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(+)-4-DIMETHYLAMINO-2,α-DIMETHYLPHENETHYLAMINE (FLA 336(+)), A SELECTIVE INHIBITOR OF THE A FORM OF MONOAMINE OXIDASE IN THE RAT BRAIN

ANNA-LENA ASK, KERSTIN HÖGBERG, LENNART SCHMIDT, HANS KIESSLING and SVANTE B. ROSS

Research and Development Laboratories, Astra Läkemedel AB, S-151 85 Södertälje, Sweden

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Abstract—(+)-4-Dimethylamino-2,α-dimethylphenethylamine (FLA 336(+)) and its *N*-demethylated secondary amino derivative FLA 788(+) were examined for their monoamine oxidase (MAO) inhibitory effects in the rat brain. They were found to be reversible and very selective inhibitors of the A form of monoamine oxidase *in vitro* and *in vivo* after oral administration. FLA 788(+) was 2–6 times more active than FLA 336(+) *in vitro* depending on the assay technique employed but the two compounds had similar potency after oral administration. Both compounds inhibited competitively the deamination of 5-hydroxytryptamine by hypothalamic mitochondria. Although the irreversible inhibitor clorgyline was 60 times more potent than FLA 336(+) *in vitro*, it was equipotent with FLA 336(+) and FLA 788(+) in the rat brain after oral administration. There was a high correlation between the log plasma concentration of FLA 788(+) and the MAO inhibition in hypothalamic slices. The plasma concentration of the metabolite FLA 788(+) exceeded that of FLA 336(+) after oral administration of the latter compound. Thus, the MAO inhibition produced by FLA 336(+) *in vivo*, appears in part to be due to the metabolite FLA 788(+).

Monoamine oxidase [MAO; amine: oxygen oxidoreductase (deaminating, flavin containing), EC 1.4.3.4], which is involved in the biochemical regulation of the neurotransmitting monoamines noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT, serotonin) in the brain, exists in two forms, MAO-A and MAO-B, with different substrate and inhibitor specificities [1–4]. Since NA and 5-HT are selective substrates for MAO-A, this enzyme form is very important in the regulation of the neuronal concentrations of these transmitters. Thus, selective inhibitors of MAO-A cause elevation of the concentrations of NA and 5-HT in the rat brain [5, 6]. Lipper *et al.* [7] recently showed that clorgyline, an irreversible MAO-A inhibitor [1], had antidepressant action, whereas pargyline, which is predominantly a MAO-B inhibitor, had a weaker effect. This finding supports the hypothesis that inhibition of the A form is essential for the antidepressant effect of MAO inhibitors.

In our search for selective and reversible inhibitors of the A form of MAO we found that some 4-dimethylamino-α-methylphenethylamine derivatives are potent and selective inhibitors of MAO-A following injection to mice [8]. Some of these compounds were further studied in rats [6]. Of these compounds 4-dimethylamino-2,α-dimethylphenethylamine (FLA 336) was almost as potent as clorgyline in inhibiting MAO-A in the rat brain after oral administration and elevated the concentration of 5-hydroxytryptamine even at lower doses than did clorgyline [6]. Since FLA 336 interacts only very weakly with orally administered tyramine [6] the compound appears to be an interesting reversible and selective MAO-A inhibitor with potential therapeutic value. The present study reports the MAO inhibitory actions *in vitro* and *in vivo* of the (+)-enantiomer of FLA 336 and its secondary amine metabolite FLA 788 (Fig. 1).

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 180–220 g were used. Compounds dissolved in distilled water, were administered orally by a metal probe. The rats were decapitated with a guillotine and the hypothalamus–thalamus region was dissected out.

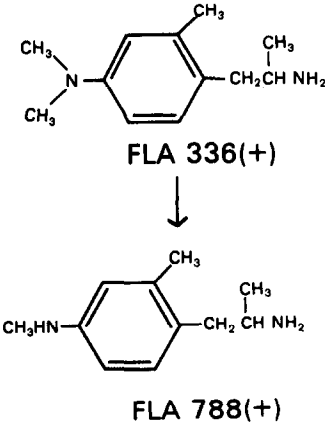

Fig. 1. Chemical structure of FLA 336(+) and its metabolite FLA 788(+).

