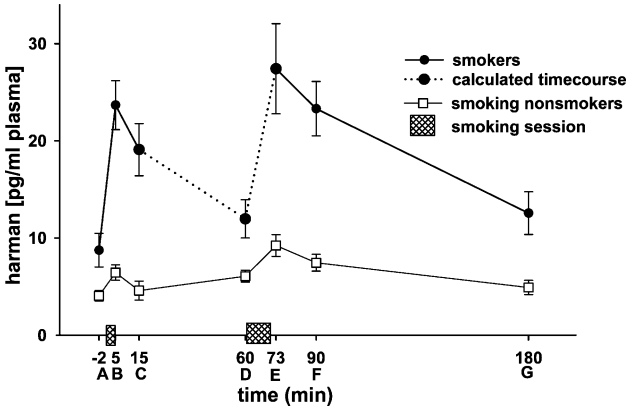

Fig. 2. Timecourse of the levels of harman (1-methyl-β-carboline) in plasma from smokers and nonsmokers after smoking one (timepoint 0 min; B) and two (timepoint 60 min; D) filterless cigarettes respectively. For details, see legend of Fig. 1.

Table 2
Pharmacokinetic parameters of harman in plasma and platelets

	n	Median	Minimum	Maximum
Plasma				
C_{max} [pg/ml]	20	27.08[a]	6.69	63.85
t_{max} [min]	20	15	–	–
λ_z [min^{-1}]	18	0.0101	0.0023	0.0264
$t_{1/2,z}$ [min]	18	68.3	26.3	302.5
Platelets				
C_{max} [pg/10^9 platelets]	13	12.21[b]	2.04	252.52
t_{max} [min]	13	15	–	–
λ_z [min^{-1}]	9	0.0047[c]	0.0009[c]	0.0240[c]
$t_{1/2,z}$ [min]	9	146.2[c]	28.8[c]	738.6[c]

[a] Geometric mean.
[b] Geometric mean.
[c] Figures represent minimum values since four subjects still showed an increase in concentration during the observation period.

the concentration was 27.4 ± 20.2 pg/ml and 30 min after that start, the concentration was 23.3 ± 12.2 pg/ml and reached almost baseline within 120 min (timepoint G, 12.6 ± 9.6 pg/ml, A to F, $P=0.096$).

The Spearman correlation coefficients between the various timepoints were significant for: B to C, $r=0.56$, $P=0.012$; B to E, $r=0.5$, $P=0.025$, B to F, $r=0.62$, $P=0.005$, C to E, $r=0.67$, $P=0.002$; and C to F, $r=0.77$, $P<0.001$.

In nonsmokers, the baseline value was 4.1 ± 2.6 pg/ml and, thus, about half of that of the smokers (difference not statistically significant). Five minutes after the start of the first smoking session, the concentration was slightly elevated (6.4 ± 4.0 pg/ml) and 15 min after the start, the concentration of harman was 4.8 ± 4.9 pg/ml. Immediately before the start of the second smoking session, the level was 6.1 ± 3.0 pg/ml. Thirteen minutes after the start of the second smoking session, the level was clearly increased (9.2 ± 5.6 pg/ml, $P=0.06$ compared to baseline levels). Thirty minutes after the start of that session, the concentration was 7.4 ± 4.3 pg/ml, which reached a statistical trend compared to baseline ($P=0.06$). At timepoint G, baseline level was reached again (4.9 ± 3.6 pg/ml).

The concentrations of the nonsmokers differed from those of smokers at all timepoints, but the baseline (timepoint B: $P=0.001$, C: $P=0.012$, E: $P=0.05$, F: $P=0.001$, G: $P=0.044$).

3.4. Pharmacokinetics of harman

For harman, also the terminal part after the second smoking session of each of the 39 graphical representations of the bioanalytical results (plasma: 20, platelets: 19) was used for selection of the data pairs suitable for λ_z determination. For the determination of λ_z in plasma, three data pairs were available except for eight subjects only two; in platelets, only two data pairs except for six subjects. Thus, the latter were not evaluable. In addition, two plasma and four platelet concentration time profiles were also not evaluable due to an increase of concentration during the observation period.

The results of the pharmacokinetic parameters are summarized with their descriptive statistics in Table 2.

