Study examining the inhibition of human monoamine oxidase (MAO) by the new psychoactive substance 5-(2-aminopropyl)indole ('5-IT')

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1. Introduction

Following an examination of the available information on 5-(2-aminopropyl)indole (5-IT) ([1]), on 3 October 2012 the EMCDDA and Europol launched a Joint Report on the substance. It was presented to the Council of the European Union, the European Commission and the European Medicines Agency on 12 December 2012 ([2,3]).

Consequently, a Technical report will be prepared as a matter of priority within a tight deadline in order to be available for the risk assessment as requested by the Council of the European Union. As identified in the Joint report, there is a lack of information on the pharmacology and toxicology of 5-(2-aminopropyl)indole. However, a single study published in the 1960s indicated that the substance may act as an inhibitor of monoamine oxidase (Cerletti et al., 1968). In some of the non-fatal intoxications and deaths associated with 5-(2-aminopropyl)indole that have been reported, symptoms typical of monoaminergic toxicity have been noted. These include hyperthermia along with dilated pupils, sweating, increased heart rate, high blood pressure, agitation, restlessness, disorientation and anxiety. The purpose of the contract therefore is to conduct *in vitro* studies on 5-(2-aminopropyl)indole to investigate its effects on monoamine oxidase in order to inform the risk assessment.

2. Objectives of the study

Experimental work was conducted to determine and evaluate the effect of 5-(2-aminopropyl)indole (5-IT) on the human monoamine oxidase (MAO) enzyme. *In vitro* assays included the use of recombinant MAO enzymes and kynuramine as substrate. Following incubation, the assays were analysed using previously validated methods based on the analysis of the reaction products by HPLC coupled to Diode Array Detector (DAD) and fluorescence detection. The two isoenzymes MAO-A and -B were considered. The inhibition parameters, IC_{50} (concentration of inhibitor that produce 50 % inhibition) and K_i (dissociation constant of enzyme and inhibitor), were determined using different concentrations of substrate and inhibitor and appropriate equations. Inhibition of MAO-A by known inhibitors was also evaluated and the inhibition

([1]) For the purpose of this report the abbreviation '5-IT' is used interchangeably with '5-(2-aminopropyl)indole'.
([2]) OJ L 127, 20.5.2005, p. 32.
([3]) EMCDDA and Europol. (2012), *EMCDDA–Europol Joint Report on a new psychoactive substance: 5-(2-aminopropyl)indole*, EMCDDA, Lisbon.

parameters (IC_{50}) determined. In addition, experimental work was undertaken on the mode of binding of 5-IT, its mechanism of inhibition and selectivity.

3. Experimental

Recombinant human monoamine oxidase-A and B were obtained from Gentest BD biosciences (Woburn, MA, USA). Kynuramine dihydrobromide, 4-hydroxyquinoline, harmaline, clorgyline, *R*-deprenyl, toloxatone (5-(hydroxymethyl)-3-(3-methylphenyl)-2-oxazolidinone), and moclobemide (4-chloro-*N*-[2-(4-morpholinyl)ethyl]benzamide) were purchased from Sigma-Aldrich. 5-IT hemisuccinate (5-API hemisuccinate; 5-(2-aminopropyl)indole hemisuccinate) was purchased from LGC standards. All test compounds were dissolved in milli-q ultrapure water and diluted appropriately in 100 mM phosphate buffer pH 7.4.

3.1. Monoamine oxidase assays (MAO-A and B)

The enzymatic activity of human MAO isozymes was studied using protein fractions containing this enzyme by using kynuramine as a non-selective substrate (Herraiz and Chaparro, 2006). The reaction velocity *v* (μM/min) was obtained from oxidative deamination of kynuramine to form under the conditions of the assay 4-hydroxyquinoline that was analysed by HPLC-DAD and its concentration calculated from a calibration curve of peak area (λ at 320 nm) against concentration (Herraiz and Caparro, 2006). To carry out the assay, protein fractions containing MAO-A or B were diluted to the desired concentrations in 100 mM potassium phosphate buffer (pH 7.4). A 0.2 ml reaction mixture containing 0.01–0.02 mg/ml protein and 0.25 mM kynuramine in 75 mM potassium phosphate (pH 7.4) was incubated at 37 ºC for 40 min. After incubation the reaction was stopped by the addition of 2N NaOH (75 μl), followed by the addition of 70 % perchloric acid (25 μl), and the sample centrifuged (10000 x g) for 6 min. An aliquot of the supernatant (20 μl) was injected into the HPLC and the deamination products of kynuramine formed during the enzymatic reaction determined by RP-HPLC-DAD and fluorescence detection.

