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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-IT), 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 μ M, whereas four analogs inhibited also MAO-B with IC50 values between 82 and 376 μ M. 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.

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

Highlights

- Study provided comprehensive in vitro data on the inhibitory potential of tryptamine-like new psychoactive substances (NPS) towards human MAO-A/B
- Studied compounds were shown to inhibit at least one MAO isoform
- 7-Me-AMT was identified to have the lowest IC₅₀ value against MAO-A and to act as competitive inhibitor
- MAO inhibition by tested NPS was shown to be of possible clinical relevance concerning serotonergic and adrenergic effects
- Interactions with drugs and drugs of abuse particularly acting as monoamine reuptake inhibitors were shown to be likely

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

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Abstract

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

1 spectrometry (HILIC-HR-MS/MS), to investigate the in vitro inhibition potential of AMT
2 and 13 ring-substituted analogs (Fig. 1) on recombinant human MAO-A or B, and to
3 determine their IC₅₀ values.
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8 **2. Materials and methods**

9 *2.1. Chemicals and enzymes*

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13 Harmine was obtained from THC Pharm (Frankfurt, Germany), amphetamine-*d*₅
14 and AMT succinate from LGC Standards (Wesel, Germany), harmaline, yohimbine,
15 selegiline, kynuramine (KYN), 4-hydroxyquinoline (4-OHC), ammonium acetate,
16 potassium dihydrogenphosphate, and dipotassium hydrogenphosphate from Sigma-
17 Aldrich (Taufkirchen, Germany), formic acid (MS grade) from Fluka (Neu-Ulm,
18 Germany), acetonitrile, methanol (both LC-MS grade), and all other chemicals from
19 VWR (Darmstadt, Germany). 5-IT was synthesized (Scott et al. 2014) and kindly
20 provided by the Department of Pharmacology and Therapeutics, Trinity Centre for
21 Health Sciences, St James's Hospital, Dublin 8, Ireland, before it was scheduled. All
22 non-scheduled AMT analogs were prepared following procedures published
23 previously (Young 1958). Details can also be found in (Brandt et al. 2004).
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34 The baculovirus-infected insect cell microsomes (Supersomes) containing
35 human complementary DNA-expressed MAO-A or MAO-B (5 mg protein/mL), wild-
36 type Supersomes as negative control without MAO activity (MAO control, 5 mg
37 protein/mL), and pooled human liver S9 (20 mg protein/mL) were obtained from
38 Corning (Amsterdam, The Netherlands). After delivery, enzyme preparations were
39 thawed at 37 °C, aliquoted, snap-frozen in liquid nitrogen, and stored at -80 °C until
40 use.
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50 *2.2. HILIC-HR-MS/MS apparatus*

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52 A Thermo Fisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000
53 Rapid Separation (RS) UHPLC system with a quaternary UltiMate 3000 RS pump
54 and an UltiMate 3000 RS autosampler was used and controlled by the TF
55 Chromeleon software version 6.80. It was coupled to a TF Q-Exacte Plus equipped
56 with a heated electrospray ionization II source (HESI-II). The gradient elution was
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1 performed on a Macherey-Nagel (Düren, Germany) HILIC Nucleodur column (125 ×
2 3 mm, 3 μm) using aqueous ammonium acetate (25 mM, eluent A) and acetonitrile
3 containing 0.1% (v/v) formic acid (eluent B). The flow rate was set to 500 μL/min and
4 the gradient was programmed as follows: 0-0.5 min hold 80% B, curve 5; 0.5-3.8 min
5 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-
6 5.1 min to 80% B, curve 5; and 5.1-6 min hold 80% B, curve 5. Chromatography was
7 performed at 60 °C maintained by a Dionex UltiMate 3000 RS analytical column
8 heater. The injection volume for all samples was 1 μL. HESI-II conditions were
9 already described before (Richter et al. 2016): sheath gas, 53 arbitrary units (AU);
10 auxiliary gas, 14 AU; sweep gas, 3 AU; spray voltage, 3.50 kV; heater temperature,
11 438 °C; ion transfer capillary temperature, 269 °C; and S-lens RF level, 60.0. Mass
12 calibration was done prior to analysis according to the manufacturer's
13 recommendations using external mass calibration. For evaluating the
14 chromatographic separation, a full scan experiment was used with the following scan
15 parameters: polarity, positive; micro scan, 1; resolution, 35,000; automatic gain
16 control (AGC) target, 3e6; maximum injection time (IT), 200 ms; and scan range, 50-
17 750. The final quantification was performed using a targeted single ion monitoring (t-
18 SIM) and a subsequent data-dependent MS² (dd-MS²) mode with an inclusion list
19 containing the exact masses of positively charged KYN (*m/z* 165.1022), 4-OHC (*m/z*
20 146.0600), and the internal standard (IS) amphetamine-*d*₅ (*m/z* 141.1434). The
21 settings for the t-SIM mode were as follows: micro scan, 1; resolution, 35,000; AGC
22 target, 5e4; maximum IT, 100 ms; and isolation window, 4 *m/z*. The settings for the
23 dd-MS² mode were as follows: micro scan, 1; resolution, 35,000; AGC target, 2e5;
24 maximum IT, 100 ms; isolation window, 4 *m/z*; and dynamic exclusion, 4 s. TF
25 Xcalibur Qual Browser 2.2 software was used for data handling. The settings for
26 automated peak integration were as follows: peak detection algorithm, ICIS; area
27 noise factor, 5; and peak noise factor, 300. GraphPad QuickCalcs (GraphPad
28 Software, San Diego, USA) was used for outlier detection
29 (<http://graphpad.com/quickcalcs/grubbs1>), while GraphPad Prism 5.00 (GraphPad
30 Software) was used for statistical evaluation.
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1 Stock solutions of the MAO substrate KYN (6.25 mM) or its metabolite 4-OHC
2 (0.1 mM) were prepared in water. Stock solutions of harmine, harmaline, 5-IT,
3 selegiline, AMT (5 mM, respectively), or yohimbine (3 mM) were used in methanol.
4 Stock solutions of the AMT analogs (5 mM, respectively) were prepared in DMSO.
5 Afterwards, they were diluted 1:5 (v/v) with methanol, gently evaporated under
6 nitrogen, and resolved in water/methanol (9:1, v/v). To obtain the solutions for
7 incubation, the stock solutions were diluted in phosphate buffer. The organic solvent
8 content in the final incubation mixtures was always below 2.5% (Chauret et al. 1998).
9 Stock solutions were aliquoted and stored at -20 °C.
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20 *2.4. Method validation*