In all evaluable subjects and both matrices, the mean maximum concentrations of harman was reached after the second dosing (median 15 min). The terminal elimination rate constants are presented in Table 2. For platelets, it has to be kept in mind that the value represents only nine subjects, whereas six subjects still had an increase in concentration so that they may be considered as 'at least' values. This also applies for the apparent terminal half-life ($n=18$ plasma, but only $n=9$ platelet). In addition, a large coefficient of variation (78% for plasma and 112% for platelets) was observed. The median $t_{1/2,z}$ in plasma was 68.3 min. The apparent terminal half-life in platelets was 'at least' approximately 2.5 h, thus longer compared to plasma.

3.5. Norharman in platelets

The levels of norharman were measured in smokers at four timepoints: baseline, 5 min after the start of the first smoking session, 13 min after the start of the second smoking session, and 120 min after the start of that session (Fig. 3). The concentrations changed over time ($\chi^2=12.6$, $n=15$, $df=3$, $P=0.004$). This was confirmed by comparison of single timepoints—A to B: $r=0.74$, $P=0.002$; A to E: $r=0.62$, $P=0.005$; B to G: $r=0.51$, $P=0.029$; E to G: $r=0.78$, $P=0.001$. Thus, smoking clearly influenced the levels of norharman in platelets acutely.

The baseline concentration of the smokers was 29.6 ± 13.1 pg in 10^9 platelets and was four times higher than in the nonsmokers (baseline 7.3 ± 4.5 pg in 10^9 platelets, $P<0.001$; Fig. 3). Five minutes after the start of the first smoking session, the concentration of norharman increased (39.1 ± 50.8 pg in 10^9 platelets, $P=0.002$). Thirteen minutes

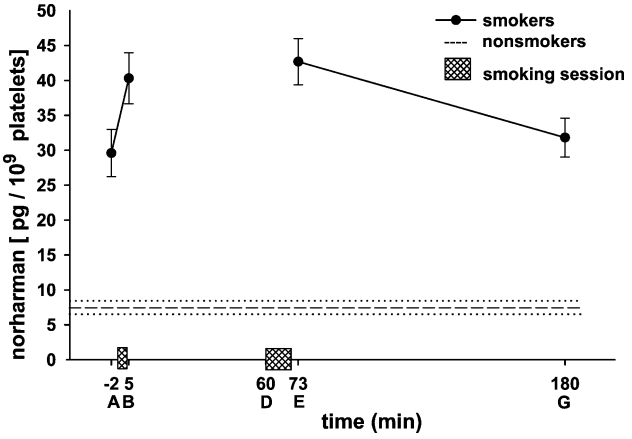

Fig. 3. Timecourse of the levels of norharman in platelets from smokers ($n=19$) after smoking one (timepoint 0 min; B) and two (timepoint 60 min; D) filterless cigarettes respectively. The baseline level from nonsmokers ($n=5$) is indicated by the hatched line. The values are the means ± S.E.M.

after the start of the second smoking session the concentration amounted to 41.3 ± 14.4 pg/10^9 platelets. This value differed from baseline ($P = 0.005$). One-hundred twenty minutes after the start of that session almost baseline concentrations were reached again (31.8 ± 12.1 pg in 10^9 platelets, significantly different from timepoint E: $P = 0.001$).

In nonsmokers, only the baseline values were determined.

3.6. Harman in platelets

The concentration of harman in platelets of smokers did not change significantly over time ($\chi^2 = 3.0$, $df = 3$, $n = 15$, $P = 0.4$; Fig. 4). The baseline mean was about four times higher than in nonsmokers (11.0 ± 19.7 pg/10^9 platelets, $n = 15$ smokers; 2.7 ± 1.5 pg/10^9 platelets, $n = 11$ nonsmokers, $P = 0.001$).

Five minutes after the start of the first smoking session, the concentration of harman was nearly unchanged (9.8 ± 20.3, $n = 16$). Thirteen minutes after the start of the second smoking session, the concentration was doubled (25.9 ± 59.1 pg in 10^9 platelets, $n = 19$) with minor changes at timepoint F (20.2 ± 51.8 in 10^9 platelets, $n = 19$).

3.7. Inhibition of monoamineoxidase B activity by norharman

Ex vivo experiments were performed to investigate the impact of smoking on monoamineoxidase B activity in smokers and nonsmokers (Figs. 5 and 6).