3.2. MAO-A and -B inhibition studies with 5-IT and other inhibitors

MAO-A or -B enzymes were incubated as above with kynuramine as a substrate and added with increasing concentrations of 5-IT or other inhibitors of MAO-A and B at the desired concentration in phosphate buffer (pH 7.4), and incubated at 37 ºC for 40 min, as above. IC_{50} values (concentration of inhibitor producing 50 per cent inhibition of enzymatic activity) were calculated by fitting (% inhibition vs. concentration of inhibitor) to non-linear regression curves or by linear regression of inhibition (%) against the log of substrate concentration. Assays were carried out at least in duplicate.

Kinetic constant of Michaelis-Menten (K_m) and the maximum velocity (V_{max}) were obtained from nonlinear regression analysis (velocity vs. concentration) using different concentrations of substrate. The mechanism of MAO inhibition by 5-IT was assessed experimentally by obtaining the corresponding double reciprocal Lineweaver-Burk plots of the enzyme activity at different concentrations of substrate and inhibitor. The secondary plot of the slope from the double reciprocal curves versus the concentration

of inhibitor was used to calculate K_i (inhibition constant) values. Kinetic assays were carried out at least in duplicate.

To determine the type of binding of 5-IT to MAO-A (i.e. reversible or irreversible inhibition), MAO-A (0.025 mg/ml protein) in 100 mM phosphate buffer (pH 7.4) was incubated at 37 ºC for 30 min with or without (control) 5-IT (100 µM), and then centrifuged (15000 x g, 15 min) to pellet the protein, that was washed with 100 mM phosphate buffer and the procedure repeated three times. Finally, the enzyme was resuspended in phosphate buffer and used to measure MAO activity, as above. Assays were carried out at least in duplicate.

3.3. Chromatographic analysis by RP-HPLC

The analysis of the kynuramine deamination product 4-hydroxyquinoline was accomplished by RP-HPLC-DAD and fluorescence detection using an HPLC 1050 (Hewlett Packard) provided with a 1100 DAD (Agilent) and a 1046A-fluorescence detector (Hewlett Packard). A 150 mm x 3.9 mm, 4 µm, Nova-pak C18 column (Waters, Milford, MA, USA) was used for separation. Chromatographic conditions were: 50 mM ammonium phosphate buffer (pH 3) (buffer A) and 20% of A in acetonitrile (buffer B). Gradient programmed from 0 % (100 % A) to 32 % B in 8 min and then 90 % B at 10 min. The flow rate was 1 ml/min, the column temperature was 40 ºC and the injection volume was 20 µl.

4. Results

A) In vitro inhibition of human MAO-A by 5-IT

Enzymatic reactions were carried out at different concentrations of substrate and the results of the enzyme activity against the concentration of substrate fitted to nonlinear regression analysis to plot Michaelis-Menten curves (Figure 1). The reaction velocity *v* (µM/min) was obtained from the deamination of kynuramine by MAO-A to form 4-hydroxyquinoline. In that way, calculated values of V_{max} of 0.8 ± 0.02 µM/min and K_m of 61.88 ± 4.6 µM were obtained for MAO-A and kynuramine. Subsequently, inhibition of human MAO-A by the substance 5-(2-aminopropyl)indole (5-IT) was studied in presence of kynuramine as the substrate (250 µM). The profile is shown in Figure 2, and it clearly suggests that 5-IT is an *in vitro* inhibitor of the human MAO-A with an IC_{50} value of 1.6 ± 0.1 µM.