Mitochondria from hypothalamus were prepared from homogenates in 10 vol. of ice-chilled 0.32 M sucrose (all-glass Potter–Elvehjem homogenizers). The homogenate was centrifuged at 800 *g* for 10 min at 2°. The supernatant was centrifuged at 10,000 *g* for 20 min at 2°. The pellet was re-homogenized in the original volume of 0.32 M sucrose and the centrifugation was repeated. The pellet obtained was re-homogenized in 0.32 M sucrose, portioned (0.5 ml) in small test tubes and kept at −20° until used. The protein content was determined according to Lowry *et al.* [9] with bovine serum albumin as standard.

Mitochondrial MAO activity was determined according to a modification of the method of Wurtman and Axelrod [10] using substrates for the A form ([^{14}C]-5-HT) and B-form [^{14}C]phenethylamine) of MAO. The incubation mixture consisted of 50 μl of the mitochondrial suspension, the inhibitor to be tested in 100 μl distilled water, 700 μl 0.1 M sodium phosphate buffer (pH 7.4), and 150 μl of the radioactive substrate at appropriate concentration. The incubation mixture was incubated at 37° for 5 min in a shaking waterbath under atmospheric conditions. The reaction was stopped by addition of 1 ml of 1 M HCl. The acid metabolite was extracted into 6 ml ethyl acetate by vigorous shaking on a Vortex mixer for 10 sec. After centrifugation 4 ml of the organic layer was transferred to a counting vial and added to 1 ml ethanol and 10 ml scintillation liquid (Econofluor, NEN). Blanks were incubated without the mitochondrial suspension.

The method used to measure the deamination of [^{14}C]-5-HT, [^{3}H]tyramine ([^{3}H]TYR) and [^{14}C]phenethylamine ([^{14}C]PEA) by rat hypothalamic slices has previously been described [6, 8]. The incubation was performed in glass vials containing hypothalamic–thalamic slices (100 mg for [^{14}C]-5-HT and [^{3}H]TYR and 40 mg for [^{14}C]PEA), 2.0 ml Krebs buffer, pH 7.4, containing 5.5 mM glucose, 1.1 mM ascorbic acid and 0.13 mM EDTA, the compound under investigation and [^{14}C]-5-HT, [^{3}H]TYR or [^{14}C]PEA, 1×10^{-7} M final concentrations. The incubation was performed at 37° in an atmosphere of 6.5% CO_2 in O_2. Under these conditions the rate of [^{14}C]-5-HT and [^{3}H]TYR deamination was linear for 10 min [6] and the two substrates did not interact with each other's deamination when incubated simultaneously [6]. Hence the double labelling technique was possible to use for these two substrates. Incubation time employed in these experiments was 5 min. The rate of [^{14}C]PEA deamination was, on the other hand, linear for 3 min [6] and the incubation time used for this substrate was therefore 90 sec. After incubation the slices were homogenized in 1.0 ml of 1 M HCl containing the appropriate substrates and acid metabolites as carriers (1 μg/ml). To 0.5 ml of the medium was added 1 ml of the HCl extraction solution. The acid metabolites were extracted both from the slices and the medium into 6 ml ethyl acetate as described above. The amines in the slice homogenate were then extracted into 6 ml of ethyl acetate after alkalization with solid Na_2CO_3 and 3.0 ml NaCl-saturated 0.5 M borate buffer, pH 10. Solid NaCl was added to facilitate the extraction.

Radioactivity was counted in a Packard Tri Carb liquid scintillator. Tritium was measured with an efficiency of 42% (gain=80%, window 20–1000) and ^{14}C with an efficiency of 63% (gain=15%, window 250–1000). During these conditions the amount of tritium in the ^{14}C-channel was negligible (≤0.01%). Since the specific activity of ^{14}C was 1/20 or less than that of tritium, the amount of ^{14}C in the tritium channel was at most 1% of the total tritium counts and could be neglected. The amount of the acid metabolite formed was expressed in nmoles per g tissue per 5 min incubation. The calculations were made with correction for the extraction recovery. The IC_{50} for MAO inhibition was defined as 50% reduction of the total amounts of the deamination products formed.

The inhibition of MAO *ex vivo* after oral administration of the test compounds to rats was measured *in vitro* with the same slice technique as described above. The dose producing 50% inhibition (ED_{50}) was determined from log dose–response curves.