The timecourse of the maximum turnover rate values did not change over time in smokers ($\chi^2 = 0.94$, $df = 3$, $n = 7$, $P = 0.85$). However, in nonsmokers, the maximum turnover rate was affected by smoking ($\chi^2 = 13$, $df = 6$, $n = 5$, $P = 0.03$).

The baseline maximum turnover rate values for smokers was 5.9 ± 2.4 μmol 4-hydroxyquinoline min^{-1} mg protein^{-1}. Five minutes after the start of the first smoking session, the maximum turnover rate value was 5.2 ± 2.7 μmol 4-hydroxyquinoline min^{-1} mg protein^{-1}, and 30 min after the start of the second smoking session, the value was 5.6 ± 3 μmol 4-hydroxyquinoline min^{-1} mg protein^{-1}, and 120 min thereafter, 5.5 ± 2.5 μmol 4-hydroxyquinoline (Fig. 5).

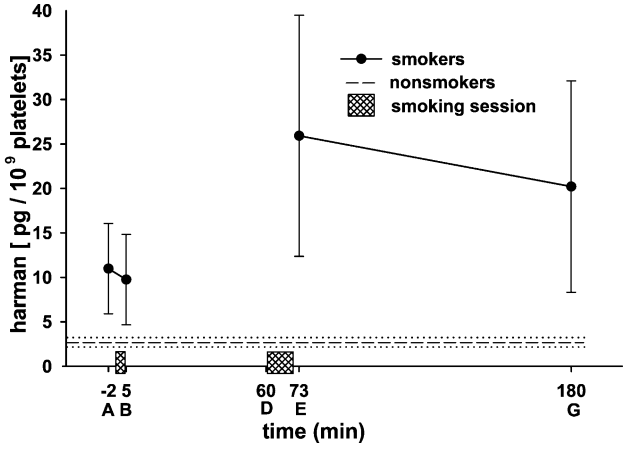

Fig. 4. Timecourse of the levels of harman in platelets from smokers ($n = 19$) after smoking one (timepoint 0 min; B) and two (timepoint 60 min; D) filterless cigarettes respectively. For details, see legend of Fig. 3.

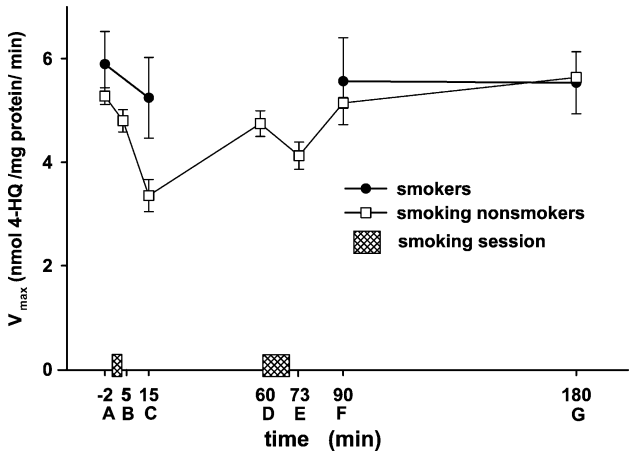

Fig. 5. Ex vivo saturation kinetic experiments. The maximum velocity of monoamineoxidase of platelets from smokers and nonsmokers was determined at various timepoints. The study design is as described in legend of Fig. 1. The values are the means ± S.E.M. from 19 (smokers) and 5 (nonsmokers) male subjects. 4-Hydroxyquinoline was used as substrate.

The baseline mean maximum turnover rate value of monoamineoxidase B activity in nonsmokers was 5.3 ± 0.8 μmol 4-hydroxyquinoline min^{-1} mg protein^{-1}. Five minutes after the start of the first smoking session, the maximum turnover rate value was 4.8 ± 1.1 μmol 4-hydroxyquinoline min^{-1} mg protein^{-1}, and 15 min thereafter, 3.4 ± 1.5 μmol 4-hydroxyquinoline min^{-1} mg protein^{-1}. Before the start of the second smoking session, the value was 4.7 ± 1.2 μmol 4-hydroxyquinoline min^{-1} mg protein^{-1}, 13 min thereafter, 4.1 ± 1.3 μmol 4-hydroxyquinoline min^{-1} mg protein^{-1}, 30 min thereafter, 5.1 ± 0.5 μmol 4-hydroxyquinoline min^{-1} mg protein^{-1}, and 120 min thereafter, 5.6 ± 0.5 μmol 4-hydroxyquinoline min^{-1} mg protein^{-1} (Fig. 5).

