Interactions of phenethylamine-derived psychoactive substances of the 2C-series with human monoamine oxidases

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Abstract
Psychoactive substances of the 2C-series (2Cs) are phenethylamine-derived designer drugs that can induce psychostimulant and hallucinogenic effects. Chemically, the classic 2Cs contain two methoxy groups in positions 2 and 5 of the phenyl ring, whereas substances of the so-called FLY series contain rigidified methoxy groups integrated in a 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b’]difuran core. One of the pharmacological features that has not been investigated in detail is the inhibition of monoamine oxidase (MAO). Inhibition of this enzyme can cause elevated monoamine levels that have been associated with adverse events such as agitation, nausea, vomiting, tachycardia, hypertension, or seizures. The aim of this study was to extend the knowledge surrounding the potential of MAO inhibition for 17 test drugs, which consisted of 12 2Cs (2C-B, 2C-D, 2C-E, 2C-H, 2C-I, 2C-N, 2C-P, 2C-T-2, 2C-T-7, 2C-T-21, bk-2C-B, and bk-2C-I) and five FLY analogs (2C-B-FLY, 2C-E-FLY, 2C-EF-FLY, 2C-I-FLY, and 2C-T-7-FLY). The extent of MAO inhibition was assessed using an established in vitro procedure based on heterologously expressed enzymes and analysis by hydrophilic interaction liquid chromatography–high resolution tandem mass spectrometry. Thirteen test drugs showed inhibition potential for MAO-A and 11 showed inhibition of MAO-B. In cases where MAO-A IC50 values were determined, values ranged from 10 to 125 μM (7 drugs) and from 1.7 to 180 μM for MAO-B (9 drugs). In the absence of detailed clinical information on most test drugs, it is concluded that a pharmacological contribution of MAO inhibition cannot be excluded and that further studies are warranted.

KEYWORDS
drugs of abuse, HILIC–HRMS/MS, IC50 value, monoamine oxidase inhibition, phenethylamines

1 | INTRODUCTION

Year after year, more and more so-called new psychoactive substances (NPS) enter the drugs of abuse (DOA) market with 803 different substances being reported to the United Nations Office on Drugs and Crime in the period 2009–2017.1,2 Many NPS show psychoactive effects similar to drugs under international control and are often sold via the Internet.3 Although NPS are initially not controlled and easily available, they can pose a significant health risk and reports about adverse effects are frequently available.3-6 One of the challenges when dealing with this phenomenon is the lack of knowledge concerning pharmacokinetics and toxicity since they are marketed without (pre)clinical safety studies.7 The 2C-series drugs of abuse (2Cs) are phenethylamine derivatives that commonly exhibit a primary amine functionality separated from the phenyl ring by two carbon atoms. This differs from their amphetamine counterparts that show a
methyl group at the alpha-position (3Cs) (Figure 1). Within a 2,5-
dimethoxyphenethylamine nucleus, a lipophilic substituent is com-
monly present in position 4.8 In the so-called FLY series, the methoxy
groups are rigidified into a 2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b’]
difuran core.9 A number of 2Cs have shown psychostimulant and hal-
lucinogenic properties and information gathered from casework sug-
gests that intoxicated patients might exhibit either sympathomimetic
toxidrome, serotonin (5-HT) toxicity, hallucinogenesis, or combina-
tions thereof.10 The available information on the pharmacology of
2Cs indicates that the interaction with 5-HT receptor subtypes is
one of the pharmacological features linked to these substances.9,11-14
Partial agonism at alpha-1 adrenergic receptors has also been
described for 4-bromo-2,5-dimethoxyphenethylamine (2C-B).15 In
addition, the re-uptake inhibition of the monoamines norepinephrine
and 5-HT into rat brain synaptosomes was described for 4-
ethyl-2,5-dimethoxyphenethylamine (2C-E) and 4-ido-2,5-
dimethoxyphenethylamine (2C-I).16 At the same time, monoamine
oxidase (MAO) inhibition may also lead to elevated monoamine levels
that have been associated with adverse events described in cases of
2C intoxications, which included agitation, nausea, vomiting, tachycar-
dia, hypertension, or seizures.10,17-19

The aim of this study was to extend the knowledge surrounding
the potential of MAO inhibition (and to determine their IC50 values)
for 17 test drugs, which consisted of 12 2Cs and five FLY analogs
(Figure 1). A previously published MAO inhibition assay based on
heterologously expressed enzymes and hydrophilic interaction
liquid chromatography–high resolution tandem mass spectrometry
(HILIC–HRMS/MS) was applied for this purpose. MAO activity
should be assessed using kynuramine (KYN) as the non-selective substrate, as
the formation of the corresponding aldehyde is catalyzed by MAO-A
and B and followed by non-enzymatic condensation to the product
4-hydroxyquinoline (4-OHC).

