In vitro monoamine oxidase inhibition potential of alpha-methyltryptamine analog new psychoactive substances for assessing possible toxic risks

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Abstract

Tryptamines have emerged as new psychoactive substances (NPS), which are distributed and consumed recreationally without preclinical studies or safety tests. Within the alpha-methylated tryptamines, some of the psychoactive effects of the prototypical alpha-methyltryptamine (AMT) have been described decades ago and a contributing factor of its acute toxicity appears to involve the inhibition of monoamine oxidase (MAO). However, detailed information about analogs is scarce. Therefore, thirteen AMT analogs were investigated for their potential to inhibit MAO. An in vitro assay analyzed using hydrophilic interaction liquid chromatography–high resolution-tandem mass spectrometry was developed and validated. The AMT analogs were incubated with recombinant human MAO-A or B and kynuramine, a non-selective MAO substrate to determine the IC₅₀ values. The known MAO-A inhibitors 5-(2-aminopropyl)indole (5-IF), harmine, harmaline, yohimbine, and the MAO-B inhibitor selegiline were tested for comparison. AMT and all analogs showed MAO-A inhibition properties with IC₅₀ values between 0.049 and 166 μM, whereas four analogs inhibited also MAO-B with IC₅₀ values between 82 and 376 μM. 7-Me-AMT provided the lowest IC₅₀ value against MAO-A comparable to harmine and harmaline and was identified as a competitive MAO-A inhibitor. Furthermore, AMT, 7-Me-AMT, and nine further analogs inhibited MAO activity in human hepatic S9 fraction used as model for the human liver which expresses both isoforms. The obtained results suggested that MAO inhibition induced by alpha-methylated tryptamines might be clinically relevant concerning possible serotonergic and adrenergic effects and interactions with drugs (of abuse) particularly acting as monoamine reuptake inhibitors. However, as in vitro assays have only limited conclusiveness, further studies are needed.

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1. Introduction

New psychoactive substances (NPS) are emerging drugs that are mainly consumed as legal and easy available substitutes for...
traditional and controlled drugs of abuse (Brandt et al., 2014; Meyer, 2016). The detection of synthetic tryptamines including alpha-methyltryptamine (AMT) has been frequently reported to the EU Early Warning System coordinated by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and continuously monitored as part of the EMCDDA’s toxicovigilance system (EMCDDA, 2015). AMT (α-MT, 5-IT, 6F-290) has been investigated in the 1960s as a potential antidepressant and its popularity as a drug of abuse came to light in the 1990s due to its hallucinogenic and stimulant properties (Araujo et al., 2015). In late 2011, an isomer of AMT, 5-(2-aminopropyl)indole (5-IT, 5-API), appeared on the European drug market (EMCDDA, 2014). After intake of AMT or 5-IT, symptoms related to monoaminergic toxicity were described, such as restlessness, agitation, disorientation, shivering, sweating, mydriasis, vomiting, tachycardia, or hyperthermia (EMCDDA, 2015; Shulgin and Shulgin, 1997). Both were also involved in several fatalities (Boland et al., 2005; Elliott and Evans, 2014). A key factor involved in the occurrence of clinical features included the inhibition of monoamine oxidase (MAO) enzymes, followed by increased monoamine levels inducing a serotonin syndrome (Boyer and Shannon, 2005; EMCDDA, 2014). In 1968, AMT, 5-IT, and four positional isomers were identified as inhibitors of guinea pig MAO (Cerletti et al., 1968). More recently, 5-IT was confirmed as highly selective and potent inhibitor of recombinant human MAO-A (Herraiz and Brandt, 2014).

Furthermore, tryptamines were identified in NPS products combining different NPS groups (UNODC, 2016). Thus, the risk of encountering monoaminergic (side) effects and drug-drug interactions is very likely although the extent to which this might occur is difficult to predict, given that systematic data are not available. In contrast to authorized medicines, NPS are marketed and consumed without preclinical or clinical studies. Procedures employed for various substrates, mostly therapeutic drugs, have been described for in vitro monitoring of MAO activity (Tipton et al., 2006). Examples of drugs of abuse tested for human MAO inhibition activity include MDMA and its metabolites, and 5-IT (Herraiz and Brandt, 2014; Steuer et al., 2016).

Therefore, the aim of the present study was to develop a MAO inhibition assay based on hydrophilic interaction liquid chromatography–high resolution-tandem mass spectrometry (HILIC–HR-MS/MS), to investigate the in vitro inhibition potential of AMT and 13 ring-substituted analogs (Fig. 1) on recombinant human MAO-A or B, and to determine their IC50 values.