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22 The method for quantification of 4-OHC was validated in accordance to the
23 “Guideline on bioanalytical method validation” published by the European Medicines
24 Agency (EMA) (EMA 2011). For validation, 30 µL samples containing 1 µg/mL MAO
25 control in phosphate buffer were used. If needed, 10 µL of the phosphate buffer was
26 replaced by appropriate amounts of the calibration or quality control (QC) stock
27 solution. These samples were diluted with the same volume of acetonitrile with or
28 without IS. Briefly, the method was tested for selectivity (using ten blank samples
29 without IS), carry-over (using a blank sample without IS following the high QC), lower
30 limit of quantification (LLOQ) defined as lowest calibration standard, within-run
31 accuracy and precision (analyzed in a single run five samples per level at four
32 concentration levels: LLOQ QC, low QC, medium QC, and high QC), between-run
33 accuracy and precision (analyzed in three different runs on two different days six
34 samples per level at four concentration levels: LLOQ QC, low QC, medium QC, and
35 high QC), matrix effect (using six samples with matrix and six samples without matrix
36 at two concentration levels: low QC and high QC), and stability of processed samples
37 in the autosampler. The calibration consisted of six concentration points (50, 200,
38 400, 600, 800, and 1000 nM) equally distributed over the whole range. The
39 concentrations of LLOQ QC, low QC, medium QC, and high QC were as follows: 50,
40 100, 500, and 900 nM. Instead of MAO-A or B, MAO control was used for sample
41 preparation. For quantification, the ratios of 4-OHC versus IS were used. The
42 analytical runs consisted of a blank sample without IS, a blank sample with IS,
43 calibration standards, three levels of QC samples (low, medium, and high) in
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1 duplicate, and the study samples. All calculations were done using GraphPad Prism
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7 *2.5. Determination of K_m values*

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10 KYN deamination is depicted in Fig. 2. The kinetic constants were derived from
11 incubations with MAO-A, MAO-B, or S9. Incubations were performed at 37 °C for 20
12 min using 1 µg/mL MAO-A or MAO-B, respectively, or 100 µg/mL S9, and the MAO
13 substrate KYN at concentrations of 0.1, 0.5, 1, 5, 10, 25, 50, 100, 150, 200, 250, and
14 500 µM. In addition, blank incubations with 1 µg/mL MAO control were prepared as
15 negative control. Besides enzyme preparations and substrate, the incubation
16 mixtures (final volume 30 µL) contained 100 mM phosphate buffer. Reactions were
17 initiated by addition of the ice-cold enzyme preparation and stopped with 30 µL of
18 ice-cold acetonitrile, containing 10 µM amphetamine- d_5 as IS. The solution was
19 centrifuged for 2 min at 10,000×g, 50 µL of the supernatant were transferred to an
20 autosampler vial, and injected onto the HILIC-HR-MS/MS apparatus for analysis.
21 Enzyme kinetic constants were estimated by non-linear curve fitting using GraphPad
22 Prism 5.00 after validated quantification of the formed metabolite 4-OHC via six-point
23 calibration. The Michaelis-Menten equation (Eq. (1)) was used to calculate apparent
24 K_m and V_{max} values for MAO-A, MAO-B, or S9, where v is the initial reaction velocity,
25 S the substrate concentration, V_{max} the maximal reaction velocity, and K_m the
26 substrate concentration at half V_{max} .
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$$41 \quad (1) \quad v = \frac{V_{max} \times S}{K_m + S}$$