2 EXPERIMENTAL

2.1 Chemicals and enzymes

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

Amphetamine-d5 was obtained from LGC Standards (Wesel,
Germany); selegiline, KYN, 4-OHC, ammonium acetate, potassium
dihydrogenphosphate, and dipotassium hydrogenphosphate 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-(2-Aminopropyl)indole (5-IT) was synthesized20 and provided by
the Department of Pharmacology and Therapeutics, Trinity Centre for
Health Sciences, St James’s Hospital (Dublin, Ireland). 2C-B tartrate
was provided for research purposes before scheduled by Hessisches
Landeskriminalamt (Wiesbaden, Germany); 2C-I hydrochloride by
Landeskriminalamt Baden-Württemberg (Stuttgart, Germany); 2C-E hydrochloride
by Bundeskriminalamt (Wiesbaden, Germany); and 4-nitro-2,5-
dimethoxyphenethylamine (2C-H) and 2,5-dimethoxy-4-nitrophene-
thylamine (2C-N) by the Department of Forensic Toxicology,
Institute of Forensic Research (Krakow, Poland). 5-IT, 2C-B, 2C-I, and
2C-T-2 were provided before they were scheduled. 4-Methyl-2,5-
dimethoxyphenethylamine (2C-D) hydrochloride, 2C-E hydrochloride,
and 4-propylthio-2,5-dimethoxyphenethylamine (2C-T-7) hydrochloride
were purchased from Lipomed AG (Weil am Rhein, Germany);
4-propyl-2,5-dimethoxyethamine (2C-P) hydrochloride from Dejachem (Schwendi, Germany); and 2-(8-bromo-2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b']difuran-4-yl)ethan-1-amine (2C-B-FLY) hydrochloride from Cayman Chemicals (Ann Arbor, MI, USA). 2-Amino-1-(4-bromo-2,5-dimethoxyphenyl)ethan-1-one (bk-2C-B) hydrochloride and 2-amino-1-(4-iodo-2,5-dimethoxyphenyl)ethan-1-one (bk-2C-I) were available from previous work. 21,22

Prior to the determinations of MAO activities, stock solutions or enzyme preparations were serially diluted using 100 mM phosphate buffer. Prior to the determinations of MAO activities, stock solutions or enzyme preparations were serially diluted using 100 mM phosphate buffer. Prior to the determinations of IC50 values, 2C stock solutions were gently evaporated under nitrogen and dissolved in water/methanol (9:1, v/v) to keep the organic solvent content in the final incubation mixtures constantly below 1%.23

2.2 Initial MAO inhibition screening

Final incubation mixtures had a volume of 30 μL and consisted of 1 μg/mL MAO-A or MAO-B, the non-selective MAO substrate KYN at concentrations comparable to its Km value (MAO-A: 43 μM, MAO-B: 23 μM), and 10 μM of one of the potential inhibitors as described before.24 Reactions were initiated by addition of the ice-cold enzyme dilution, incubated for 20 minutes at 37°C, and stopped with 30 μL of ice-cold acetonitrile containing 10 μM amphetamine-d5 as internal standard (IS). The mixture was centrifuged for 2 minutes at 10 000 g. 50 μL of the supernatant were transferred to an autosampler vial, and injected onto the HILIC-HRMS/MS apparatus for analysis. All incubations were performed in triplicate (n = 3). In addition to these samples, reference samples without inhibitor, positive control samples with known inhibitors (MAO-A: 5-IT, MAO-B: selegiline, 10 μM each), blank samples without MAO activity, and interfering samples were also prepared in triplicate. Interfering samples were incubated reference samples without inhibitor and terminated with ice-cold acetonitrile containing the IS and the test drugs each at a concentration of 10 μM. A simplified scheme of the initial inhibition screening procedure is given in Figure 2. The 4-OHC amount, given as the peak area ratio of 4-OHC and the IS, in blank samples without MAO activity was subtracted from the 4-OHC amount detected in all other samples. The 4-OHC amount detected in reference samples without inhibitor was set at 100% MAO activity and compared to all other incubations. For statistical analysis, 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 (GraphPad Software, San Diego, CA, USA) was used.