2. Materials and methods

2.1. Chemicals and enzymes

Harmane was obtained from THC Pharm (Frankfurt, Germany), amphetamine-ds and AMT succinate from LGC Standards (Wesel,
Germany), harmaline, yohimbine, selegiline, kynuramine (KYN), 4-hydroxyquinoline (4-OHC), ammonium acetate, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate from Sigma-Aldrich (Taufkirchen, Germany), formic acid (MS grade) from Fluka (Neu-Ulm, Germany), acetonitrile, methanol (both LC-MS grade), and all other chemicals from VWR (Darmstadt, Germany). 5-IT was synthesized (Scott et al., 2014) and kindly provided by the Department of Pharmacology and Therapeutics, Trinity Centre for Health Sciences, St James’s Hospital, Dublin 8, Ireland, before it was scheduled. All non-scheduled AMT analogs were prepared following procedures published previously (Young, 1958). Details can also be found in (Brandt et al., 2004).

The baculovirus-infected insect cell microsomes (Supersomes) containing human complementary DNA-expressed MAO-A or MAO-B (5 mg protein/mL), wild-type Supersomes as negative control without MAO activity (MAO control, 5 mg protein/mL), and pooled human liver S9 (20 mg protein/mL) were obtained from Corning (Amsterdam, The Netherlands). After delivery, enzyme preparations were thawed at 37 °C, aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C until use.

2.2. HILIC–HR-MS/MS apparatus

A Thermo Fisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 Rapid Separation (RS) UHPLC system with a quaternary UltiMate 3000 RS pump and an UltiMate 3000 RS autosampler was used and controlled by the TF Chromelon software version 6.80. It was coupled to a TF Q-Exactive Plus equipped with a heated electrospray ionization liquid source (HESI-II). The gradient elution was performed on a Macherey-Nagel (Düren, Germany) HILIC Nucleodur column (125 × 3 mm, 3 μm) using aqueous ammonium acetate (25 mM, eluent A) and acetonitrile containing 0.1% (v/v) formic acid (eluent B). The flow rate was set to 500 μL/min and the gradient was programmed as follows: 0–0.5 min hold 80% B, curve 5; 0.5–3.8 min to 50% B, curve 5; 3.8–3.9 min to 40% B, curve 5; 3.9–5 min hold 40% B, curve 5; 5–5.1 min to 80% B, curve 5; and 5.1–6 min hold 80% B, curve 5. Chromatography was performed at 60 °C maintained by a Dionex UltiMate 3000 RS analytical column heater. The injection volume for all samples was 1 μL. HESI-II conditions were already described before (Richter et al., 2016): sheath gas, 53 arbitrary units (AU); auxiliary gas, 14 AU; sweep gas, 3 AU; spray voltage, 3.50 kV; heater temperature, 438 °C; ion transfer capillary temperature, 269 °C; and S-lens RF level, 60.0. Mass calibration was done prior to analysis according to the manufacturer’s recommendations using external mass calibration. For evaluating the chromatographic separation, a full scan experiment was used with the following scan parameters: polarity, positive; micro scan, 1; resolution, 35,000; automatic gain control (AGC) target, 36e5; maximum injection time (IT), 200 ms; and scan range, 50–750. The final quantification was performed using a targeted single ion monitoring (t-SIM) and a subsequent data-dependent MS² (dd-MS²) mode with an inclusion list containing the exact masses of positively charged KYN ([m/z] 165.1022), 4-OHC ([m/z] 146.0600), and the internal standard (IS) amphetamine-d₃ ([m/z] 141.1434). The settings for the t-SIM mode were as follows: micro scan, 1; resolution, 35,000; AGC target, 5e4; maximum IT, 100 ms; and isolation window, 4 m/z. The settings for the dd-MS² mode were as follows: micro scan, 1; resolution, 35,000; AGC target, 2e5; maximum IT, 100 ms; isolation window, 4 m/z; and dynamic exclusion, 4 s. TF Xcalibur Qual Browser 2.2 software was used for data handling. The settings for automated peak integration were as follows: peak detection algorithm, IC5; area noise factor, 5; and peak noise factor, 300. GraphPad QuickCalcs (GraphPad Software, San Diego, USA) was used for outlier detection (http://graphpad.com/quickcalcs/grubbs1), while GraphPad Prism 5.00 (GraphPad Software) was used for statistical evaluation.

2.3. Preparation of stock solutions

Stock solutions of the MAO substrate KYN (6.25 mM) or its metabolite 4-OHC (0.1 mM) were prepared in water. Stock solutions of harmaline, harmaline, 5-IT, selegiline, AMT (5 mM, respectively), or yohimbine (3 mM) were used in methanol. Stock solutions of the AMT analogs (5 mM, respectively) were prepared in DMSO. Afterwards, they were diluted 1:5 (v/v) with methanol, gently evaporated under nitrogen, and resolved in water/methanol (9:1, v/v). To obtain the solutions for incubation, the stock solutions were diluted in phosphate buffer. The organic solvent content in the final incubation mixtures was always below 2.5% (Chauvet et al., 1998). Stock solutions were aliquoted and stored at −20 °C.