42 43 44 45 46 47 48 *2.6. Initial inhibition screening and determination of IC_{50} values and inhibition* 49 *constants* 50

51 *2.6.1. Initial inhibition screening*

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54 For inhibition studies, 10 µL of the phosphate buffer was replaced by
55 appropriate amounts of the potential inhibitor solution. KYN was used in
56 concentrations comparable to its K_m value. All other incubation conditions were the
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1 same as described above. To test for inhibition capability, MAO-A or B was incubated
2 with 10 μM of the potential inhibitor in triplicate ($n = 3$). In addition to these samples,
3 control samples without inhibitor, positive control samples with model inhibitors (10
4 μM 5-IT for MAO-A or 10 μM selegiline for MAO-B), blank samples without MAO
5 activity, and interfering samples were also prepared in triplicates. For preparation of
6 the interfering samples, control samples without inhibitor were incubated and the
7 reaction was terminated with ice-cold acetonitrile containing the IS and the potential
8 inhibitors. The 4-OHC formation in test samples was then compared to metabolite
9 formation in control samples. For statistical analysis of data, a one-way ANOVA
10 followed by Dunnett's multiple comparison test (significance level, $P < 0.001$, 99.9%
11 confidence intervals) by GraphPad Prism 5.00 was used.
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23 *2.6.2. Determination of IC_{50} values*

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26 Inhibitors were incubated at least at ten different concentrations (0.00004,
27 0.0002, 0.0006, 0.002, 0.01, 0.04, 0.2, 0.6, 2.5, 10, 40 160 640, 2560 μM),
28 depending on their expected inhibition strengths after initial inhibition screening. All
29 other incubation conditions were the same as described above. Control samples
30 were also prepared as described above. The IC_{50} values were calculated by plotting
31 the metabolite formation (relative to the control samples) over the logarithm of the
32 inhibitor concentration using GraphPad Prism 5.00.
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42 *2.6.3. Determination of inhibition constants (K_i values)*

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45 For experimental determination, four concentration levels of the test inhibitor
46 were incubated with KYN at five different concentrations (0.5, 5, 25, 100, and 500
47 μM). Inhibitor concentration levels depended on the previously determined IC_{50} value.
48 For selegiline and 7-Me-AMT, inhibitor concentration levels were: 0, 0.01, 0.05, and
49 0.1 μM , for 5-IT, 0, 0.1, 0.5, and 1 μM . All other incubation conditions were the same
50 as described above. 7-Me-AMT and 5-IT were incubated with MAO-A, whereas
51 MAO-B was used in the case of selegiline. The inhibition constants (K_i values) were
52 calculated using GraphPad Prism 5.00. Michaelis-Menten plots were transferred to
53 Lineweaver-Burk plots ($1/V$ versus $1/S$), and fit factors for different inhibition models
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1 were compared to determine the most probable inhibition mode. If competitive
2 inhibition was identified as most probable inhibition model, the equation of Cheng
3 and Prusoff (Eq. (2)) (Cheng and Prusoff 1973) was additionally used to calculate K_i
4 from IC_{50} and K_m values.
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$$7 \quad (2) \quad IC_{50} = K_i \times \left(1 + \frac{S}{K_m}\right)$$

8 9 10 11 12 13 14 15 *2.7. MAO inhibition in S9 fraction*

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17 Three concentration levels of the inhibitors were incubated with KYN at
18 concentrations at its K_m value. Inhibitor concentration level one was the lowest
19 concentration and chosen based on plasma concentrations reported after intake
20 (Table 1). Concentration levels two and three were calculated to be ten and one
21 hundred times concentration level one, respectively. For selegiline, inhibitor
22 concentration levels were 0.005, 0.05, and 0.5 μ M; for harmine, harmaline, and
23 yohimbine, 0.1, 1, and 10 μ M inhibitor were chosen, and for all other compounds, 1,
24 10, and 100 μ M. All other incubation conditions were the same as described above.
25 In addition, control samples without inhibitor and blank samples were prepared. All
26 incubations were performed in triplicate ($n = 3$). For evaluation, the metabolite
27 formation in test samples was compared to metabolite formation in control samples.
28 For statistical analysis of the data, a one-way ANOVA followed by Dunnett's multiple
29 comparison test (significance level, $P < 0.05$, 95% confidence intervals) by GraphPad
30 Prism 5.00 was used.
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49 **3. Results**