2.3 Determination of IC50 values

MAO-A or B were incubated with the following 10 inhibitor concentrations: 2, 5, 10, 20, 39, 78, 156, 313, 625, and 1250 μM, with the exception of 2C-H that was used in lower concentrations (0.04, 0.08, 0.15, 0.3, 0.6, 1, 2, 5, 10, and 20 μM). All incubations were performed in duplicate (n = 2). All other incubation conditions were the same as described in section 2.2. Reference and blank samples were also prepared as described in section 2.2. The IC50 values were calculated by plotting the 4-OHC formation expressed as MAO activity (relative to reference samples) over the logarithm of the inhibitor concentration using GraphPad Prism 5.00.

2.4 HILIC-HRMS/MS conditions

Apparatus and conditions were the same as already described.24 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 were used and controlled by TF Chromeleon software version 6.80. The chromatographic system was coupled to a TF Q-Exactive Plus equipped

![FIGURE 2](https://wileyonlinelibrary.com)
3 | RESULTS AND DISCUSSION

3.1 | Initial MAO inhibition screening

Formation of 4-OHC was measured using HILIC–HRMS/MS and the complete analytical procedure was already previously successfully validated. The results of the initial MAO inhibition screening of the 2Cs are summarized in Figure 3. Suitable incubation conditions were verified using positive control samples with known inhibitors (Figure 2). 5-IT reduced MAO-A activity by more than 90%, while selegiline almost completely inhibited MAO-B activity, which was in agreement with a previous study. As only 4-OHC, KYN, and the IS were detected by the analytical method, it was mandatory to exclude analytical interferences, such as ion suppression or enhancement caused by potentially co-eluting test drugs. Therefore, MAO activity determined from the interfering samples was compared to that in the reference samples. The test compounds caused no analytical interferences.

For 13 test drugs, MAO-A inhibition was observed to some degree. Seven of these drugs (2C-B, 2C-I, 2C-T-7, 2C-B-FLY, 2C-EFLY, 2C-I-FLY, and 2C-T-7-FLY) provided a statistically significant reduction of MAO-A activity (***, P < 0.001). However, none of these drugs was able to reduce the MAO-A activity by more than 50%, which meant that IC50 values could not be determined below 10 μM, as this was the concentration used in the initial inhibition screening. Eleven test drugs resulted in some MAO-B inhibitions and nine substances (2C-B, 2C-D, 2C-E, 2C-H, 2C-L, 2C-N, 2C-T-7, bk-2C-B, and bk-2C-L) induced a reduction in MAO-B activity that was highly significant (***, P < 0.001). However, only 2C-H reduced MAO-B activity by more than 50%, which resulted in a corresponding IC50 value below 10 μM (Table 1).

3.2 | Determination and comparison of IC50 values

To save time and costs, IC50 values for MAO-A and B inhibition were only determined in cases where the test drugs revealed a statistically highly significant (***, P < 0.001) inhibition during the initial screening phase. All incubations for the IC50 value determinations were performed in duplicate, in contrast to triplicate incubations during the initial MAO inhibition screening, to reduce workload and number of samples in accordance with the previous study. In total, seven IC50
values of MAO-A inhibitors and nine IC₅₀ values of MAO-B inhibitors were determined and listed in Table 1. For MAO-A inhibition, IC₅₀ values were determined between 10 (2C-T-7-FLY) and 125 μM (2C-B and 2C-I). For MAO-B inhibition, IC₅₀ values were found to range between 1.7 (2C-H) and 180 μM (2C-T-7).