2.4. Method validation

The method for quantification of 4-OHC was validated in accordance to the “Guideline on bioanalytical method validation” published by the European Medicines Agency (EMA, 2011). For validation, 30 μL samples containing 1 μg/mL MAO control in phosphate buffer were used. If needed, 10 μL of the phosphate buffer were replaced by appropriate amounts of the calibration or quality control (QC) stock solution. These samples were diluted with the same volume of acetonitrile with or without IS. Briefly, the method was tested for selectivity (using ten blank samples without IS), carry-over (using a blank sample without IS following the high QC), lower limit of quantification (LLOQ) defined as lowest calibration standard, within-run accuracy and precision (analyzed in a single run five samples per level at four concentration levels: LLOQ QC, low QC, medium QC, and high QC), between-run accuracy and precision (analyzed in three different runs on two different days six samples per level at four concentration levels: LLOQ QC, low QC, medium QC, and high QC), matrix effect (using six samples with matrix and six samples without matrix at two concentration levels: low QC and high QC), and stability of processed samples in the autosampler. The calibration consisted of six concentration points (50, 200, 400, 600, 800, and 1000 nM) equally distributed over the whole range. The concentrations of LLOQ QC, low QC, medium QC, and high QC were as follows: 50, 100, 500, and 900 nM. Instead of MAO-A or B, MAO control was used for sample preparation. For quantification, the ratios of 4-OHC versus IS were used. The analytical runs consisted of a blank sample without IS, a blank sample with IS, calibration standards, three levels of QC.

![Fig. 2. Deamination of kynuramine catalyzed by MAO-A or MAO-B providing an aldehyde, followed by non-enzymatic condensation to 4-hydroxyquinoline.](image-url)
samples (low, medium, and high) in duplicate, and the study samples. All calculations were done using GraphPad Prism 5.00.

2.5. Determination of \( K_m \) values

KYN deamination is depicted in Fig. 2. The kinetic constants were derived from incubations with MAO-A, MAO-B, or 59. Incubations were performed at 37 °C for 20 min using 1 µg/mL MAO-A or MAO-B, respectively, or 100 µg/mL 59, and the MAO substrate KYN concentrations of 0.1, 0.5, 1, 5, 10, 25, 50, 100, 150, 200, 250, and 500 µM. In addition, blank incubations with 1 µg/mL MAO control were prepared as negative control. Besides enzyme preparations and substrate, the incubation mixtures (final volume 30 µL) contained 100 mM phosphate buffer. Reactions were initiated by addition of the ice-cold enzyme preparation and stopped with 30 µL of ice-cold acetonitrile, containing 10 mM amphetamine-\( \text{d}_5 \) as IS. The solution was centrifuged for 2 min at 10,000g. 50 µL of the supernatant were transferred to an autosampler vial, and injected onto the HILIC–HR-MS/MS apparatus for analysis. Enzyme kinetic constants were estimated by non-linear curve fitting using GraphPad Prism 5.00 after validated quantification of the formed metabolite 4-OHC via six-point calibration. The Michaelis–Menten equation (Eq. (1)) was used to calculate apparent \( K_m \) and \( V_{\text{max}} \) values for MAO-A, MAO-B, or 59, where \( v \) is the initial reaction velocity, \( S \) the substrate concentration, \( V_{\text{max}} \) the maximal reaction velocity, and \( K_m \) the substrate concentration at half \( V_{\text{max}} \).

\[
v = \frac{V_{\text{max}} \times S}{K_m + S}
\] (1)

2.6. Initial inhibition screening and determination of \( IC_{50} \) values and inhibition constants

2.6.1. Initial inhibition screening

For inhibition studies, 10 µL of the phosphate buffer was replaced by appropriate amounts of the potential inhibitor solution. KYN was used in concentrations comparable to its \( K_m \) value. All other incubation conditions were the same as described above. To test for inhibition capability, MAO-A or B was incubated with 10 µM of the potential inhibitor in triplicate (\( n = 3 \)). In addition to these samples, control samples without inhibitor, positive control samples with model inhibitors (10 µM 5-IT for MAO-A or 10 µM selegiline for MAO-B), blank samples without MAO activity, and interfering samples were also prepared in triplicates. For preparation of the interfering samples, control samples without inhibitor were incubated and the reaction was terminated with ice-cold acetonitrile containing the IS and the potential inhibitors. The 4-OHC formation in test samples was then compared to metabolite formation in control samples. For statistical analysis of data, a one-way ANOVA followed by Dunnett's multiple comparison test (significance level, \( P < 0.001, 99.9\% \) confidence intervals) by GraphPad Prism 5.00 was used.