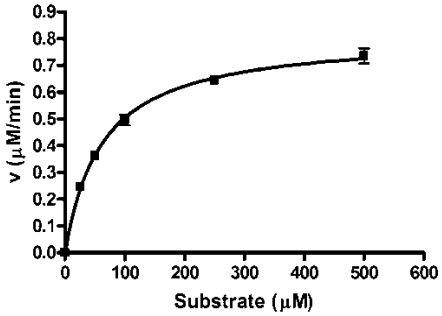

Figure 1. Michaelis-Menten curve of kynuramine deamination to form 4-hydroxyquinole by MAO-A (0.01 mg/ml MAO-A protein and kynuramine in 0.2 ml phosphate buffer. 37 ºC, 40 min).

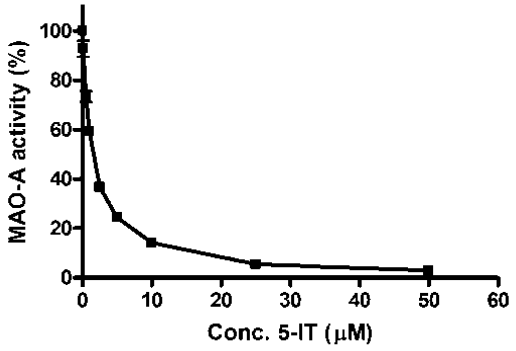

Figure 2. Inhibition of human MAO-A in presence of 5-IT

B) In vitro inhibition of human MAO-B by 5-IT

Inhibition of human MAO-B by the substance 5-IT was studied in the presence of kynuramine as substrate (250 µM). The profile obtained is shown in Figure 3 and clearly shows that 5-IT was devoid of activity as an inhibitor of recombinant human MAO-B. Indeed, in the range used (0–500 µM, 5-IT) no inhibition of MAO-B was detected. Instead, under the same conditions, *R*-deprenyl, a selective inhibitor of MAO-B, highly inhibited this enzyme at sub-micromolar concentrations (Figure 4). As a result of these data, no further studies were carried out with MAO-B isoenzyme and 5-IT.

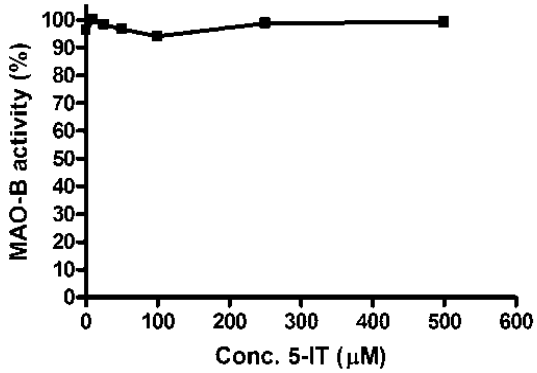

Figure 3. Activity of human MAO-B in presence of 5-IT.

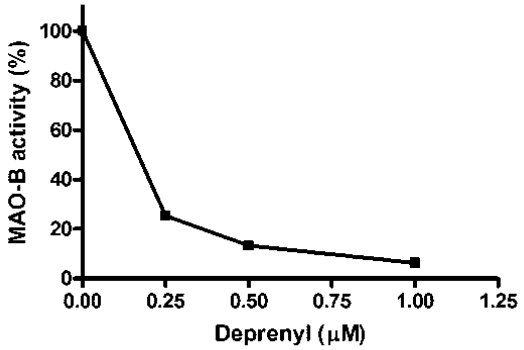

Figure 4. Inhibition of human MAO-B by (*R*)-deprenyl.

C) Kinetic study of human MAO-A inhibition and determination of K_i (inhibition constant) values

As 5-IT was an inhibitor of human MAO-A, kinetic assays corresponding to the activity of this enzyme in the presence of increasing concentrations of 5-IT (0–2 μM) were accomplished by using various concentration of substrate. The corresponding double reciprocal curves (i.e. Lineweaver-Burk plots) were obtained experimentally and are given in Figure 5, showing that 5-IT is a competitive inhibitor of MAO-A. Thus, in the presence of increasing concentrations of 5-IT, the enzyme had similar V_{max} (velocity in high concentrations of substrate) whereas K_m increased with the concentration of inhibitor. The inhibition constant K_i (dissociation constant of the enzyme-inhibitor complex) was calculated from a secondary plot of the slopes of curves in Figure 5 against the concentration of inhibitor giving a value of K_i of 0.25 μM (Figure 6) (intercept on the x-axis).

