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EFFECTS OF ORG 6582 ON MONOAMINE UPTAKE *IN VITRO*

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Abstract—*In vivo* studies have indicated that Org 6582 is a potent long acting selective inhibitor of 5HT uptake. The objective of this study was to investigate the effects of Org 6582 on the uptake of [^{3}H]DA into synaptosome-rich homogenates of rat corpus striatum and on the uptake of [^{3}H]NA or [^{3}H]5HT into synaptosome-rich homogenates of rat hypothalamus. Two experimental approaches were adopted. In one, drugs were directly added to the incubation medium. In the other, rats were injected interperitoneally (i.p.) with the drugs under study at various times prior to death and synaptosomal [^{3}H]monoamine uptake subsequently determined. Org 6582 was a competitive inhibitor of [^{3}H]5HT uptake with a K_i value of 8.9×10^{-8}M. The ability of Org 6582 to inhibit [^{3}H]5HT uptake was sixteen and seventy-two times greater than its effect on[^{3}H]NA and [^{3}H]DA uptake respectively. At 1 hr after injection, Org 6582 equalled fluoxetine and was more potent than chlorimipramine at blocking [^{3}H]5HT uptake. The duration of inhibition of [^{3}H]5HT uptake by chlorimipramine was appreciably shorter than that of either Org 6582 or fluoxetine. In contrast to both desipramine and chlorimipramine, Org 6582 was essentially devoid of effect on [^{3}H]NA uptake at 1 hr after injection. [^{3}H]DA uptake was also unaffected by 1 hr pretreatment with Org 6582. The findings of this study confirm that Org 6582 is a potent long acting selective inhibitor of 5HT uptake.

Uptake into the presynaptic neurone is considered to be the major mechanism by which monoamines are inactivated at the neural synapse [1]. A characteristic feature of the tricyclic antidepressants is their ability to block the uptake of noradrenaline (NA) and 5-hydroxytryptamine (5HT) and various studies *in vitro* have revealed that tertiary tricyclics, such as imipramine and amitriptyline, have a greater inhibitory effect on 5HT uptake whereas their corresponding desmethylated analogues, namely desipramine and nortriptyline, preferentially block NA uptake [2–6]. This partial selectivity of uptake inhibition by tricyclic antidepressants has stimulated the search for agents with a greater specificity of action and recent studies in this laboratory have revealed that Org 6582 (*d,l*-8-chloro-11-anti-amino-benzo-(b)-bicyclo-[3.3.1]-nona-3,6a-(10a)-diene hydrochloride) is a potent long acting selective inhibitor of 5HT uptake *in vivo* (Fig. 1) [7, 8]. The objective of this study was to investigate the effects of Org 6582 on monoamine uptake mechanisms *in vitro*. Two experimental approaches were used. In the first, drugs were directly added to the incubation medium at the start of the preincubation period (*in vitro* experiments). In the second, rats were injected i.p. with the agent under study at various times prior to sacrifice and synaptosomal monoamine uptake subsequently determined (*ex vivo* experiments). A preliminary account of some of the findings has been published in abstract form [9].

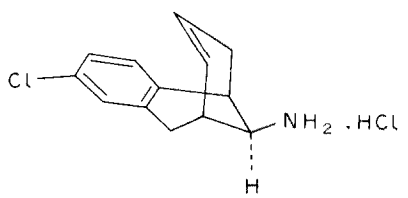

Fig. 1. Structure of Org 6582.

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MATERIALS AND METHODS

Materials. Org 6582 was synthesized by the Chemical R and D Laboratories of Organon Labs Ltd. The following compounds were generously donated: *d*-amphetamine sulphate (Smith, Kline and French), benztropine hydrobromide (Duncan Flockhart), chlorimipramine hydrochloride and desipramine hydrochloride (Geigy), fluoxetine hydrochloride (Eli Lilly), mazindol (Sandoz) and nomifensine hydrogen maleate (Hoechst). Pargyline hydrochloride was purchased from Regis Chemical Co. and [G-^{3}H] 5-hydroxytryptamine creatinine sulphate (14.0 Ci/m-mole), L-[7-^{3}H] noradrenaline (5.8 Ci/m-mole) and [ethylamine-1,2-^{3}H] dopamine hydrochloride (8.5 Ci/m-mole) were obtained from the Radiochemical Centre, Amersham, U.K. All other chemicals were obtained from BDH Chemicals Ltd., Poole, U.K.