2.6.2. Determination of \( IC_{50} \) values

Inhibitors were incubated at least at ten different concentrations (0.00004, 0.0002, 0.0006, 0.002, 0.01, 0.04, 0.2, 0.6, 2.5, 10, 40 160 640, 2560 µM), depending on their expected inhibition strengths after initial inhibition screening. All other incubation conditions were the same as described above. Control samples were also prepared as described above. The \( IC_{50} \) values were calculated by plotting the metabolite formation (relative to the control samples) over the logarithm of the inhibitor concentration using GraphPad Prism 5.00.

\[\text{IC}_{50} = \frac{IC_{50} \times S}{K_m + S}
\]

Table 1

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>Reference plasma concentrations</th>
<th>( IC_{50} ) values, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/L</td>
<td>µM</td>
</tr>
<tr>
<td><strong>AMT-type NPS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMT</td>
<td>440 (Ferec et al., 2015)</td>
<td>0.38 (8)</td>
</tr>
<tr>
<td>160–1300 (PM) (Elliot and Evans, 2014)</td>
<td>0.09–7.5</td>
<td></td>
</tr>
<tr>
<td>4-MeO-AMT</td>
<td></td>
<td>1.4 (11)</td>
</tr>
<tr>
<td>5-MeO-AMT</td>
<td></td>
<td>31 (4)</td>
</tr>
<tr>
<td>1-Me-AMT</td>
<td></td>
<td>51 (3)</td>
</tr>
<tr>
<td>2-Me-AMT</td>
<td></td>
<td>11 (7)</td>
</tr>
<tr>
<td>5-CL-AMT</td>
<td></td>
<td>15.1 (13)</td>
</tr>
<tr>
<td>7-Me-AMT</td>
<td></td>
<td>0.049 (2)</td>
</tr>
<tr>
<td>5-F-AMT</td>
<td></td>
<td>0.45 (23)</td>
</tr>
<tr>
<td>6-F-AMT</td>
<td></td>
<td>1.8 (19)</td>
</tr>
<tr>
<td>5-Cl-AMT</td>
<td></td>
<td>0.25 (7)</td>
</tr>
<tr>
<td>5-Br-AMT</td>
<td></td>
<td>1.3 (7)</td>
</tr>
<tr>
<td>5-F-2-Me-AMT</td>
<td></td>
<td>4.1 (7)</td>
</tr>
<tr>
<td>1-Me-2-Ph-AMT</td>
<td></td>
<td>223 (3)</td>
</tr>
<tr>
<td>2-Ph-AMT</td>
<td></td>
<td>166 (2)</td>
</tr>
<tr>
<td>Known inhibitors</td>
<td></td>
<td>3.7 (18)</td>
</tr>
<tr>
<td>5-IT</td>
<td>15–590 (Backberg et al., 2014)</td>
<td>0.1–3.4</td>
</tr>
<tr>
<td>700–5100 (PM) (EMCODA, 2014)</td>
<td>0.20 (1)</td>
<td></td>
</tr>
<tr>
<td>Selegiline</td>
<td>0.3–1.5 (Barrett et al., 1996)</td>
<td>0.002–0.01</td>
</tr>
<tr>
<td>Harmine</td>
<td>36–222 (Baselt, 2008)</td>
<td>0.006 (2)</td>
</tr>
<tr>
<td>1.0–15.6 (Oliveira et al., 2012)</td>
<td>0.005–0.07</td>
<td></td>
</tr>
<tr>
<td>2.7–15.7 (Oliveira et al., 2012)</td>
<td>0.01–0.07</td>
<td></td>
</tr>
<tr>
<td>3.7–171 (Baselt, 2008)</td>
<td>0.01–0.5</td>
<td></td>
</tr>
</tbody>
</table>

\( ^* \)No plasma concentrations reported, thus concentrations estimated according to the structure-related compound AMT.
2.6.3. Determination of inhibition constants (K\textsubscript{i} values)

For experimental determination, four concentration levels of the test inhibitor were incubated with KYN at five different concentrations (0.5, 5, 25, 100, and 500 \textmu M). Inhibitor concentration levels depended on the previously determined IC\textsubscript{50} value. For selegiline and 7-Me-AMT, inhibitor concentration levels were 0, 0.01, 0.05, and 0.1 \textmu M, for 5-IT, 0, 0.1, 0.5, and 1 \textmu M. All other incubation conditions were the same as described above. 7-Me-AMT and 5-IT were incubated with MAO-A, whereas MAO-B was used in the case of selegiline. The inhibition constants (K\textsubscript{i} values) were calculated using GraphPad Prism 5.00. Michaelis-Menten plots were transferred to Lineweaver-Burk plots (1/V versus 1/S), and fit factors for different inhibition models were compared to determine the most probable inhibition mode. If competitive inhibition was identified as most probable inhibition model, the equation of Cheng and Prusoff (Eq. (2)) (Cheng and Prusoff, 1973)

Fig. 1. Initial inhibition screening results using 10 \textmu M each of the inhibitor. Percentage of activity represented the percentage of metabolite formation in relation to control incubations without inhibitor (100%). Values are expressed as mean and were tested for significance (n = 3, ***, \( P < 0.001 \), **, \( P < 0.01 \), *, \( P < 0.1 \) for 4-hydroxyquinoline formation in incubations with the test inhibitor versus 4-hydroxyquinoline formation in control incubations).
was additionally used to calculate \( K_i \) from \( IC_{50} \) and \( K_m \) values.