On the other hand, as the inhibition of 5-IT over MAO-A is competitive and the inhibitor is reversible (see below), the equation of Cheng-Prusoff (i.e. $IC_{50} = K_i (1 + S/K_m)$ could be used to determine K_i from the IC_{50} and K_m values (Cheng and Prusoff, 1973). The K_i obtained was 0.32 μM which is in good agreement with the one calculated experimentally from the double reciprocal curves and secondary plot (Figures 5 and 6).

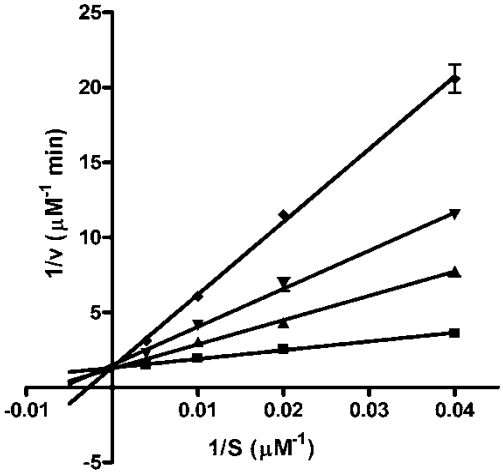

Figure 5. Lineweaver-Burk plot of the MAO-A enzymatic reaction in presence of increased concentrations of 5-IT and kynuramine used as substrate. Control without 5-IT (■); 0.5 µM 5-IT (▲); 1 µM 5-IT (▼); 2 µM 5-IT (♦).

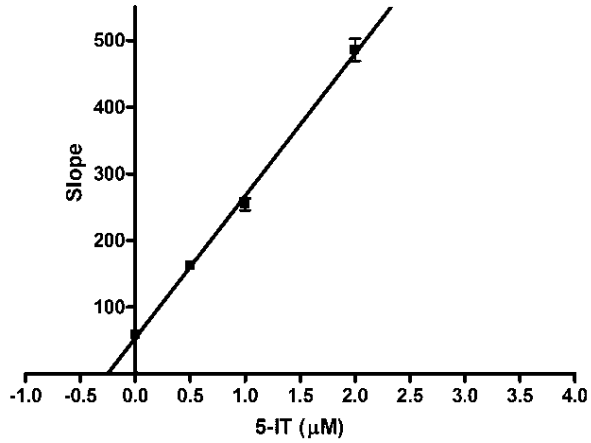

Figure 6. Secondary plot of slope of the curves of Lineweaver-Burk plot against the concentration of 5-IT as an inhibitor that was used to calculate K_i.

D) Type of binding (reversibility) of 5-IT with MAO-A

Following incubation of the enzyme MAO-A with 5-IT, and after washing to completely remove the 5-IT, the activity of the enzyme was fully recovered when compared with a control incubated in absence of 5-IT. Therefore, 5-IT binds to MAO-A under a reversible type of binding (Figure 7).

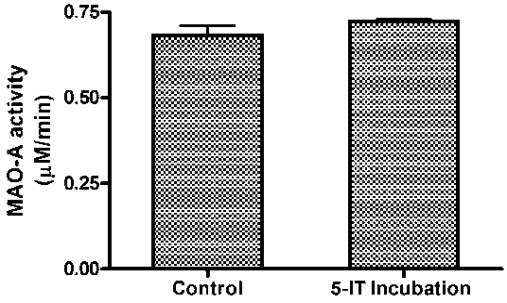

Figure 7. Human MAO-A (0.025 mg/ml, protein conc.) was incubated in duplicate with 0 or 100 µM 5-IT at 37 ºC, 30 min. The 5-IT removed through successive washing and centrifugation and then enzymatic assay was conducted.