As far as the potential clinical relevance of MAO inhibition based on IC₅₀ values was concerned, plasma concentrations obtained from the case report literature (Table 1) might be worthy of consideration even though only limited information is available. Usually, case reports involving fatal or non-fatal intoxications are the only information source and difficult to interpret due to individual variation and/or poly-drug intoxication. In those reports, dosage, time of ingestion, and route of application remain often unclear. Postmortem concentrations are particularly problematic as they can be affected by postmortem redistribution. Although the presence of some 2Cs on the DOA market is documented, few case reports involving drug consumption appear to be available. For example, plasma concentrations from individual patients have only been reported for 2C-P and 2C-T-7. In the first case, a 19-year-old male ingested approximately 25 mg 2C-P, which was sold as 2C-B, and was admitted to the emergency department with severe hallucinations, mydriasis, tachycardia, agitation, and confusion. In the second case, a 20-year-old male died after he had been insufflating approximately 35 mg of 2C-T-7 and quantification was subsequently performed in postmortem samples. The IC₅₀ values determined in this study were higher than the plasma concentrations reported for 2C-P and 2C-T-7. However, as they were measured in individual cases, their significance remains unclear.

Shulgin and Shulgin published their experiences with numerous phenethylamines, which included information about synthesis, dosage, and duration of effects. These data are available for all 2Cs tested in this study with exception of the two bk-2Cs and FLY analogs. For some compounds, such as 2C-D or 2C-N, higher doses were described than those estimated in the previously mentioned case reports. Consequently, higher doses are expected to lead to higher plasma concentrations. However, it must be considered that concentrations detectable in certain tissues are often higher than in plasma due to lipophilicity or active transport processes. A brain-to-plasma concentration ratio of 13.9 was described for 2C-B in rats. Elevated concentrations in the liver, the main metabolizing organ, are also more likely to be encountered. From this perspective, a contribution to the clinical effects of MAO inhibition can perhaps not be excluded.

2C-B, 2C-I, and 2C-T-7 were identified as moderately potent MAO-A inhibitors with IC₅₀ values of 46 (2C-T-7) and 125 μM (2C-B and 2C-I). However, the corresponding FLYs were shown to be more potent MAO-A inhibitors with IC₅₀ values of 10 (2C-T-7-FLY), 13 (2C-I-FLY), and 19 μM (2C-B-FLY). As the deamination of 5-HT is predominantly catalyzed by MAO-A, its inhibition is likely to contribute to increasing 5-HT levels, which could be clinically relevant. For example, in the case of 2C-I intoxication, 5-HT toxicity has been observed as a clinical feature. The MAO-A activity related to different 2C-B or 2C-B-FLY concentrations is depicted in Figure 4. 2C-E-FLY provided an IC₅₀ value of 18 μM comparable to the above-mentioned FLYs, whereas 2C-EFLY showed only weak MAO-A inhibition during the initial inhibition screening procedure.

### Table 1

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>Reference Plasma Concentration, μM</th>
<th>IC₅₀ Value, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MAO-A</td>
</tr>
<tr>
<td>2C-B</td>
<td>*</td>
<td>125 (1.1)</td>
</tr>
<tr>
<td>2C-D</td>
<td>*</td>
<td>n.d.</td>
</tr>
<tr>
<td>2C-E</td>
<td>*</td>
<td>n.d.</td>
</tr>
<tr>
<td>2C-H</td>
<td>*</td>
<td>n.d.</td>
</tr>
<tr>
<td>2C-I</td>
<td>*</td>
<td>125 (1.2)</td>
</tr>
<tr>
<td>2C-N</td>
<td>*</td>
<td>n.d.</td>
</tr>
<tr>
<td>2C-P</td>
<td>18¹⁷</td>
<td>0.1</td>
</tr>
<tr>
<td>2C-T-2</td>
<td>*</td>
<td>n.d.</td>
</tr>
<tr>
<td>2C-T-7</td>
<td>57 and 100 (heart and femoral blood, PM)¹⁹</td>
<td>0.2 and 0.4</td>
</tr>
<tr>
<td>2C-T-21</td>
<td>*</td>
<td>n.d.</td>
</tr>
<tr>
<td>bk-2C-B</td>
<td>*</td>
<td>n.d.</td>
</tr>
<tr>
<td>bk-2C-I</td>
<td>*</td>
<td>n.d.</td>
</tr>
<tr>
<td>2C-B-FLY</td>
<td>*</td>
<td>19 (1.1)</td>
</tr>
<tr>
<td>2C-E-FLY</td>
<td>*</td>
<td>18 (1.1)</td>
</tr>
<tr>
<td>2C-EF-FLY</td>
<td>*</td>
<td>n.d.</td>
</tr>
<tr>
<td>2C-I-FLY</td>
<td>*</td>
<td>13 (1.1)</td>
</tr>
<tr>
<td>2C-T-7-FLY</td>
<td>*</td>
<td>10 (1.1)</td>
</tr>
<tr>
<td>Known inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-IT</td>
<td>15–590²⁴ 700–5,100 (PM)²⁵</td>
<td>0.1–3.4</td>
</tr>
<tr>
<td>Selegiline</td>
<td>0.3–1.5²⁶</td>
<td>0.002–0.01</td>
</tr>
</tbody>
</table>