Pairwise comparisons of the means of smokers and nonsmokers revealed no statistically significant differences.

The affinity constant, K_m, of the monoamineoxidase B did change in samples from nonsmokers, but not from smokers during the experiment: $\chi^2 = 12.3$, $df = 6$, $n = 5$, $P = 0.04$ (nonsmokers) and $\chi^2 = 5.8$, $df = 3$, $n = 15$, $P = 0.13$ (smokers; Fig. 6).

The baseline K_m-value from smokers was 33.1 ± 5.4 μM. Fifteen minutes after the start of the first smoking session, the mean value was unchanged (33.1 ± 4.2 μM). Thirty minutes after the start of the second smoking session, the K_m-value was 34.1 ± 8.2 μM, and 120 min thereafter, the K_m-value was 30.9 ± 2.8 μM.

The baseline K_m-value of the monoamineoxidase B in platelets from nonsmokers was 28.0 ± 2.4 μM. Five minutes

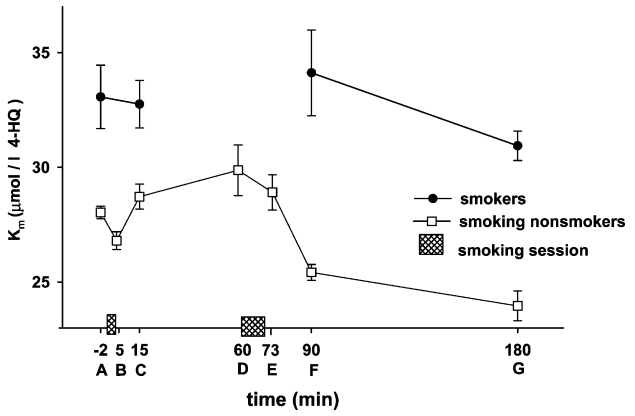

Fig. 6. Ex vivo saturation kinetic experiments. The K_m-value of the monoamine oxidase was determined at various timepoints in smokers ($n = 19$) and nonsmokers ($n = 5$). For details, see legend of Fig. 5.

after the start of the first smoking session, the K_m-value was 26.8 ± 2.0 µM, and 15 min thereafter, 28.7 ± 2.7 µM. Immediately before the second smoking section, the K_m-value was 29.9 ± 5.5 µM. Five minutes after the start of the second smoking session, the K_m-value was 28.9 ± 3.8 µM, 30 min thereafter, 25.4 ± 1.7 µM, and 120 min thereafter, 24.0 ± 3.2 µM.

The affinity of the monoamineoxidase B as assessed by the K_m-value in nonsmokers was always higher than that in smokers (timepoints A: $P = 0.001$, C: $P = 0.004$, F: $P < 0.001$, G: $P < 0.001$).

4. Discussion

Smoking causes a reduction of monoamineoxidase activity in male smokers (Von Knorring and Oreland, 1985; Norman et al., 1987). The activity of the enzyme normalized after cessation of smoking, e.g. in abstinent smokers (Norman et al., 1987; Fowler et al., 1996). This suggests reversible inhibition by a single or several constituents of the tobacco smoke. The mean inhibition of monoamineoxidase activity was 40%, while for single individuals, the difference in monoamineoxidase activity ranged from 24% to 113% (Norman et al., 1987). Others found in a group of 88 active smokers (one-third females two thirds males), a decrease of monoamineoxidase B activity of 53%. The K_m-values were not determined (Berlin et al., 1995).

Most previous studies used for the investigation of monoamineoxidase B a single substrate concentration near saturation. The advantage of the present study was the application of six substrate concentrations (range: 1/7 to 4.5 fold of K_m), which allows to assess the effects of smoke on both maximum turnover rate and affinity as assessed by the K_m-value. We found that the maximum turnover rate was not different in smokers and nonsmokers and did not change in smokers at all investigated timepoints. The K_m-values were higher in smokers. Acute smoking affected the monoamineoxidase affinity in nonsmokers only. These findings are surprising because nonsmokers inhaled less smoke as indicated by the lower increase of the levels of norharman and harman in plasma after smoking in nonsmokers compared to smokers. The findings suggest that compounds of the cigarette smoke interact with monoamineoxidase in both competitive and noncompetitive manner. In vitro studies (unpublished own findings) have demonstrated that low concentrations of norharman inhibit the enzyme competitively (shift of the affinity constant to higher values) and high concentrations noncompetitively (upper micromolar range, lowering of the maximum turnover rate). Therefore, it was expected that the K_m-value is increased and the maximum velocity reduced by acute smoking of smokers rather than of nonsmokers. The only explanation would be that the binding sites of the enzyme are blocked by compounds during previous smoking sessions of smokers and that the additional supply of compounds by acute smoking are not effective enough to cause measurable reduction of affinity. However, such mechanisms do not explain the lack of change of the maximum velocity in smokers.