In vitro *experiments.* In all studies male Wistar rats (ALA/CFHB strain) weighing 180–220 g were used. *In vitro* monoamine uptake was studied using a crude synaptosomal fraction from either rat corpus striatum or hypothalamus. The corpus striatum or hypothalamus was dissected from the rat brain as described by Glowinski and Iversen [10]. Corpus striatum was used for studies on [^{3}H]DA uptake and hypothalamus for studies on [^{3}H]5HT and [^{3}H]NA uptake. The tissue was weighed and homogenized in 19 volumes of 0.32 M sucrose using a slowly rotating (less than 200 rpm) Teflon pestle in

a glass homogenizer, clearance 0.1 mm, until a uniform suspension was obtained. The resulting homogenate was centrifuged at 1000 *g* for 10 min at 4°, the supernatant liquid decanted and gently stirred to give a uniform suspension. A 0.1 ml aliquot of suspension (equivalent to 5 mg original tissue) was added to beakers containing 2 ml Krebs–bicarbonate buffer with the following composition (g/l): NaCl, 6.92; KCl. 0.354; $MgSO_4 \cdot 7H_2O$, 0.294; $CaCl_2 \cdot 6H_2O$, 0.282; KH_2PO_4, 0.162; $NaHCO_3$, 2.1; glucose, 2.0; pargyline, 0.03; ascorbic acid, 0.02; and gassed with 95% oxygen–5% carbon dioxide for at least 15 min prior to use. After 10 min preincubation in a Dubnoff metabolic shaker at 37° under an atmosphere of 95% O_2–5% CO_2 with shaking, 100 μl [^{3}H]monoamine were added to give a final concentration of 2.6×10^{-8} M. Incubation was continued for a further 10 min, 5 min or 2 min for [^{3}H]NA, [^{3}H]5HT or [^{3}H]DA, respectively, and was terminated by the addition of 5 ml of ice-cold saline and by standing the beakers in ice for 10 min. The cellular particles were separated from the incubation medium by filtration under vacuum using Millipore filters (24 mm diameter, pore size 0.45 μm) [11]. The beakers were rinsed with 5 ml ice-cold saline and the washings filtered. The filters were then rinsed with a further 5 ml ice-cold saline to remove any remaining radioactivity which was not associated with tissue. The filter discs were placed in counting vials and 15 ml Bray's scintillant added to each. After 4 hr at room temperature the filter discs had disintegrated and the contents of the vials were ready for counting. Samples were counted in a Nuclear-Chicago Isocap 300 scintillation counter with a counting efficiency of 20 per cent for tritium.

Incubations were carried out in the presence of four concentrations of test drug, control at 37° and control at 0°. The concentration of radioactivity in the incubation media was obtained by preparing incubation vessels as above but adding 0.1 ml 0.32 M sucrose instead of 0.1 ml homogenate, and counting 1 ml of the resulting mixture. Each incubation was performed in quadruplicate. The concentration of monoamine taken up was calculated in terms of dpm/g original tissue divided by dpm/ml medium and was corrected for non-specific binding by subtracting the amount taken up at 0° and was expressed in terms of the tissue–medium ratio (T/M).

Under these conditions of incubation at least 85 per cent of the radioactivity is associated with the parent amine [2, 12, 13]. Control T/M ratios for the uptake of [^{3}H]5HT, [^{3}H]NA, and [^{3}H]DA were 13.2±0.2, 3.8±0.2 and 51.5±1.3 respectively.

Percent inhibition was calculated thus:

$$\text{Per cent inhibition} = \frac{(\text{Control T/M} - \text{treated T/M})}{\text{Control T/M}} \times 100 \text{ per cent}$$

The molar concentration of drug required to inhibit uptake by 50 per cent (IC_{50}) was determined from log concentration: probit graphs of the inhibition data.

A kinetic analysis of the inhibition of [^{3}H]5HT uptake by Org 6582 was studied using homogenates of hypothalamus which were prepared and incubated as described above in the presence of either 2.6×10^{-8}M or 10.4×10^{-8}M 5HT. The reciprocal of the amount of 5HT taken up (expressed in terms of nanomoles 5HT per g of fresh tissue per 5 min) was plotted against the concentration of drug according to the method of Dixon [14].