The 5-HT levels in rat hypothalamus was determined fluorometrically according to Curzon and Green [11]. The tissues were stored at −70° until assayed.

The plasma and brain concentrations of FLA 336(+) and FLA 788(+) were determined by a mass fragmentographic method (Högberg *et al.*, to be published).

Compounds. (+)-4-Dimethylamino-2,α-dimethylphenethylamine (FLA 336(+)) bitartrate, the corresponding (−)-enantiomer (FLA 336(−)), (+)-4-methylamino-2,α-dimethylphenethylamine (FLA 788(+)) bitartrate and clorgyline hydrochloride were synthetized by Dr. L. Florvall (Astra Läkemedel AB, Södertälje, Sweden.) L-Deprenyl hydrochloride was kindly donated by Professor J. Knoll (Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary). 5-Hydroxytryptamine [side chain-2-^{14}C] creatinine sulfate (sp. act. 54 Ci/mmole) was purchased from The Radiochemical Centre (Amersham, U.K.) and tyramine [^{3}H(G)]hydrochloride (sp. act. 9 Ci/mmole) and β-phenethylamine [ethyl-1-^{14}C] hydrochloride (sp. act. 48 mCi/mmole) from New England Nuclear (Boston, MA).

RESULTS

In vitro *inhibition*

Mitochondrial preparation. The selective action of FLA 336(+) and FLA 788(+) on the A type of MAO was shown in the mitochondrial preparation, in which the deamination of [^{14}C]-5-HT was inhibited at 1000 times lower concentration than that of [^{14}C]PEA (Fig. 2). FLA 788(+) was about 6 times more potent than FLA 336(+) in this assay (Table 1). The irreversibly acting clorgyline was 200–1000 times more active as a MAO-A inhibitor *in vitro* compared with other compounds tested.

Kinetic experiments indicated that FLA 336(+) and FLA 788(+) inhibited the deamination of [^{14}C]-5-HT competitively (Fig. 3A and B). The K_i values estimated from the slopes of the double reciprocal plots were 3.2×10^{-7} M (FLA 336(+)) and 1.6×10^{-7} M (FLA 788(+)). The inhibition of

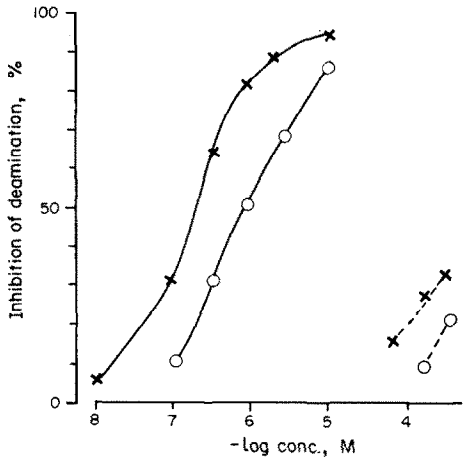

Fig. 2. Inhibition of the deamination of [^{14}C]-5-HT (solid lines) and [^{14}C]phenethylamine (broken lines) by FLA 336(+), (○) and FLA 788(+), (×). Mitochondria were prepared from rat hypothalamus. The incubation was performed with 50 μM of [^{14}C]-5-HT and 2.5 μM of [^{14}C]PEA. Each point is a single determination.

the [^{14}C]PEA deamination appeared also to be competitive (Fig. 3C). The inhibitor constants estimated were 5.2×10^{-4} M (FLA 336(+)) and 1.3×10^{-3} M (FLA 788(+)). Thus, the selectivity of FLA 788(+) was 4 times larger than that of FLA 336(+).

The reversibility of the MAO inhibition was examined by preincubation of a mitochondrial preparation with the inhibitors in a small volume (100 μl). After dilution the inhibition was the same as that found with the inhibitors of the same final concentrations without preincubation (Table 2).