\[
IC_{50} = K_m \left(1 + \frac{S}{K_m} \right)
\]  

(2)

2.7. MAO inhibition in S9 fraction

Three concentration levels of the inhibitors were incubated with KYN at concentrations at its \( K_m \) value. Inhibitor concentration level one was the lowest concentration and chosen based on plasma concentrations reported after intake (Table 1). Concentration levels two and three were calculated to be ten and one hundred times concentration level one, respectively. For selegiline, inhibitor concentration levels were 0.005, 0.05, and 0.5 \( \mu \)M; for harmine, harmaline, and yohimbine, 0.1, 1, and 10 \( \mu \)M inhibitor were chosen, and for all other compounds, 1, 10, and 100 \( \mu \)M. All other incubation conditions were the same as described above. In addition, control samples without inhibitor and blank samples were prepared. All incubations were performed in triplicate (\( n = 3 \)). For evaluation, the metabolite formation in test samples was compared to metabolite formation in control samples. For statistical analysis of the data, a one-way ANOVA followed by Dunnett’s multiple comparison test (significance level, \( P < 0.05 \), 95% confidence intervals) by GraphPad Prism 5.00 was used.

3. Results

3.1. Method validation

The analytical procedure was based on HILIC–HR-MS/MS in t-SIM mode with a subsequent dd-MS\(^2\) mode. The method was successfully validated in accordance to the criteria of the (EMA, 2011). The method was selective at LLOQ levels, with a response of interfering components less than 20% or 5% compared to the response of the analyte at the LLOQ and the response of the IS, respectively. No carry-over problems could be observed and the LLOQ for 4-OHC was defined as 50 nM. The mean for within-run and between-run accuracies ranged from 3% to 19% but were within 20% of the nominal values for the LLOQ QC and within 15% for the low, medium, and high QC samples. The mean within-run and between-run precisions ranged from 1% to 13%. Precisions were within 20% for the LLOQ QC and within 15% for the low, medium, and high QC samples. The coefficients of variation (CVs) of matrix factors were 9% and 11% for the analytes and the IS at low QC level and 3% and 7% at high QC level. The CVs of IS-normalized matrix factor were 14% for low and 9% at high QC level and thus not greater than 15%. Processed samples showed sufficient stability in the autosampler for at least 10 h, corresponding to the maximum duration of the analytical runs.

3.2. Determination of \( K_i \) values

The kinetics for KYN deamination using MAO-A, MAO-B, or 50 followed classic Michaelis–Menten behavior. Calculated \( K_m \) values were 43 ± 5 \( \mu \)M, 23 ± 4 \( \mu \)M, and 34 ± 2 \( \mu \)M for MAO-A, MAO-B, and 50, respectively. \( V_{max} \) values were 75 ± 2 nmol/min/mg, 56 ± 2 nmol/min/mg, and 7 ± 0.1 nmol/min/mg for MAO-A, MAO-B, and 50, respectively. \( K_i \) values were 0.9910, 0.9760, and 0.9980, respectively.

3.3. Initial inhibition screening results

The results are summarized in Fig. 3. All test tryptamines showed significant inhibition of MAO-A resulting in residual activities below 50%. MAO-B was additionally inhibited by 5-F-AMT, 6-F-AMT, 5-CI-AMT, and 5-F-2-Me-AMT, resulting in residual activities between 60 and 80%. Harmine, harmaline, and yohimbine also showed inhibition of MAO-A in this assay. whereas harmine and harmaline inhibited MAO-A activity almost completely, yohimbine inhibition resulted in residual activities of about 25%. No analytical interferences could be detected for all incubation sets and the positive control samples showed almost complete inhibition of MAO-A or B with residual activities of about 5%.

3.4. Determination of \( IC_{50} \) values

All results are given in Table 1. \( IC_{50} \) values of AMT and its analogs for MAO-A inhibition were between 0.049 (7-Me-AMT) and 166 \( \mu \)M (1-Me-2-Ph-AMT), for MAO-B inhibition, \( IC_{50} \) values of 5-CI-AMT, 6-F-AMT, 5-F-2-Me-AMT, and 5-F-AMT were 82, 126, 223, and 376 \( \mu \)M, respectively. The known MAO-A inhibitors harmine, harmaline, 5-IT, and yohimbine resulted in \( IC_{50} \) values of 0.006, 0.011, 0.20, and 6.0 \( \mu \)M, respectively, while the \( IC_{50} \) value of seleagine towards MAO-B was 0.017 \( \mu \)M.