Ex vivo *experiments*. Rats were injected i.p. with the test-drug (dose refers to base) or vehicle alone (5% v/v mulgofen in distilled water). At the end of the pretreatment period the rats were stunned, killed by decapitation and the brain quickly removed and dissected as described above. The tissue under study was homogenized and centrifuged as described above except that homogenization was in 9 volumes of 0.32 M sucrose. The rest of the procedure was as described in the section above except that 0.1 ml stirred supernatant liquid was added to 0.95 ml drug-free Krebs–bicarbonate buffer and 50 μl [^{3}H] monoamine was added after preincubation. Results were expressed either as per cent of control values or per cent inhibition of uptake, each result being the mean ± S.E.M. of at least four determinations. In some instances, results were expressed as the ED_{50} value which is defined as the dose of drug (mg/kg, i.p.) required to block uptake by 50 per cent and was obtained from log dose/response curves constructed by the method of least squares. Statistical significances were determined by means of Student's '*t*' test (two-tailed).

RESULTS

In vitro *experiments*. Org 6582 was more potent at inhibiting [^{3}H]5HT uptake than either [^{3}H]NA or [^{3}H]DA uptake (Table 1). Org 6582 was the least potent of the compounds studied for inhibition of [^{3}H]NA or [^{3}H]DA uptake. Comparing the IC_{50} values for the inhibition of [^{3}H]5HT uptake with those for the inhibition of [^{3}H]NA uptake Org 6582 was found to be sixteen times more potent at inhibiting [^{3}H]5HT uptake than [^{3}H]NA uptake while the corresponding values for chlorimipramine and fluoxetine were thirteen and sixty times respectively. Similarly, Org 6582, fluoxetine and chlorimipramine were 72, 275 and 278 times more potent at inhibiting [^{3}H]5HT uptake than [^{3}H]DA uptake respectively.

The nature of Org 6582 inhibition of [^{3}H]5HT uptake into hypothalamic synaptosomes was investigated by the method of Dixon [14] and the result is illustrated in Fig. 2. It can be seen that the lines obtained from the reciprocal of the uptake of [^{3}H]5HT plotted against concentration of Org 6582 for two concentrations of [^{3}H]5HT intersect above the abscissa. This indicates that Org 6582 is a competitive inhibitor of [^{3}H]5HT uptake *in vitro* with a K_i value of 8.9 ± 10^{-8} M.

Ex vivo *experiments*. Drugs were injected i.p. 1 hr before killing and their effect on *in vitro* [^{3}H]NA uptake assessed. A comparison of the effect of desipramine and Org 6582 was carried out and the results are summarised in Table 2. Pretreatment with desipramine reduced uptake in a dose dependent manner with an i.p. ED_{50} value of 20.4 mg/kg. In contrast Org 6582 given at a dose of 80 mg/kg i.p.

Table 1. *In vitro* inhibition of [^{3}H]monoamine uptake

Drug	IC_{50} (M)		
	[^{3}H]5HT	[^{3}H]NA	[^{3}H]DA
Org 6582	1.8×10^{-7}	2.9×10^{-6}	1.3×10^{-5}
Fluoxetine	9.8×10^{-9}	5.9×10^{-7}	2.7×10^{-6}
Chlorimipramine	7.9×10^{-9}	1.1×10^{-7}	2.2×10^{-6}
Desipramine	3.1×10^{-7}	3.2×10^{-8}	8.0×10^{-6}
d-Amphetamine	2.1×10^{-5}	4.7×10^{-7}	2.8×10^{-7}

Rat striatal synaptosomes were used for [^{3}H]DA studies and hypothalamic synaptosomes for [^{3}H]NA and [^{3}H]5HT studies. Drugs were preincubated with synaptosomes for 10 min after which [^{3}H] monoamine (final concentration 2.6×10^{-8} M) was added and incubation continued for 10 min, 5 min or 2 min for [^{3}H]NA, [^{3}H]5HT or [^{3}H]DA respectively. IC_{50} values, i.e. concentration of drug required to produce 50 per cent inhibition of [^{3}H]monoamine uptake were obtained from log dose/inhibition probit graphs at four concentrations of drug in quadruplicate.