Brain slices. Slices from the rat hypothalamus–thalamus region were used in a series of experiments to evaluate and compare the *in vitro* and *ex vivo* inhibitory effects of the new compounds on brain MAO [6, 8]. In order to imitate physiological conditions low concentrations (1×10^{-7} M) of [^{14}C]-5-HT, [^{3}H]TYR and [^{14}C]PEA were used. The inhibitory effects on the deamination of [^{14}C]-5-HT were similar to those found in the mitochondrial experiments, i.e. clorgyline was considerably more potent than the new compounds, of which FLA 788(+) was 4 times more potent than FLA 336(+), (Table 1). The inhibition of the [^{3}H]TYR deamination was somewhat weaker compared with that of [^{14}C]-5-HT. The compounds had very poor activity on the

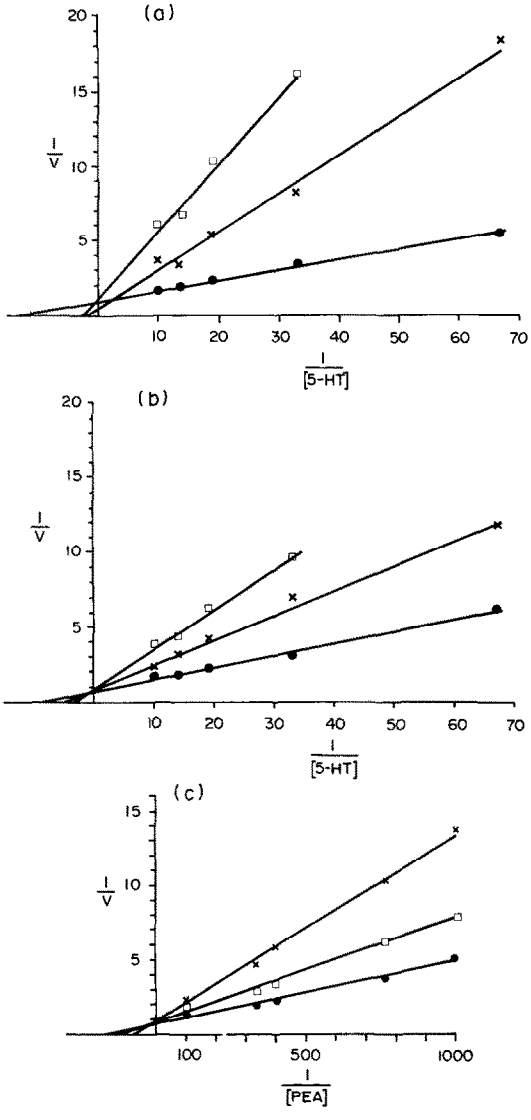

Fig. 3. Lineweaver–Burk plots of the inhibition of the deamination of [^{14}C]-5-HT (A and B) and [^{14}C]PEA (C) by rat hypothalamic mitochondria. (A) FLA 336(+) 0.8 (□) and 1.6 μM (×). (B) FLA 788(+) 0.2 μM (□) and 0.4 μM (×). (C) FLA 336(+), 1 mM (×), FLA 788(+) 1 mM (□). v = nmoles [^{14}C]-5-HIAA or [^{14}C]phenylacetic acid formed per 5 min incubation. Substrate concentrations are given in μM. The lines are drawn by the least square method. Each point is the mean of triplicate determinations.

Table 1. Inhibitory potencies of FLA 336(+), FLA 336(−), FLA 788(+) and clorgyline on the deamination of [^{14}C]-5-HT, [^{3}H]tyramine (TYR) and [^{14}C]phenethylamine (PEA) *in vitro* and *in vivo*

	In vitro, IC_{50} (μM)					*In vivo*, ED_{50} (μmole/kg p.o.)		
	Mitochondria		Slices			Slices		
Compound	[^{14}C]-5-HT	[^{14}C]PEA	[^{14}C]-5-HT	[^{3}H]TYR	[^{14}C]PEA	[^{14}C]-5-HT	[^{3}H]TYR	[^{14}C]PEA
FLA 336(+)	0.8	≥1000	0.6	4.0	≥100	7	29	≥117
FLA 336(−)	3.0	NT	NT	NT	NT	18	≥36	NT
FLA 788(+)	0.13	≥1000	0.15	≥100	NT	9	28	≥120
Clorgyline	0.0007	0.7	0.01	0.04	20	9	16	≥130

NT, not tested.
See legends of Figs. 2 and 6 for explanation of the experimental conditions.