50 *3.1. Method validation*

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52 The analytical procedure was based on HILIC-HR-MS/MS in t-SIM mode with a
53 subsequent dd-MS² mode. The method was successfully validated in accordance to
54 the criteria of the EMA (EMA 2011). The method was selective at LLOQ levels, with a
55 response of interfering components less than 20% or 5% compared to the response
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1 of the analyte at the LLOQ and the response of the IS, respectively. No carry-over
2 problems could be observed and the LLOQ for 4-OHC was defined as 50 nM. The
3 mean for within-run and between-run accuracies ranged from 3% to 19% but were
4 within 20% of the nominal values for the LLOQ QC and within 15% for the low,
5 medium, and high QC samples. The mean within-run and between-run precisions
6 ranged from 1% to 13%. Precisions were within 20% for the LLOQ QC and within
7 15% for the low, medium, and high QC samples. The coefficients of variation (CVs)
8 of matrix factors were 9% and 11% for the analytes and the IS at low QC level and
9 3% and 7% at high QC level. The CVs of IS-normalized matrix factor were 14% for
10 low and 9% at high QC level and thus not greater than 15%. Processed samples
11 showed sufficient stability in the autosampler for at least 10 h, corresponding to the
12 maximum duration of the analytical runs.
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25 *3.2. Determination of K_m values*

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27 The kinetics for KYN deamination using MAO-A, MAO-B, or S9 followed classic
28 Michaelis-Menten behavior. Calculated K_m values were $43 \pm 5 \mu\text{M}$, $23 \pm 4 \mu\text{M}$, and 34
29 $\pm 2 \mu\text{M}$ for MAO-A, MAO-B, and S9, respectively. V_{max} values were 75 ± 2
30 $\text{nmol}/\text{min}/\text{mg}$, $56 \pm 2 \text{ nmol}/\text{min}/\text{mg}$, and $7 \pm 0.1 \text{ nmol}/\text{min}/\text{mg}$ for MAO-A, MAO-B, and
31 S9, respectively. R^2 values were 0.9910, 0.9760, and 0.9980, respectively.
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41 *3.3. Initial inhibition screening results*

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43 The results are summarized in Fig. 3. All test tryptamines showed significant
44 inhibition of MAO-A resulting in residual activities below 50%. MAO-B was
45 additionally inhibited by 5-F-AMT, 6-F-AMT, 5-CI-AMT, and 5-F-2-Me-AMT, resulting
46 in residual activities between 60 and 80%. Harmine, harmaline, and yohimbine also
47 showed inhibition of MAO-A in this assay. Whereas harmine and harmaline inhibited
48 MAO-A activity almost completely, yohimbine inhibition resulted in residual activities
49 of about 25%. No analytical interferences could be detected for all incubation sets
50 and the positive control samples showed almost complete inhibition of MAO-A or B
51 with residual activities of about 5%.
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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 μ M (1-Me-2-Ph-AMT), for MAO-B inhibition, IC_{50} values of 5-Cl-AMT, 6-F-AMT, 5-F-2-Me-AMT, and 5-F-AMT were 82, 126, 223, and 376 μ 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 μ M, respectively, while the IC_{50} value of selegiline towards MAO-B was 0.017 μ 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 μ 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 μ M. For 5-IT and selegiline, experimentally determined K_i values were 0.18 and 0.011 μ M, respectively. Calculated K_i values were 0.10 and 0.009 μ M, 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-*d*₅, which provided comparable properties to 4-OHC. The whole analytical procedure was successfully validated in accordance with international guidelines (EMA 2011).

For determination of K_i values, the substrate concentration should be used below or at its K_m value. Different K_m 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, K_m 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 9,000xg 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).

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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 K_m value of KYN transformation in S9 was determined. Resulting K_m 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 μ M 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.

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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 inhibition of MAO-A or B, respectively. A one-way ANOVA followed by Dunnett's multiple comparison test was used to decide whether 4-OHC formation 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 μ M) 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 μ M 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.

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In accordance to Cerletti et al. and Zirkle and Kaiser (Cerletti et al. 1968; Zirkle and Kaiser 1964), AMT was identified as MAO-A inhibitor. All tested analogs also

1 revealed MAO-A inhibition properties, whereas 5-F-AMT, 6-F-AMT, 5-CI-AMT, and 5-
2 F-2-Me-AMT inhibited MAO-B as well. Furthermore, harmine, harmaline, and
3 yohimbine showed MAO-A inhibition consistent with previous investigations e.g. (Dos
4 Santos et al. 2013). The initial inhibition screening was useful to realize that most
5 tested compounds inhibited only one MAO isoform. Hence, time and costs for further
6 IC₅₀ determinations could be saved. Consequently, only for compounds showing a
7 statistically significant inhibition in the initial inhibition screening, IC₅₀ values were
8 determined.