Another finding of the study is noteworthy, namely the lack of a difference of the maximum turnover rate between smokers and nonsmokers. In contrast, chronic alcoholics have a reduced maximum turnover rate of monoamineoxidase B, which is regarded as a nosological trait (Rommelspacher et al., 1994a).

The inhibition of monoamineoxidase by tobacco smoke has been confirmed by in vitro studies (Yu and Boulton, 1987). Rat lung mitochondria monoamineoxidase were incubated in a phosphate buffer containing cigarette smoke. At higher concentrations of cigarette smoke in solution, the enzyme activity was totally inhibited. Fractionation of the solution revealed that only those fractions which contained "low molecular weight compounds" inhibited both isoforms of monoamineoxidase. The inhibiting compound(s) was (were) not identified. It is remarkable that both norharman (12.6 µg/g tobacco smoked) and harman (3.6 µg/g) are present in tobacco smoke in high concentrations (Poindexter and Carpenter, 1962) and that norharman inhibits specifically monoamineoxidase B ($K_i = 730$ nM), whereas harman inhibits monoamineoxidase A in vitro ($K_i = 220$ nM, May et al., 1991) and in vivo (Rommelspacher et al., 1994b). L-Deprenyl inhibited specific [^{3}H]norharman binding to crude mitochondrial membranes from rat brain with a K_i-value of 130 nM, suggesting high affinity binding of norharman to monoamineoxidase B in brain tissue (Pawlik and Rommelspacher, 1988). The observation of Yu and Boulton (1987) that small compounds of tobacco smoke inhibit both isoforms are compatible with the notion that norharman and harman are contributing to this actions. Furthermore, dopamine, the most affected neurotransmitter in Parkinson's disease is metabolized by both forms of monoamineoxidase in human brain tissue. The ratio of activities vary considerably from brain region to brain region, from about 1:1 in cerebral and

cerebellar cortex to about 1:2 in pons and medulla oblongata. In cerebral cortex, the K_m-values for monoamineoxidase A and B towards dopamine were found to be 240 and 230 μM, respectively (O'Carroll et al., 1983). Therefore, provided the inhibition of monoamineoxidase contributes to the postulated protective effect of cigarette smoke, the high concentration of the two beta-carbolines in tobacco smoke with the differing affinity to both isoforms would make norharman and harman two attractive candidates for the responsible compounds. By comparing the K_i-values for norharman and harman (both beta-carbolines are no substrates for the enzyme) with the K_m-values for dopamine, it is noteworthy that the affinity of norharman was about 330 and of harman 1000 times higher than that of dopamine.

In view of the observation of the Parkinson Study Group (1989) that the irreversible monoamineoxidase B inhibitor selegiline does not act neuroprotective at least in L-dihydroxyphenylalanine (L-DOPA)-treated patients, it is tempting to speculate that for the neuroprotective action, the inhibition of both isoforms is necessary. This increases the plausibility of our hypothesis that the beta-carbolines contribute to the neuroprotection of tobacco smoke. It is remarkable that the levels of both norharman and harman are elevated in the cerebrospinal fluid (CSF) from untreated (de novo) and L-DOPA-treated patients with Parkinson's disease (Kuhn et al., 1996). Because of the difficulties to get access to monoamineoxidase A-containing tissue, we focussed in this study on monoamineoxidase B, which is expressed in platelets.