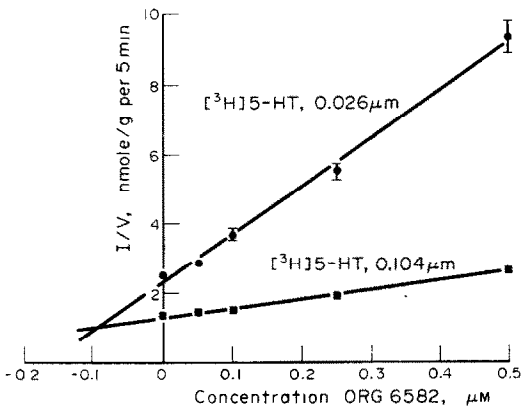

Fig. 2. Kinetics of inhibition of [^{3}H]5HT uptake of Org 6582. Rat hypothalamic synaptosomes were incubated in the presence of either 2.6×10^{-8} M or 10.4×10^{-8} M [^{3}H]5HT and varying concentrations of Org 6582. Amine uptake is expressed as nanomole/g original tissue/5 min. The method of graphical analysis was that of Dixon [14]. Each point is the mean of at least six determinations and best fitting lines were obtained by the method of least squares.

had no effect on *in vitro* [^{3}H]NA uptake. 1 hr pretreatment with chlorimipramine (80 mg/kg) inhibited [^{3}H]NA uptake by 53.2±12.1 per cent.

The uptake of [^{3}H]DA by rat striatal synaptosomes 1 hr after pretreatment with various drugs was studied and the results are summarised in Table 3. Org 6582, chlorimipramine or desipramine given at a dose of 80 mg/kg i.p. had no effect on the uptake of [^{3}H]DA into striatal synaptosomes. However, pretreatment with benztropine (40 mg/kg i.p.), nomifensine (27 mg/kg i.p.) or mazindol (40 mg/kg i.p.) caused a significant inhibition of striatal synaptosomal [^{3}H]DA uptake.

Table 3. Effect of 1 hr pretreatment on the uptake of [^{3}H]DA into rat striatal synaptosomes

Drug	Dose (mg/kg i.p.)	% Control
Org 6582	80	94.0 ± 3.3
Chlorimipramine	80	96.3 ± 8.7
Desipramine	80	106.8 ± 5.0
Benztropine	40	74.1 ± 2.4*
Nomifensine	27	61.8 ± 1.2*
Mazindol	40	54.0 ± 1.4*

Striatal synaptosomes from rats injected 1 hr prior to killing were incubated for 2 min in [^{3}H]DA (2.6×10^{-8} M). Each result, expressed as per cent control, is the mean ± S. E. M. of at least four determinations.

* Significantly different from control ($P < 0.001$).

The effect of 1 hr pretreatment with Org 6582, chlorimipramine or fluoxetine on rat hypothalamic [^{3}H]5HT uptake was studied and the results are summarised in Table 4. Org 6582 and fluoxetine were equipotent in inhibiting [^{3}H]5HT uptake, their i.p. ED_{50} values being 7.8 and 8.7 mg/kg respectively. Both drugs were approximately 2.5 times more potent than chlorimipramine which had an i.p. ED_{50} value of 21.8 mg/kg.

Table 2. Effect of 1 hr pretreatment on the uptake of [^{3}H]NA into rat hypothalamic synaptosomes

Drug	Dose (mg/kg i.p.)	% Control	ED_{50} (mg/kg)	95% Confidence limits
Org 6582	80	102.1 ± 21.9	> 80	
Desipramine	60	32.7 ± 2.6		
	40	36.7 ± 9.1	20.4	4.2–38.2
	20	49.5 ± 11.7		
	10	63.4 ± 12.1		
Chlorimipramine	80	53.2 ± 12.1		

Hypothalamic synaptosomes from rats injected i.p. 1 hr prior to killing were incubated for 10 min in [^{3}H]NA (2.6×10^{-8} M). Each result, expressed as per cent control, is the mean ± S. E. M. of at least four determinations. Doses of drugs (mg/kg) which cause 50 per cent inhibition of uptake (ED_{50}) and 95 per cent confidence limits were calculated by the method of least squares regression.

Table 4. Effect of 1 hr pretreatment on the uptake of [^{3}H]5HT into rat hypothalamic synaptosomes

Drug	Dose (mg/kg i.p.)	% Control	ED_{50} (mg/kg)	95% Confidence limits
Org 6582	20	27.1 ± 0.7		
	10	37.7 ± 1.6	7.8	7.1–8.5
	5	63.3 ± 1.4		
	2.5	82.5 ± 1.9		
Fluoxetine	20	36.1 ± 1.1		
	10	42.5 ± 1.4	8.7	7.5–10.3
	5	62.9 ± 2.8		
	2.5	75.6 ± 4.7		
Chlorimipramine	80	22.7 ± 0.9	21.8	19.1–24.5
	40	36.2 ± 2.5		
	20	49.0 ± 1.0		
	10	69.1 ± 3.7		

Hypothalamic synaptosomes from rats injected i.p. 1 hr prior to killing were incubated for 5 min in [^{3}H]5HT (2.6×10^{-8} M). For remainder of legend, see Table 2.