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

16 7-Me-AMT showed an IC₅₀ value against MAO-A activity comparable to the
17 strong inhibitors harmine and harmaline. For 7-Me-AMT, plasma levels were not yet
18 published, but described plasma concentrations for AMT (Elliott and Evans 2014;
19 Ferec et al. 2015) were much higher than published harmine and harmaline plasma
20 concentrations (Baselt 2008; Oliveira et al. 2012). For AMT, Shulgin and Shulgin
21 (Shulgin and Shulgin 1997) described oral intake of 15 to 30 mg or smoking of 5 to
22 20 mg as common dosage. These amounts were comparable to oral dosages of 5-IT
23 (Shulgin and Shulgin 1997). In comparison, 5-MeO-AMT, however, was found to be
24 significantly more potent with an active oral dose in the 1.5-4.5 mg range (Kantor et
25 al. 1980; Shulgin 1979; Shulgin and Nichols 1978; Shulgin and Shulgin 1997).
26 Concerning other AMT analogs, no data about dosage or plasma concentrations are
27 available so far, but similar doses followed by similar plasma concentrations may be
28 expected. Considering assumed plasma levels, clinical relevance of the MAO-A
29 inhibition after intake of 7-Me-AMT could not be excluded. The same should be true
30 for 5-CI-AMT, AMT, and 5-F-AMT, as their IC₅₀ values against MAO-A were in the
31 same range as the IC₅₀ value of 5-IT. Given that the other AMT analogs showed
32 higher IC₅₀ values against MAO-A, clinical relevance should be unlikely. Comparing
33 the structural properties and IC₅₀ values, a methylation in the 7-position of the indole
34 ring appeared to result in increased potency whereas compounds with a methylation

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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 combination of a methyl 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 isoforms and thus, clinical relevance could not be excluded.

Furthermore, the K_i values of 7-Me-AMT, 5-IT, and selegiline were experimentally determined and additionally calculated from the IC₅₀ values resulting in comparable K_i values. Both K_i values for 5-IT were similar to those published by Herraiz and Brandt (Herraiz 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 isoforms 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 isoform, which means that the effect might be more pronounced for a

1 MAO-A specific substrate such as serotonin or noradrenalin. As tryptamines were
2 often identified in NPS products containing different NPS groups (UNODC 2016), an
3 intake with substances that increase monoamine levels or inhibit MAO could lead to
4 severe intoxications as described by (Brush et al. 2004). They reported about an
5 adolescent who suffered from a severe intoxication exhibiting hyperthermia,
6 tachycardia, and massive agitation after consumption of the combination of a
7 hallucinogenic tryptamine and the monoamine oxidase inhibitor harmaline (Brush et
8 al. 2004). Therefore, clinical effects due to MAO inhibition by AMT or its analogs
9 could not be excluded for most of the tested compounds.
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11 As controlled human trials for NPS are not feasible due to ethical reasons, in
12 vitro approaches for studying their toxicokinetics have to be used (Caspar et al. 2015;
13 Dinger et al. 2016b; Meyer 2016; Meyer et al. 2015; Michely et al. 2015; Wagmann et
14 al. 2016). Concerning MAO inhibition by drugs of abuse, only few studies were
15 published. Cerletti et al. identified AMT, 5-IT, and four positional isomers as inhibitors
16 of guinea pig MAO in an assay based on the manometric determination of the
17 oxygen uptake of guinea pig liver homogenates with serotonin as substrate (Cerletti
18 et al. 1968). Herraiz and Brandt confirmed 5-IT as highly selective and potent
19 inhibitor of recombinant human MAO-A by measuring the KYN deamination using
20 high-performance liquid chromatography coupled to diode array detection (Herraiz
21 and Brandt 2014). Steuer et al. investigated the MAO inhibition potential of MDMA
22 and its metabolites on the deamination of the neurotransmitters dopamine and
23 serotonin using HILIC-MS/MS. MDMA and MDA were identified as inhibitors of
24 recombinant human MAO-A (Steuer et al. 2016). However, MAO inhibition is not
25 always an effect of the abused drug itself. Users also intentionally consume MAO
26 inhibitors in order to enhance the activity of substances such as *N,N*-
27 dimethyltryptamine (DMT) that otherwise would be metabolically inactivated.
28 Ayahuasca for example, a hallucinogenic beverage, is the combination of DMT and
29 the β -carbolines MAO inhibitors harmine and harmaline (Araujo et al. 2015).
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56 **5. Conclusion**

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

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24 The authors declare that they have no conflict of interest.
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Table 1 Test compounds, reference plasma concentrations, and IC₅₀ values (percentage errors in brackets) of AMT-type new psychoactive substances (NPS) and known MAO inhibitors. Plasma concentrations in μM were calculated from the published data (in mg/L or μg/L). Probable clinically relevant IC₅₀ values for AMT-type NPS are given in bold. PM: post-mortem cases