3.5. Determination of \( K_i \) values

Michaelis–Menten and Lineweaver–Burk plots after incubation of 7-Me-AMT are given in Fig. 4. The \( K_i \) value was 0.026 \( \mu \)M. A competitive inhibition model was preferred, which was in line with the visual inspection of the intersection in the Lineweaver–Burk plot. \( K_i \) value calculated from \( IC_{50} \) and \( K_m \) values was 0.025 \( \mu \)M. For 5-IT and seleagine, experimentally determined \( K_i \) values were 0.18 and 0.011 \( \mu \)M, respectively. Calculated \( K_i \) values

![Fig. 4. Michaelis-Menten plot (left part) of the deamination of kynuramine in absence or presence of three given concentrations of 7-Me-AMT for determination of the inhibition constant. Data points represent means and ranges (error bars) of duplicate measurements. Transformation of data to a Lineweaver-Burk plot (right part) for determination of the inhibition mode.](image-url)
were 0.10 and 0.009 μM, respectively. Visual inspection and software-based evaluation preferred competitive inhibition model for both compounds.

3.6. MAO inhibition in S9 fraction

Results are summarized in Fig. 5. 7-Me-AMT exerted significant inhibition of KYN deamination by MAO in S9 for all concentration levels. AMT, 5-Cl-AMT, and 5-Br-AMT inhibited MAO activity when incubated at concentration levels two or three. 4-MeO-AMT, 2-Me-AMT, 5-Me-AMT, 5-F-AMT, 6-F-AMT, 5-F-2-Me-AMT, and 2-Ph-AMT showed significant inhibition only when incubated in concentration level three. 5-MeO-AMT, 1-Me-AMT, and 1-Me-2-Ph-AMT showed no MAO inhibition in S9, even at the highest concentration level. Harmine and harmaline showed significant inhibition of MAO-A at all concentration levels, while in case of 5-IT and selegiline concentration levels two or three led to significant inhibition, and for yohimbine only concentration level three.

4. Discussion

MAO activity was assessed using KYN, a non-selective substrate for MAO-A and B, which was transformed to the corresponding aldehyde, followed by non-enzymatic condensation to 4-OHC (Fig. 2) (Weissbach et al., 1960). 4-OHC formation was analyzed using HILIC–HR-MS/MS. Although literature described many MAO activity assays (Tipton et al., 2006), Herraiz and Chaparro found that the needed selectivity for analyzing 4-OHC could only be achieved by chromatographic separation before detection (Herraiz and Chaparro, 2006). Since HILIC was shown to provide sufficient retention and separation of small and polar analytes (Steuer et al., 2016), it was anticipated that HILIC separation would be a suitable method in the present study. Due to its high flexibility and sensitivity, HR-MS/MS required comparatively low levels of recombinant MAO enzymes and thus reduction of the total protein content, which can lead to non-specific protein binding of analytes (Baranczewski et al., 2006). The t-SIM mode with a subsequent dd-MS² acquisition allowed simultaneous quantification and identification, respectively. The t-SIM provided approximately 50 scans per peak by far sufficient for quantification, whereas the dd-MS² provided additional selectivity for further studies without the need for revalidation. Experimental variability during sample preparation and analysis was corrected by addition of the internal standard amphetamine-d₆, which provided comparable properties to 4-OHC. The whole analytical procedure was successfully validated in accordance with international guidelines (EMA, 2011).

For determination of Kᵢ values, the substrate concentration should be used below or at its Kᵢ value. Different Kᵢ values of KYN deamination by MAO-A or B derived from diverse enzyme sources were published (Herraiz and Brandt, 2014; Naoi and Nagatsu, 1988; Parikh et al., 2002; Ro et al., 2001) and none for S9. Therefore, Kᵢ values were also determined in this study. The question as to whether the S9 fraction would show MAO activity remained inconsistently answered. As enzymes of the outer mitochondrial membrane, MAO was expected to be removed by centrifugation at 9000g during production of S9 fraction (Lee and Zhu, 2011). However, Salva et al. reported NADPH-independent formation of metabolites even in pooled human liver microsomes, which could be abolished by MAO inhibitors. They ascribed this MAO activity to a contamination with mitochondrial enzymes (Salva et al., 2003). Furthermore, the guidance for industry by the Food and Drug Administration recommended the addition of the MAO inhibitor pargyline to the S9 fraction to identify MAO contribution in the oxidative biotransformation of any given tested drug (FDA, 2006). As 4-OHC formation in S9 was also observed in the present study, the Kᵢ value of KYN transformation in S9 was determined. Resulting Kᵢ values for MAO-A and MAO-B were similar, as expected for a non-selective substrate, and comparable to published values (Herraiz and Brandt, 2014; Naoi and Nagatsu, 1988; Parikh et al., 2002; Ro et al., 2001). Initial experiments were conducted to choose incubation time and enzyme concentration (data not shown) to be within the linear range of metabolite formation. Substrate concentrations between 0.1 and 500 μM KYN allowed modeling of enzyme kinetics. Less than 20% of substrate was metabolized in all incubations, except of the lowest substrate concentrations. To avoid non-specific protein binding, the protein concentrations were chosen as low as analytically possible as recommended by (Baranczewski et al., 2006). Blank incubations should be used to consider MAO-independent formation of 4-OHC.