Smoking induced a dramatic increase of norharman and harman in smokers. The increase in nonsmokers was smaller probably because these individuals were not able to inhale the smoke as deeply as the smokers. The profile of the timecourse of norharman and harman levels was quite similar, suggesting that both compounds are present in the smoke in a certain portion at least in the brand used in this study. However, the absolute amount of norharman was higher than that of harman, which is consistent with the report of Poindexter and Carpenter (1962). To evaluate the pharmacokinetic findings, it has to be considered that only a very limited number of data points were available for the determination of the elimination rate constant and, thus, half-life. The plasma half-life of norharman was longer than that reported by others (51 vs. 15–35 min; Breyer-Pfaff et al., 1996). The differences may be explained by the number of subjects analysed ($n = 19$ vs. $n = 3$), the longer time period investigated (60-min first smoking session, 120-min second smoking session vs. 60-min first smoking session, 70-min second smoking session). Finally, the values reported by Breyer Pfaff et al., who metabolized norharman relatively fast (Table 1, minimum 26.8 min), are in the range of subjects of this study.

Compared to plasma (median: 51 min), the apparent terminal half-life of norharman in platelets was almost fourfold higher with a high interindividual variability for all parameters especially in platelets. Therefore, the results represent a first approach in estimating pharmacokinetic parameters. Especially, the half-life of harman in platelets should not be interpreted any further. Additional investigations should be performed to further elucidate the pharmacokinetics of norharman and harman and should integrate mixed-effect modeling data analysis.

The volume of a single platelet is in the order of magnitude of 8.8 fl on average. Provided an even distribution of norharman, the baseline concentration amounts to 4.94 nM. The concentration is higher than in blood plasma (0.057 nM), confirming the accumulation of norharman in platelets and probably in the brain. The respective calculations for harman were—plasma nonsmokers: 0.023 nM; platelets: 1.68 nM—which suggests an accumulation of harman in platelets.

In smokers, the baseline concentration of norharman in platelets was 20.02 nM and after smoking two cigarettes, 27.93 nM. The respective values for harman in platelets were 6.8 and 16.2 nM. It can be assessed from these findings that two cigarettes cause an increase of the norharman levels in platelets by approximately 10 nM. Because of the slow elimination observed in this study, a heavy smoker can reach a level of 100 to 150 nM norharman by smoking 10 cigarettes within a few hours.

In rat forebrain, the K_i-value for the inhibition of monoamineoxidase B by norharman amounted to 730 nM (May et al., 1991). This yields an IC_{50}-value of approximately 1 μM (competitive interaction). If one assumes that the concentration of the inhibitor is in a simple one-site model active in a 100-fold range from one-tenth the IC_{50}-value to 10 times the IC_{50}-value, then one-tenth of the IC_{50} concentration inhibits the enzyme by 10%. In this case, norharman levels of heavy smokers would be sufficient to inhibit 10% of the enzyme activity. However, it is possible that the degree of monoamine oxidase B inhibition could have been underestimated. Turkish et al. (1988) determined behavioural effects and compared the degree of inhibition by an irreversible (L-deprenyl) and a reversible inhibitor (MD 240928) of monoamineoxidase B. They concluded that due to dilution effects during work up, the underestimation of enzyme inhibition of reversible inhibiting compounds is at least 6% and possibly 17%. Another point to be considered is the amount of norharman remaining in the tissue. Norharman is a lipophilic compound which may be trapped in the layer between the phospholipid membrane of the mitochondrion and the enzyme protein. This would lead to the accumulation of norharman in the microenvironment of the mitochondrial membrane where the monoamine oxidase is located.

If one considers the 10% inhibition of monoamineoxidase B by norharman in heavy smokers and adds a further 10% due to diluting effects during work up, one can conclude that norharman contributes substantially to the tobacco smoke-derived inhibition of the enzyme (see Introduction).

The levels of harman in platelets increased less due to the lower levels of harman in cigarette smoke. The inhibiting potency of harman of monoamineoxidase A is about three times higher than that of norharman to the B-form

(K_i = 220–730 nM). Therefore, in heavy smokers, the levels of harman in the brain (which have not been measured directly in the study) should be high enough to inhibit monoamineoxidase A.

In conclusion, dopamine is metabolized in human brain by both isoforms of monoamineoxidase. Inhibition of the B-form by selegiline is not sufficient to protect dopaminergic neurones from neurodegeneration in patients with Parkinson's disease. On the other hand, smoking protects patients to a certain degree as found consistently in epidemiological studies. It could very well be that both isoforms have to be inhibited to reach the degree of protection which is observed in those studies. Norharman and harman are present in high concentrations in tobacco smoke. The present study provides indirect evidence that the amount of the beta-carbolines inhaled in the smoke contribute substantially to the inhibition of the enzyme as observed in positron emission tomography studies in smokers.

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