Test compounds	Reference plasma concentrations,		IC ₅₀ values, μM	
	μg/L	μM	MAO-A	MAO-B
AMT-type NPS				
AMT	440 (Ferec et al. 2015) 160-1,300 (PM) (Elliott and Evans 2014)	2.5 0.9-7.5	0.38 (8)	
4-MeO-AMT	*	0.8-6.4	1.4 (11)	
5-MeO-AMT	*	0.8-6.4	31 (4)	
1-Me-AMT	*	0.9-6.9	51 (3)	
2-Me-AMT	*	0.9-6.9	11 (7)	
5-Me-AMT	*	0.9-6.9	1.5 (13)	
7-Me-AMT	*	0.9-6.9	0.049 (2)	
5-F-AMT	*	0.8-6.8	0.45 (23)	376 (1)
6-F-AMT	*	0.8-6.8	1.8 (19)	126 (2)
5-Cl-AMT	*	0.8-6.2	0.25 (7)	82 (2)
5-Br-AMT	*	0.6-5.2	1.3 (7)	
5-F-2-Me-AMT	*	0.8-6.3	4.1 (7)	223 (3)
1-Me-2-Ph-AMT	*	0.6-4.9	166 (2)	
2-Ph-AMT	*	0.6-5.2	3.7 (18)	
Known inhibitors				
5-IT	15-590 (Backberg et al. 2014) 700-5,100 (PM) (EMCDDA 2014)	0.1-3.4 4.0-29	0.20 (1)	
Selegiline	0.3-1.5 (Barrett et al. 1996)	0.002-0.01		0.017 (2)
Harmine	36-222 (Baselt 2008) 1.0-15.6 (Oliveira et al. 2012)	0.2-1.0 0.005-0.07	0.006 (2)	

Harmaline	2.7-15.7 (Oliveira et al. 2012)	0.01-0.07	0.011 (2)
Yohimbine	3.7-171 (Baselt 2008)	0.01-0.5	6.0 (7)

* No plasma concentrations reported, thus concentrations estimated according to the structure-related compound AMT.

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Legends to the figures

Fig. 1 Chemical structures of tested alpha-methyltryptamines. Structural variations in comparison to AMT were given in red (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of the article).

Fig. 2 Deamination of kynuramine catalyzed by MAO-A or MAO-B providing an aldehyde, followed by non-enzymatic condensation to 4-hydroxyquinoline.

Fig. 3 Initial inhibition screening results using 10 μ 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).

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.

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).

Figure 1
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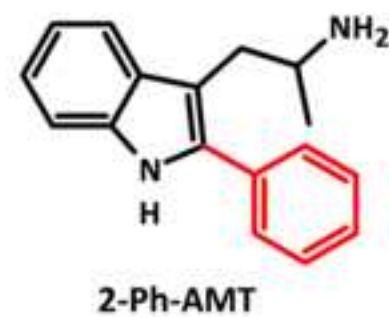
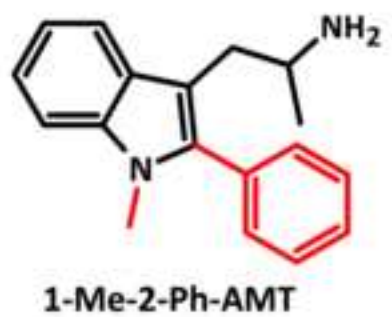
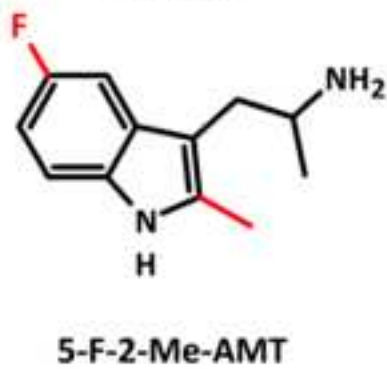
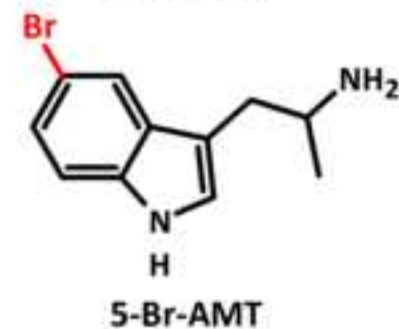
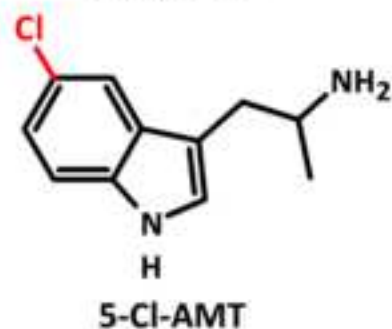
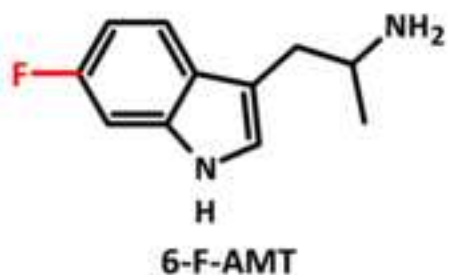
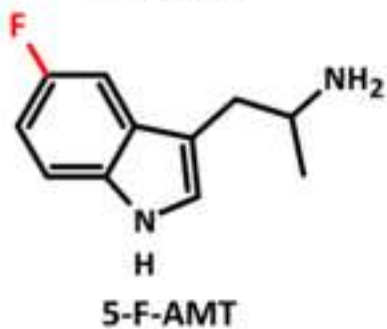
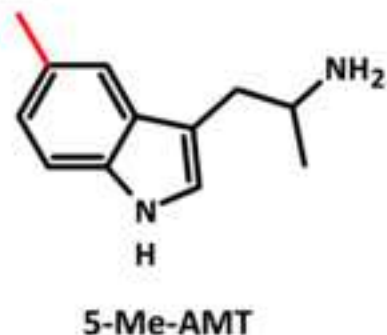
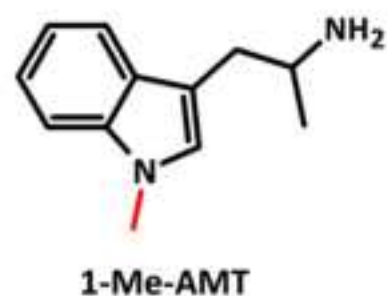
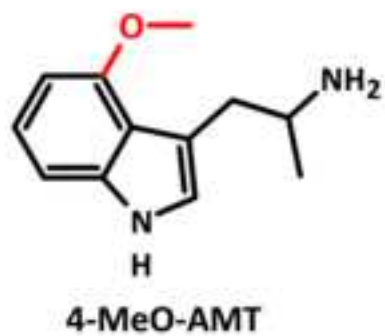
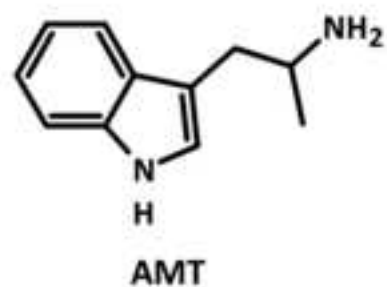