¹No plasma concentrations reported.

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2C-B and 2C-T-7 were measured in individual cases, their significance remains unclear.
The FLYs were found to be inactive as MAO-B inhibitors with the exception of 2C-E-FLY, which resulted in weak MAO-B inhibition during the initial inhibition screening. In contrast, almost all classic 2Cs (apart from 2C-T-21), and the two bk-2Cs showed MAO-B inhibition potential. While 2C-B and 2C-I moderately inhibited MAO-B with IC\textsubscript{50} values of 58 and 55 \(\mu\text{M}\), respectively, the inhibition by bk-2C-B and bk-2C-I was found to be more potent resulting in IC\textsubscript{50} values of 14 and 15 \(\mu\text{M}\), respectively. These findings revealed that the replacement of bromine by iodine did not have a strong impact on MAO-A or B inhibition. The MAO-B activity related to different 2C-I or bk-2C-I concentrations is depicted in Figure 5.

2C-H was shown to be the most potent MAO-B inhibitor with an IC\textsubscript{50} value of 1.7 \(\mu\text{M}\). However, Shulgin and Shulgin reported 2C-H to only result in weak psychoactive effects in animal assays.\textsuperscript{8} In comparison, 2C-D and 2C-E revealed higher IC\textsubscript{50} values of 24 and 124 \(\mu\text{M}\), respectively, whereas 2C-P only provided weak MAO-B inhibition potential during the initial inhibition screening. The MAO-B inhibitory potency of 2C-N (IC\textsubscript{50} value 66 \(\mu\text{M}\)) was comparable to that of 2C-B or 2C-I.

A previous study identified the MAO and cytochrome P450 isoforms involved in the deamination of 2C-B, 2C-D, 2C-E, 2C-I, 2C-T-2, and 2C-T-7.\textsuperscript{33} MAO-A and B were shown to be the predominant enzymes involved in formation of their aldehydes and the studied 2Cs had a higher affinity to MAO-A than B.\textsuperscript{33} As the present study identified these compounds with exception of 2C-T-2 as inhibitors of MAO-B, a competitive inhibition mechanism might be possible.

Nevertheless, as some 2Cs and 2C-B-FLY were described to have affinities to 5-HT receptor subtypes within the nM range,\textsuperscript{9,11-14} MAO IC\textsubscript{50} values within the \(\mu\text{M}\) range are expected to play only a minor role in the pharmacological effects after consumption of recreational doses. However, a contribution of MAO inhibition to the clinical effects observed in intoxication cases cannot be excluded.

4 | CONCLUSIONS

The presented study identified various 2C- and FLY-related test drugs as MAO inhibitors. A previously published inhibition assay was successfully applied for initial inhibition screening followed by IC\textsubscript{50} value determinations. The FLYs were identified as MAO-A inhibitors, whereas the classic 2Cs with or without beta-keto functionality exhibited MAO-B inhibition potential. 2C-T-7-FLY and 2C-H were identified as the most potent MAO-A or B inhibitors with IC\textsubscript{50} values of 10 and 1.7 \(\mu\text{M}\), respectively. Estimation of the clinical relevance of MAO inhibition based on IC\textsubscript{50} values, however, was challenging given the lack of information regarding plasma concentrations of these test drugs. Nevertheless, the extent to which the clinical pharmacology of the evaluated test drugs involves MAO inhibition warrants further investigation.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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