E) Inhibition of MAO-A by substances used as reference and established inhibitors

Experimental data of the substance 5-IT as an inhibitor of MAO-A were compared with data obtained from other known inhibitors. For that, several substances including established inhibitors of MAO-A were studied under the experimental conditions used here. Clorgyline, a well-known irreversible inhibitor of MAO-A, demonstrated strong

inhibition of human MAO-A with an IC_{50} under the experimental conditions used of 16 ± 2.6 nM (Figure 8). Similarly, the β-carboline harmaline, that is a reversible and potent inhibitor of MAO-A provided an IC_{50} of 20 nM (Figure 9).

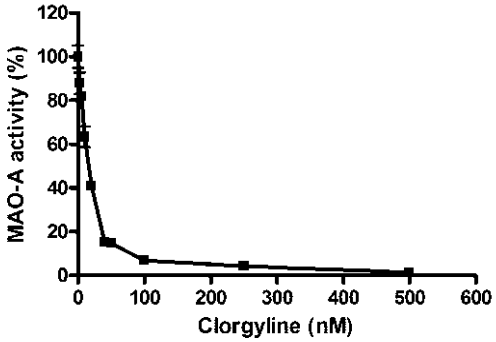

Figure 8. Inhibition of MAO-A by clorgyline.

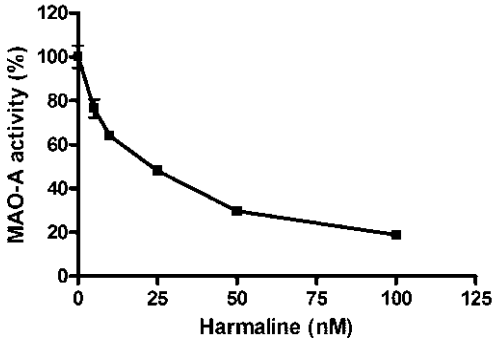

Figure 9. Inhibition of MAO-A by harmaline.

Toloxatone (Humoryl), an antidepressant launched in 1984 for the treatment of depression and which acts as a selective reversible inhibitor of MAO-A (Berlin et al.,1990) gave an IC_{50} of 6.71 ± 0.42 µM (Figure 10). Finally, moclobemide, another antidepressant that is authorised as a medicinal product in some countries and also a selective reversible MAO-A inhibitor, gave a poor inhibition of MAO-A *in vitro* with a IC_{50} value higher than 500 µM in our assay system (Figure 11). Results obtained with moclobemide suggest that it may be a poor inhibitor of MAO-A *in vitro* although a good inhibitor of MAO-A *in vivo*, probably resulting from a metabolite of the substance (Kettler et al., 1990; Fritze et al., 1989).

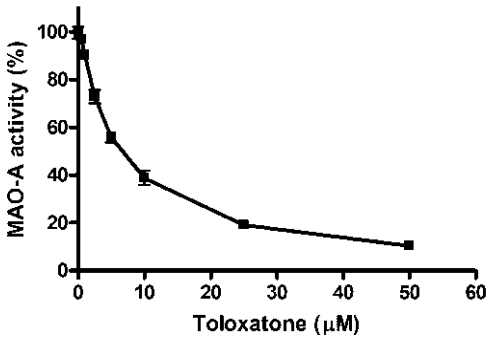

Figure 10. Inhibition of MAO-A by toloxatone.

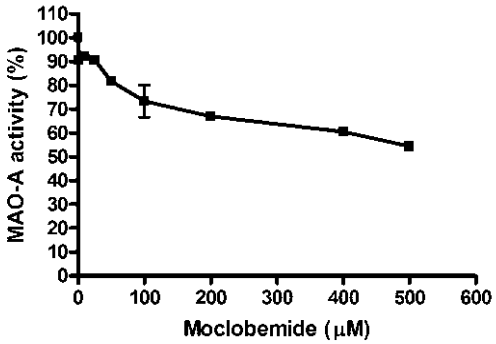

Figure 11. Inhibition of MAO-A by moclobemide.

According to the Cheng-Prusoff equation, the K_i obtained for the mentioned compounds were 0.016 µM (clorgyline), 0.004 µM (harmaline), and 1.34 µM (toloxatone), respectively. Results obtained for harmaline and toloxatone were in good agreement with previous reports (Herraiz et al., 2010; Strolin-Benedetti et al., 1983).