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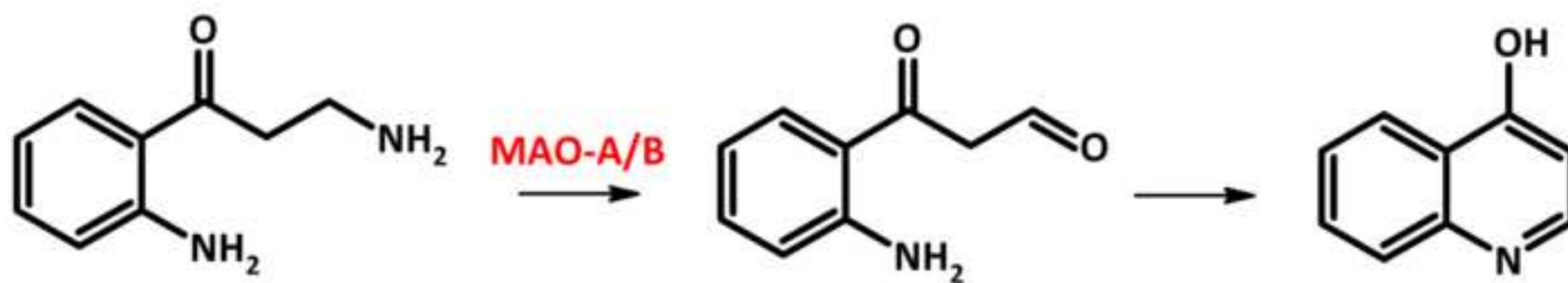