Applicability of the initial inhibition screening procedure to assess the inhibition by either MAO-A or MAO-B was tested using positive control samples containing 5-IT or selegiline, known for

![Fig. 5. Inhibition of MAO activity in S9 fraction by different inhibitor concentration levels (level 3 represented highest inhibitor concentration). Percentage of activity represented the percentage of metabolite formation in relation to control incubations without inhibitor (100%). Values are expressed as mean and were tested for significance \( n = 3, **, P < 0.001, *, P < 0.01, \), P < 0.1 for 4-hydroxyquinoline formation in incubations with the test inhibitor versus 4-hydroxyquinoline formation in control incubations.](image-url)
inhibition of MAO-A or B, respectively. A one-way ANOVA followed by Dunnett’s multiple comparison test was used to decide whether 4-OH-AMT, notably in test samples was statistically significantly different from 4-OHC formation in control samples. Such an initial inhibition screening strategy was already described by Dinger et al. for studying the cytochrome P450 (CYP) inhibition potential of 3,4-methylenedioxy-derived designer drugs (Dinger et al., 2016a).

In the present study, a set of blank samples was also analyzed. A lower inhibitor concentration (10 μM) in the positive control samples was chosen because plasma concentrations of known MAO inhibitors were expected to be lower than those of CYP inhibitors. No significant inhibition at 10 μM was thus considered as weak and probably not clinically relevant. Interfering samples were used to exclude mass spectral ion suppression or enhancement effects, caused by co-eluting analytes (Remane et al., 2010). These samples were mandatory as only KYN, 4-OHC, and the IS were monitored by the analytical method and co-eluting compounds could lead to false positive (in case of ion suppression) or false negative (in case of ion enhancement) results.

In accordance to Cerletti et al. (1968) and Zirkle and Kaiser (1964), AMT was identified as MAO-A inhibitor. All tested analogs also revealed MAO-A inhibition properties, whereas 5-F-AMT, 6-F-AMT, 5-Cl-AMT, and 5-F-2-Me-AMT inhibited MAO-B as well. Furthermore, harmine, harmaline, and yohimbine showed MAO-A inhibition consistent with previous investigations e.g. (Dos Santos et al., 2013). The initial inhibition screening was useful to realize that most tested compounds inhibited only one MAO isofrom. Hence, time and costs for further IC50 determinations could be saved. Consequently, only for compounds showing a statistically significant inhibition in the initial inhibition screening, IC50 values were determined.

To predict a potential clinical relevance of the MAO inhibition based on IC50 values, the expected plasma levels of inhibitors (given in Table 1) of inhibitors should be considered. In case of NPS, only scarce information is available, concerning plasma concentrations after intake. The only information source was case reports of fatal or non-fatal intoxications but particularly postmortem data is difficult to interpret, due to postmortem redistribution and unclear cause of death. But also data of non-fatal cases is problematic, as dosage and time of ingestion remain often unclear.

7-Me-AMT showed an IC50 value against MAO-A activity comparable to the strong inhibitors harmine and harmaline. For 7-Me-AMT, plasma levels were not yet published, but described plasma concentrations for AMT (Elliott and Evans, 2014; Ferec et al., 2015) were much higher than published harmine and harmaline plasma concentrations (Baselt, 2008; Oliveira et al., 2012). For AMT, Shulgin and Shulgin (1997) described oral intake of 15–30 mg or smoking of 5–20 mg as common dosage. These amounts were comparable to oral dosages of 5-IT (Shulgin and Shulgin, 1997). In comparison, 5-MeO-AMT, however, was found to be significantly more potent with an active oral dose in the 1.5–4.5 mg range (Kantor et al., 1980; Shulgin, 1979; Shulgin and Nichols, 1978; Shulgin and Shulgin, 1997). Concerning other AMT analogs, no data about dosage or plasma concentrations are available so far, but similar doses followed by similar plasma concentrations may be expected. Considering assumed plasma levels, clinical relevance of the MAO-A inhibition after intake of 7-Me-AMT could not be excluded. The same should be true for 5-Cl-AMT, AMT, and 5-F-AMT, as their IC50 values against MAO-A were in the same range as the IC50 value of 5-IT. Given that the other AMT analogs showed higher IC50 values against MAO-A, clinical relevance should be unlikely. Comparing the structural properties and IC50 values, a methylation in the 7-position of the indole ring appeared to result in increased potency whereas compounds with a methylation in position 1, 2, or 5 provided a higher IC50 value than AMT itself. The two compounds carrying a methyl group in position 1 provided the highest IC50 values. Therefore, an unchanged position 1 might be essential for the unhindered binding to MAO-A and especially the inhibition of a methoxy group in position 1 with a phenyl group in position 2 reduced the inhibition potential. Furthermore, a methoxy group in the 5-position led to an increased IC50 value, whereas the methoxy group located at position 4, or insertion of a halogen atom in position 5, had nearly no effect on the IC50 value, compared to AMT. Concerning MAO-B inhibition, determined IC50 values of four AMT analogs were hundred to one thousand times higher than the IC50 value of selegiline but also expected plasma concentrations of the AMT analogs were hundred to thousand times higher than published selegiline plasma concentrations. Additionally, these AMT analogs inhibited both MAO isofroms and thus, clinical relevance could not be excluded.