Table 1. MAO-A inhibition values of compounds.

Compound	**IC_{50} (µM)**	**K_i (µM)**	**K_i (µM) from IC_{50}**
5-(2-Aminopropyl)indole (5-IT)	1.6	0.25	0.32
Clorgyline	0.016	-	0.016
Harmaline	0.020	-	0.004
Toloxatone	6.7	-	1.3
Moclobemide	>500	-	-

5. Discussion

The results indicate that 5-IT is an inhibitor of human MAO-A (K_i of 0.25 μM) (Table 1). Nevertheless, this value suggests that its potency as a MAO-A inhibitor is at least ten times lower than that of clorgyline (i.e. K_i 0.016 μM) that is an irreversible inhibitor of this enzyme. It is also lower than that of the β-carboline harmaline that is also a potent reversible inhibitor of MAO-A (IC_{50} of 1.6 μM for 5-IT vs. 0.020 μM for harmaline). However, 5-IT was apparently a more potent inhibitor *in vitro* than toloxatone and moclobemide. It is known that MAO-A inhibitors and antidepressants working as MAO inhibitors result in an increase of serotonin levels *in vivo*. It cannot be ruled out that recreational use of 5-IT could elevate serotonin levels by itself or in combination with other substances.

These results indicate that 5-IT is a highly selective inhibitor of MAO-A, which is a property also shared by α-methyltryptamine (Tipton et al., 1982). Although the 5-IT structure contains a phenethylamine moiety it did not inhibit MAO-B. The ability of 5-IT to potentiate the hypertensive effects ('cheese effect') related to consumption of tyramine containing-foods cannot be fully excluded (Finberg and Gillman, 2011).

In a previous report, Cerletti et al., (1968) studied the inhibition of MAO by 5-IT and its positional isomers. These authors reported a value of IC_{50} of 22 μM for 5-IT which was higher (i.e. less potent as an inhibitor) than the value reported here. Those differences might be attributed to the different assays as well as enzyme sources and fractions used. The assay of Cerletti et al., was based on the ability of guinea pig liver homogenate to uptake oxygen in an assay that used serotonin as the substrate of MAO, whereas recombinant human MAO enzymes and kynuramine deamination were used in the current study. In the same study, Cerletti et al., (1968) reported an antagonist effect of pentylenetetrazole/reserpine (an antihypertensive drug) that might also be related to MAO inhibition. Previous results of Cerletti et al., (1968) and those reported here point to 5-IT as a selective inhibitor of MAO-A in the low micromolar range. Those results suggest that 5-IT by itself or in combination with other substances could potentiate serotonergic effects. However, further pharmacological and *in vivo* studies are needed to clarify this action and its relevance to the toxicological effects of 5-IT

6. Conclusions

The results from this study lead to the following conclusions concerning 5-(2-aminopropyl)indole (5-IT):

1) 5-IT is an inhibitor of MAO-A with an IC_{50} of 1.6 μM and K_i of 0.25 μM.
2) 5-IT is a reversible inhibitor of MAO-A.
3) 5-IT is a competitive inhibitor of MAO-A.
4) 5-IT is a highly selective inhibitor of MAO-A, and does not inhibit MAO-B.
5) Under the experimental conditions used here, other established inhibitors of MAO-A and antidepressants provided the following IC_{50} values: clorgyline 0.016 μM, harmaline 0.020 μM, toloxatone 6.7 μM and moclobemide > 500 μM. In other words,

5-IT was less potent than clorgyline and harmaline and more potent than toloxatone and moclobemide.

In summary, 5-IT is a relatively potent, reversible and selective inhibitor of MAO-A *in vitro*. In this regard it might increase monoamine levels, particularly serotonin. However, in order to clarify the significance of these results, further pharmacological and *in vivo* studies are needed to demonstrate MAO-A inhibition *in vivo* as well as an increase of serotonin and potential monoaminergic toxicity due to the use of 5-IT–containing drugs.

7. References

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