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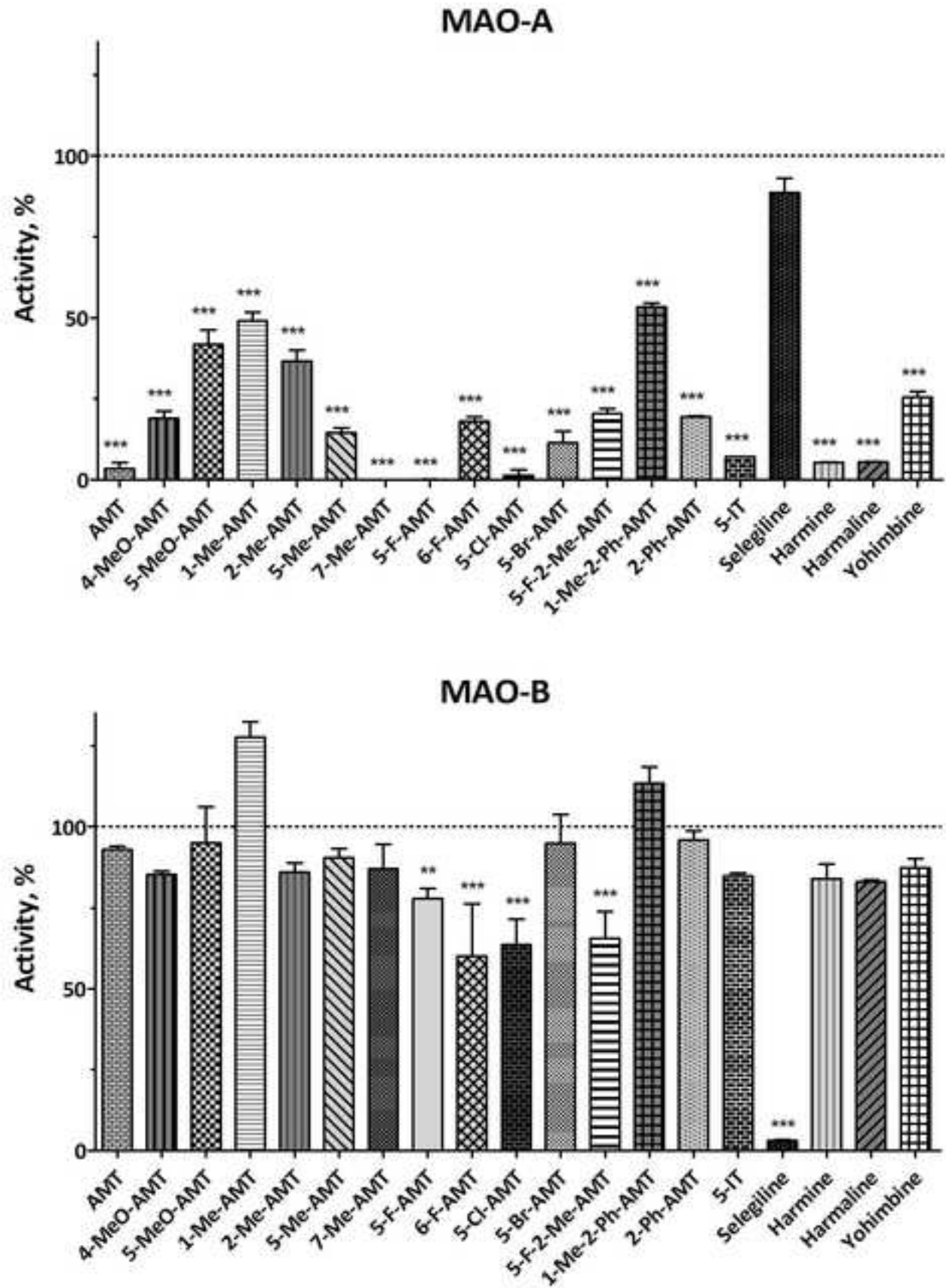


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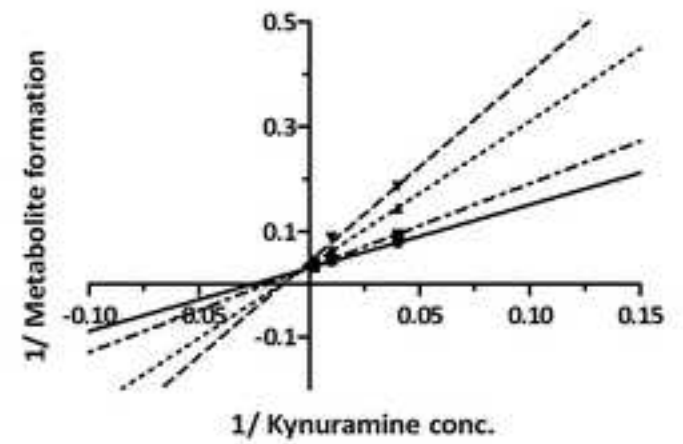
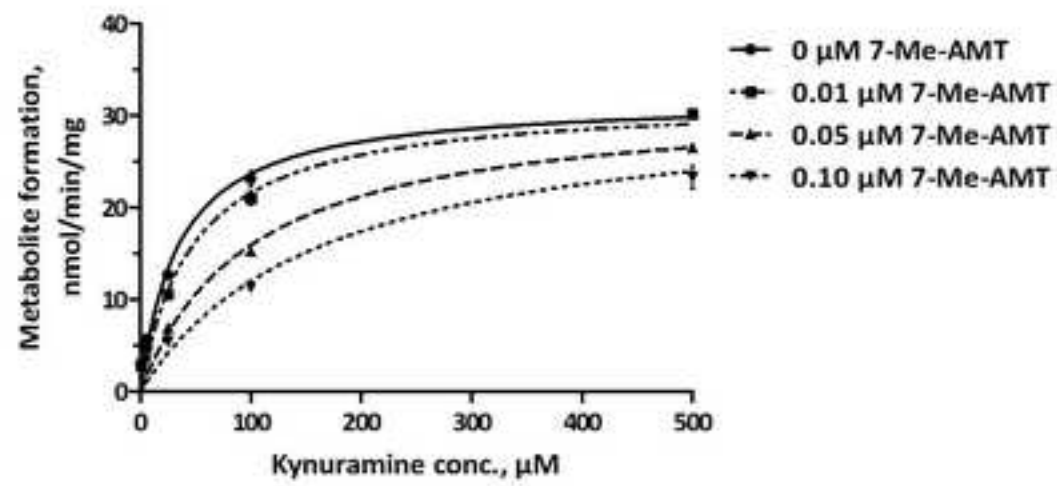


Figure 5
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