Furthermore, the IC50 values of 7-Me-AMT, 5-IT, and selegiline were experimentally determined and additionally calculated from the IC50 values resulting in comparable Kf values. Both Kf values for 5-IT were similar to those published by Herrera and Brandt (2014).

In the current study, all three compounds were identified as competitive inhibitors and 7-Me-AMT confirmed as potent MAO-A inhibitor, far stronger than 5-IT.

Moreover, MAO inhibition was tested in human hepatic S9 fraction given that MAO-B is slightly overexpressed in human liver (Nishimura and Naito, 2006; Wang et al., 2013). In contrast to the artificial system of recombinant human MAO expressed in baculovirus-infected insect cells, the S9 fraction represents an in vitro model of the human liver, containing both isofroms in their natural environment and different abundances. The test drugs were used at three different concentration levels. Concentration level one represented expected plasma concentrations, as actual concentrations in the liver were difficult to estimate. Being the main metabolizing organ, a higher concentration than in plasma is more than likely (concentration levels two and three). Obtained results correlated well with the determined IC50 values. 7-Me-AMT, with the lowest IC50 value, showed significant inhibition of MAO activity for all concentration levels, while 5-MeO-AMT, 1-Me-AMT, and 1-Me-2-Ph-AMT, with the highest IC50 values towards MAO-A, showed no inhibition at all. Residual MAO activity towards KYN in presence of the test inhibitor could be explained by inhibition of only one isofrom, which means that the effect might be more pronounced for a MAO-A specific substrate such as serotonin or noradrenaline. As tryptamines were often identified in NPS products containing different NPS groups (UNODC, 2016), an intake with substances that increase monoamine levels or inhibit MAO could lead to severe intoxications as described by (Brush et al., 2004). They reported about an adolescent who suffered from a severe intoxication exhibiting hyperthermia, tachycardia, and massive agitation after consumption of the combination of a hallucinogenic tryptamine and the monoamine oxidase inhibitor harmaline (Brush et al., 2004). Therefore, clinical effects due to MAO inhibition by AMT or its analogs could not be excluded for most of the tested compounds.

As controlled human trials for NPS are not feasible due to ethical reasons, in vitro approaches for studying their toxicokinetics have to be used (Caspar et al., 2015; Dinger et al., 2016b; Meyer, 2016; Meyer et al., 2015; Michely et al., 2015; Wagmann et al., 2016). Concerning MAO inhibition by drugs of abuse, only few studies were published. Cerletti et al. identified AMT, 5-IT, and four positional isomers as inhibitors of guinea pig MAO in an assay based on the manometric determination of the oxygen uptake of guinea pig liver homogenates with serotonin as substrate (Cerletti et al., 1968). Herrera and Brandt confirmed 5-IT as highly selective and potent inhibitor of recombinant human MAO-A by measuring the KYN deamination using high-performance liquid chromatography coupled to diode array detection (Herrera and Brandt, 2014). Steuer et al. investigated the MAO inhibition potential of MDMA.
and its metabolites on the deamination of the neurotransmitters dopamine and serotonin using HILIC-MS/MS. MDMA and MDA were identified as inhibitors of recombinant human MAO-A (Steuer et al., 2016). However, MAO inhibition is not always an effect of the abused drug itself. Users also intentionally consume MAO inhibitors in order to enhance the activity of substances such as N,N-dimethyltryptamine (DMT) that otherwise would be metabolically inactivated. Ayahuasca for example, a hallucinogenic beverage, is the combination of DMT and the β-carbolines MAO inhibitors harmine and harmaline (Araujo et al., 2015).

5. Conclusion

The presented study was the first to describe the MAO inhibition for a broad range of NPS of the alpha-methylated tryptamine type. The workup and analysis based on HILIC–HR–MS/MS were validated according to international guidelines. Due to its high sensitivity, only minimal amounts of recombinant MAO enzymes were needed, thus, reducing the risk of non-specific protein binding, as well as material costs. All tested AMT analogs inhibited MAO-A activity, whereas four compounds inhibited MAO-B as well. 7-Me-AMT was identified as potent and competitive inhibitor of MAO-A. MAO inhibition by AMT and its analogs are expected to be clinically relevant. Plasma levels of other MAO substrates, for example neurotransmitters or drugs, could increase, especially if these substances were selective MAO-A substrates. Further clinical studies are warranted to facilitate a more complete assessment.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors like to thank Achim T. Caspar, Julia Dinger, Andreas G. Helfer, Sascha K. Manier, Julian A. Michely, Lilian H. J. Richter, and Armin A. Weber for their support and fruitful discussion.

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