Experiments were undertaken to compare the duration of action of Org 6582, fluoxetine and chlorimipramine. Figure 3 shows the time course of inhibition of [^{3}H]5HT uptake following a single injection of Org 6582 (20 mg/kg i.p.), fluoxetine (20 mg/kg i.p.) or chlorimipramine (80 mg/kg i.p.). The time courses for Org 6582 and fluoxetine were similar, at 1 hr after administering Org 6582 or fluoxetine maximum inhibition of uptake had occurred (approximately 75 per cent for both drugs at 20 mg/kg i.p.). At 5 hr after injection, the level of inhibition by both drugs was similar to the 1 hr level. At 24 hr after injection [^{3}H]5HT uptake was inhibited by 55 and 59 per cent by Org 6582 and fluoxetine, respectively. At 48 hr after injection of Org 6582 or fluoxetine [^{3}H]5HT uptake was inhibited by 34 and 29 per cent respectively. In contrast, the duration of action of chlorimipramine was shorter than that of Org 6582 or fluoxetine. [^{3}H]5HT uptake was inhibited 77 per cent 1 hr after injection of chlorimipramine. This inhibition is similar to that which had occurred 1 hr after injection of Org 6582 or fluoxetine. However, there is a rapid reduction in the effect of chlorimipramine. For example, 5 hr after chlorimipramine injection, a 39 per cent inhibition of [^{3}H]5HT uptake occurred compared with approximately 70 per cent obtained at this time with Org 6582 or fluoxetine. 24 hr after injection of chlorimipramine there was no apparent inhibition of [^{3}H]5HT uptake.

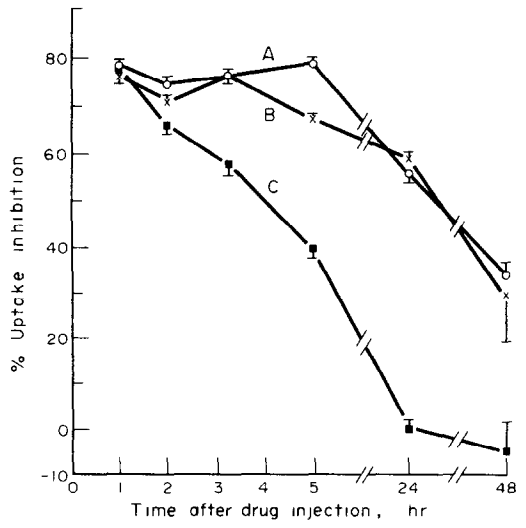

Fig. 3. Duration of inhibition of [^{3}H]5HT uptake (2.6×10^{-8} M) into rat hypothalamic synaptosomes following i.p. injections of Org 6582 (20 mg/kg) [A], fluoxetine (20 mg/kg) [B] or chlorimipramine (80 mg/kg) [C]. Each point is the mean ± S.E.M. of at least four determinations. For clarity, only one bar of the S.E.M. is shown.

DISCUSSION

Previous studies have revealed that Org 6582 is a potent long acting selective inhibitor of 5HT uptake *in vivo*. Inhibition of 5HT uptake was assessed by determining the effect of drug pretreatment on the ability of *p*-chloroamphetamine to lower rat brain 5HT levels [7, 8]. Org 6582 was observed to be approximately two, five and fourteen times more potent than fluoxetine, chlorimipramine and desipramine respectively. Org 6582 was also found to have a longer duration of action than either fluoxetine or chlorimipramine. In contrast to both chlorimipramine and desipramine, large doses of Org 6582 had essentially no demonstrable effect in NA and DA uptake as assessed by determining the effect of drug pretreatment on the ability of 6-hydroxydopamine to lower rat brain NA and DA levels and on the ability of either 6-hydroxydopamine or L-metaraminol to lower the concentration of NA in the rat heart. Others have confirmed that Org 6582 is a potent selective inhibitor of 5HT uptake *in vivo* [15–17].