Table 2. Reversibility of the MAO-A inhibitory actions of FLA 336(+) and FLA 788(+) *in vitro*

Compound	Concentration after dilution (μM)	MAO activity, per cent of control	
		Preincubation	No preincubation
FLA 336(+)	0.1	88.7 ± 1.7	88.1 ± 1.7
	0.3	75.9 ± 0.7	76.1 ± 1.0
	1.0	53.3 ± 0.7	51.2 ± 0.6
FLA 788(+)	0.05	79.6 ± 2.0	75.8 ± 1.0
	0.17	53.8 ± 1.2	49.3 ± 1.1
	0.5	26.4 ± 1.8	27.8 ± 0.6

Preincubation: the inhibitors were preincubated with a hypothalamic mitochondrial preparation in 100 μl sodium phosphate buffer, pH 7.4 at 37° for 10 min, after which the incubation mixture was diluted to 1.0 ml final volume with the buffer. The incubation with [^{14}C]-5-HT (50 μM) was then performed at 37° for 6 min.
The values are means ± S.E.M. of triplicates.

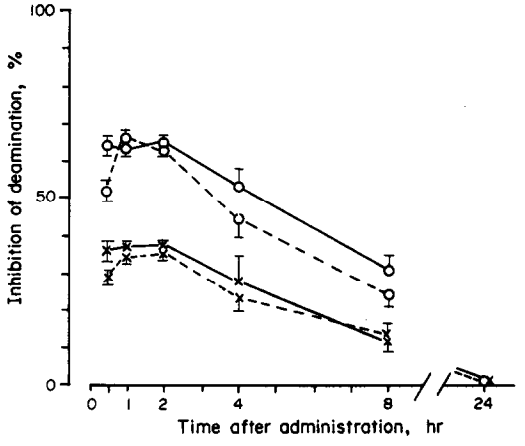

Fig. 4. Time courses of FLA 336(+) (solid lines) and FLA 788(+) (broken lines) in inhibiting the deamination of [^{14}C]-5-HT (○) and [^{3}H]tyramine (×) by hypothalamic-thalamic slices. The compounds were given orally at 15 μmoles/kg. Each value is the mean ± S.E.M. (vertical bar) from 4 rats. The concentration of the labelled amines was 1×10^{-7} M and the incubation time 5 min.

[^{14}C]PEA deamination. The uptake of [^{14}C]-5-HT and [^{3}H]TYR was not inhibited at concentrations producing 50% inhibition of the deamination of the amines.

Ex vivo *inhibition*

FLA 336(+) and FLA 788(+) (15 μmoles/kg) were given orally to rats which were killed at various intervals thereafter. The deamination of [^{14}C]-5-HT and [^{3}H]TYR by brain slices was examined with the same method as that used in the *in vitro* assay. As shown in Fig. 4, the two compounds caused almost the same degree of inhibition. Maximal effect was obtained 1–2 hr after administration. No effect persisted 24 hr after the administration.

Dose–response studies performed 2 hr after administration of the compounds showed that FLA 336(+), FLA 788(+) and clorgyline had about the same inhibitory activity on the [^{14}C]-5-HT deamination in this test (Fig. 5, Table 1). Clorgyline appeared to be slightly more active on the [^{3}H]TYR deamination compared with the other compounds. The (−)-enantiomer of FLA 336 had about half of the activity of the (+)-enantiomer, which indicates a stereoselective inhibition of MAO-A. None of the compounds inhibited the uptake of [^{14}C]-5-HT or [^{3}H]TYR at the doses producing 50% inhibition of the amine deamination.

Correlation between plasma concentration and MAO inhibition

The secondary amine metabolite FLA 788(+) is rapidly formed from FLA 336(+) in rats (J. Lundström, to be published). Two hours after oral administration of various doses of FLA 336(+) the concentration of FLA 788(+) in plasma exceeded that of FLA 336(+) (Table 3). The correlation between the plasma concentration of FLA 788(+) and the inhibition of the deamination of [^{14}C]-5-HT was very high ($r = 0.97$, $P \leq 0.001$, $n = 15$), (Fig. 6).