The ability of rat hypothalamic synaptosomes to accumulate [^{3}H]5HT was markedly impaired 1 hr after pretreatment with Org 6582. Fluoxetine and Org 6582 were comparable in potency 1 hr after injection and both agents were approximately two and a half times more potent than chlorimipramine. Moreover, the duration of action of both Org 6582 and fluoxetine was appreciably longer than that of chlorimipramine as indicated by the observation that hypothalamic synaptosomal uptake of [^{3}H]5HT was significantly impaired at 24 and 48 hr after the i.p. injection of 20 mg/kg of either Org 6582 or

fluoxetine. On the other hand, [^{3}H]5HT uptake was unaffected at these times by chlorimipramine (80 mg/kg, i.p.). Using a similar experimental approach, others have also observed that fluoxetine has a much longer duration of action than chlorimipramine [18]. The short duration of action of chlorimipramine is not unexpected since it is rapidly metabolized in the rat to chlordesipramine which is not a potent inhibitor of [^{3}H]5HT uptake [19]. In contrast to the tricyclics desipramine and chlorimipramine, Org 6582, at a large dose of 80 mg/kg injected i.p. 1 hr prior to sacrifice, was devoid of effect on the uptake of [^{3}H]NA by rat hypothalamic synaptosomes. The lack of effect of Org 6582 in this paradigm is in agreement with *in vivo* observations [8, 16]. The lack of effect of pretreatment with Org 6582, desipramine or chlorimipramine on striatal synaptosomal [^{3}H]DA uptake is in agreement with *in vivo* experiments which revealed that none of the agents prevented the fall in rat brain DA levels following the intraventricular injection of 6-hydroxydopamine [8]. *In vivo* and *in vitro* experiments have shown that DA uptake is blocked by both mazindol and nomifensine and the observation that 1 hr pretreatment with either drug blocks the subsequent uptake of [^{3}H]DA by striatal synaptosomes is in agreement with these findings [6, 20–24]. Benztropine is an inhibitor of [^{3}H]DA uptake by rat striatal synaptosomes and the observation that synaptosomal [^{3}H]DA uptake is inhibited by benztropine pretreatment agrees with the findings of others [6, 25–27].

Of the agents studied for *in vitro* inhibition of [^{3}H]5HT uptake by rat hypothalamic synaptosomes chlorimipramine $\geqslant$ fluoxetine > Org 6582 $\geqslant$ desipramine > *d*-amphetamine. The observation that fluoxetine and chlorimipramine are approximately equipotent, and that both agents are more potent than desipramine in blocking synaptosomal uptake of [^{3}H]5HT agrees with the finding of others [18]. Somewhat surprisingly Org 6582 was less potent than either fluoxetine or chlorimipramine in inhibiting the *in vitro* uptake of [^{3}H]5HT by rat hypothalamic synaptosomes. This finding is at variance with *in vivo* and *ex vivo* findings cited above [7, 8]. The possibility that the greater effect of Org 6582 on 5HT uptake observed both *in vivo* and *ex vivo* is due to the conversion of Org 6582 to an active metabolite is unlikely since Org 6582 is not only not extensively metabolized but also has a long plasma half life in the rat, a finding which is in agreement with the long duration of action of the agent observed in both the *in vivo* and *ex vivo* experiments [28]. The anomaly between the *ex vivo* and *in vitro* findings may stem from differences in the availability of the various agents at uptake sites as a consequence of differences in such physico-chemical factors as pK_a values, solubilities and partition co-efficients. It is of interest to note that the potency of pirandamine, another selective inhibitor of 5HT uptake, relative to that of chlorimipramine was greater *in vivo* than *in vitro* [29]. Moreover, orphenadrine and amatadine are potent inhibitors of 5HT uptake by rat brain *in vivo* yet their ability to block [^{3}H]5HT uptake by rat forebrain synaptosomes is weak [30].

Org 6582 was the least potent of the compounds studied for inhibition of [^{3}H]NA and [^{3}H]DA uptake by rat hypothalamic and striatal synaptosomes respectively. Comparing the IC_{50} values for the inhibition of [^{3}H]5HT uptake with those for the inhibition of [^{3}H]NA uptake and [^{3}H]DA uptake Org 6582 was found to be sixteen and seventy-two times more potent at inhibiting [^{3}H]5HT uptake. Although the ability of Org 6582 to inhibit 5HT uptake *in vivo* and *ex vivo* is long lasting, experiments undertaken to characterize the nature of uptake inhibition revealed that the compound is a competitive inhibitor of [^{3}H]5HT uptake by rat hypothalamic synaptosomes with a K_i value of 8.9×10^{-8}M.

In summary, the results of this study confirm that Org 6582 is a potent selective competitive inhibitor of 5HT uptake with a long duration of action.

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