Table 3. Plasma and brain concentrations of FLA 336(+) and FLA 788(+) 2 hr after oral administration of FLA 336(+) to rats

Dose (μmole/kg p.o.)	Plasma (nmole/ml)		Brain (nmole/g)	
	FLA 336(+)	FLA 788(+)	FLA 336(+)	FLA 788(+)
3	<0.010	0.082 ± 0.007	NT	NT
7	0.040 ± 0.005	0.307 ± 0.023	2.70 ± 0.15	4.64 ± 0.14
29	0.391 ± 0.054	1.009 ± 0.096	17.13 ± 1.52	11.48 ± 0.74

NT, not determined.
Each value is the mean ± S.E.M. of 5 animals.

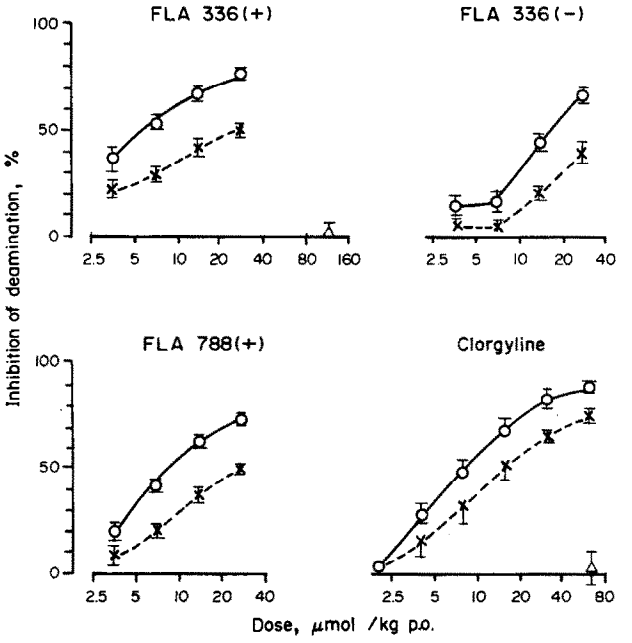

Fig. 5. Dose–response curves for the inhibitory effect of FLA 336(+), FLA 336(−), FLA 788(+) and clorgyline on the deamination of [^{14}C]-5-HT (○), [^{3}H]tyramine (×) and [^{14}C]phenethylamine (△) by hypothalamic–thalamic slices. The compounds were given orally to rats 2 hr before decapitation. The incubation was performed with 1×10^{-7} M of the labelled substrates and 100 mg brain tissue for 5 min for [^{14}C]-5-HT and [^{3}H]TYR and 90 sec for [^{14}C]PEA. Each value is the mean ± S.E.M. (vertical bars) from 4 rats.

Determination of the brain concentrations of FLA 336(+) and FLA 788(+) 2 hr after 7 μmole/kg p.o. of FLA 336(+) showed that the concentration of the metabolite was 1.7 times higher than that of the parent compound (Table 3). At 29 μmole/kg p.o. of FLA 336(+) the brain concentration of the administered compound exceeded that of the metabolite.

5-HT concentration in rat hypothalamus

The concentration of 5-HT in rat hypothalamus was determined 2 hr after administration of 3 different doses of FLA 336(+) (Table 4). The 5-HT level was significantly increased at all three doses examined.

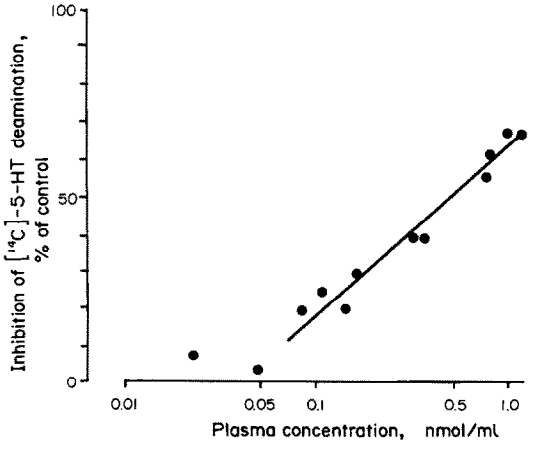

Fig. 6. Correlation between the plasma concentration of FLA 788(+) and the inhibition of the deamination of [^{14}C]-5-HT by hypothalamic–thalamic slices after oral administration of FLA 336(+) at 3, 7 and 29 μmoles/kg.

DISCUSSION

The results obtained in the present study and those by Fowler and Oreland [12] show that FLA 336(+) and its metabolite FLA 788(+) are very selective, competitive and reversible inhibitors of MAO-A in rat and human brain mitochondrial preparations. The ratios between the potencies in inhibition of MAO-A and MAO-B were in our experiments 1600 for FLA 336(+) and 8100 for FLA 788(+). Thus the metabolite was even more selective than the parent compound. The inhibition was also stereoselective since FLA 336(−) was less active compared with FLA 336(+). Similar stereoselectivity has been observed for amphetamine [13].

Table 4. 5-HT levels in rat hypothalamus 2 hr after oral administration of FLA 336(+)

Dose (μmole/kg p.o.)	5-HT (μg/g)	Per cent increase
Control	1.20 ± 0.06	
3	1.74 ± 0.03*	145
7	1.83 ± 0.07*	153
29	2.01 ± 0.07*	168

* $P < 0.001$ compared with the control.

5-HT was determined according to Curzon and Green [11].

Each value is the mean ± S.E.M. from 5 rats.

Direct determinations of the MAO inhibition in the rat brain after administration of reversible inhibitors contain some experimental difficulties. Conventional methods with homogenates and high substrate concentrations will give severe underestimation of the real degree of inhibition since the inhibitor becomes highly diluted in the homogenate and high substrate concentrations do not reflect the actual free concentrations of the amine substrate in the brain. In order to overcome these obstacles we have used the slice technique in which the dilution factor is less marked. By using low concentrations of the labelled substrate we tried to imitate the real free substrate concentration of the amines under *in vivo* conditions. In a previous study we found that the MAO inhibition in mouse brain slices determined with this technique was obtained in the same dose range of the test compounds which produced behaviour changes related to the MAO inhibition [8]. We also have found that the increase in the 5-HT concentration in the whole rat brain after administration of various reversible MAO inhibitors was obtained at similar or even lower doses than those producing 50% inhibition of MAO-A when measured *ex vivo* with the slice technique [6].

An alternative method to determine the degree of *in vivo* inhibition of MAO is to measure the antagonism of the irreversible MAO inhibition produced by, for example, phenelzine [14]. Comparison of these two methods shows a good correlation (to be published).

A third method is to determine the increase in the endogenous amine concentration and/or the decrease in their deaminated metabolites. However, the activity in the monoaminergic neuron systems is rapidly regulated by feedback mechanisms which may influence the amine and metabolite concentrations. Additional effects of the test compounds, e.g. amine release, may cause false estimations of the degree of MAO inhibition by this method. In order to have a complete picture of the actions of a reversible MAO inhibitor several methods have to be used.

The time course of the inhibition of the deamination of [^{14}C]-5-HT and [^{3}H]TYR showed that the inhibition was absent 24 hr after a single administration of FLA 336(+) and FLA 788(+). Although the increased concentrations of endogenous amines may have influenced the measured MAO activity, these experiments show that the effect of FLA 336(+) is fully reversible within 24 hr.

The two FLA compounds were 200–1000 times less potent than clorgyline when examined in the mitochondrial MAO system *in vitro*. However, this difference in activity was markedly reduced in the brain slices, primarily due to decreased inhibitory potency of clorgyline. The FLA compounds had, on the other hand, similar potencies in the two preparations. After oral administration the difference was completely eliminated. These observations indicate that clorgyline is largely bound to other tissue components [15] and/or has a different distribution or rate of metabolism than the FLA compounds, i.e. only a minor part reaches mitochondria in intact tissues.

The observations that the plasma and brain concentrations of FLA 788(+) particularly at lower doses exceeded that of FLA 336(+), and that FLA 788(+) *in vitro* was 2–6 times more active than FLA 336(+) indicate that a part of the MAO inhibitory effect of FLA 336(+) *in vivo* is due to the main metabolite FLA 788(+). The high correlation between the plasma concentration of FLA 788(+) and the inhibition of the deamination of [^{14}C]-5-HT in brain slices also supports the role of the metabolite for the MAO inhibition in the brain under *in vivo* conditions.

The results obtained in this study indicate that FLA 336(+) and its metabolite FLA 788(+) are potent and selective inhibitors of MAO-A in the rat brain. Since these compounds have similar inhibitory activities on MAO-A of mitochondrial preparations from human brain [12] and since they have low tyramine potentiating actions in rats ([6] and unpublished observations) they may have therapeutic values for treatment of depressive disorders.

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