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SUBSTRATE-SELECTIVE INHIBITION OF MONOAMINE OXIDASE BY SOME CYCLOPROPYLAMINO SUBSTITUTED OXADIAZOLES

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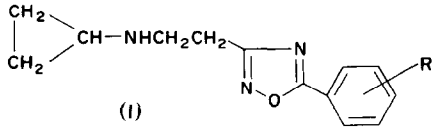
Abstract—1. Inhibition of rat monoamine oxidase by 5-phenyl-3-(*N*-cyclopropyl) ethylamine-1,2,4-oxadiazole (PCO) has been studied *in vitro* and *in vivo*. The compound is an irreversible inhibitor showing selectivity for the serotonin oxidising site (A-site) of the enzyme. 2. Substitution in the 5-phenyl ring of PCO gives compounds which may also be selective for the A-site such as the 2-chlorophenyl compound, may show no selectivity or may, like the 3-nitrophenyl compound, be selective for the B-site of the enzyme. 3. The selectivity found when these inhibitors react with monoamine oxidase *in vitro* is also apparent when the enzyme from rats which have received single doses of the compounds is studied, but is less clearcut after repeated administration.

The complex nature of mitochondrial monoamine oxidase (MAO) in many mammalian tissues is now well recognised and has been revealed, both in direct purification and separation experiments and by analysis of the action of substrate specific inhibitors. Evidence for this complexity has been reviewed recently by Youdim [1]. This paper describes a series of substituted cyclopropylamines (I) which, like the cyclopropylamines studied by Fuller [2], inhibited rat liver



(I)

MAO irreversibly but showed different degrees of inhibition depending on the substrate used. Relatively minor structural variations in the molecule had a marked effect on the degree and type of selectivity seen. The results obtained are consistent with the existence of two types of MAO in rat liver, similar to the A and B enzymes first proposed by Johnston [3] to account for the interaction of rat brain MAO with the substrate specific inhibitor clorgyline (*N*-methyl-*N*-propynyl-3-[2,4-dichlorophenoxy]propylamine). This concept of MAO as a two site enzyme has been developed by Squires [4] who characterised the enzyme from a number of sources using selective inhibitors.

EXPERIMENTAL

Materials

Tyramine hydrochloride was obtained from BDH Ltd. Radioactive substrates, [1-^{14}C]tyramine and [1-^{14}C]serotonin creatinine sulphate were obtained from the Radiochemical Centre, Amersham. Tranylcypromine sulphate was obtained from Smith, Kline and French Ltd. Other enzyme inhibitors used and the serotonin creatinine sulphate were synthesised in the Chemistry Department, Roche Products Ltd., by Drs. P. G. Philpott and B. Heath-Brown.

Reagents used in the preparation of buffers, etc., were of analytical reagent grade.

Methods

Enzyme preparation. Livers from male Sprague–Dawley rats (Roche Products Animal Farm, Welwyn Garden City) were homogenised for 30 sec at 0° in water (10 vol) using a Silverson homogeniser followed by a Potter–Elvehjem glass homogeniser and centrifuged, first at 800 *g* for 10 min and subsequently at 8000 *g* for 30 min. The pellet from the second centrifugation was washed twice with water (10 vol) and resuspended in 67 mM sodium potassium phosphate buffer (10 mg/g of liver). Aliquots were kept frozen and thawed immediately before use. Solubilised MAO was made from rat liver by the method described by Youdim and Collins [5].

Determination of enzyme activity

(i) *Manometric assay.* A Gilson respirometer was used to measure rates of O_2 uptake. Flasks with two side arms were used, one containing substrate in 0·25 ml of 67 mM phosphate buffer and the other, inhibitor in the same buffer. The flask contained 2·0 ml of enzyme suspension. The final concentration of substrate in the flask was 5 mM unless otherwise stated. Inhibitor studies were normally carried out by equilibrating the flasks for 5 min, adding the inhibitor to the enzyme, preincubating for 30 min, adding the substrate, equilibrating for 5 min and reading the manometer at 5 min intervals for a further 30 min, the enzyme concentration used being such that O_2 uptake

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was linear during this period. The mean rate of O_2 consumption in μl/min was calculated. The bath temperature was 37° and the gas phase was air.

(ii) *Assay using radioactive substrate.* The method used was essentially that developed by Otsuka and Kobayashi for tyramine [6] and described in detail by Mantle *et al.* [7].

Experiments with whole animals

a. *Rats.* Male rats of the same strain and age as those used to prepare enzyme for the *in vitro* work were used. Drugs were administered by i.p. injection in isotonic saline and control animals dosed with saline alone.

(i) *Tryptamine excretion.* The animals were placed individually in metabolic cages with free access to food and water, and urine collected during 24 hr after drug administration. Tryptamine excretion was measured by the method of Sjoerdsma *et al.* [8].

(ii) *MAO activity in treated animals measured in vitro.* Animals treated with drugs as described above were killed, livers and brains were removed and enzyme preparations made from individual organs in the manner described for the bulk liver preparation. The MAO activities were compared with those of saline treated controls using manometric assay.

b. *Mice*

(i) *Effect of drugs on metabolism of L-DOPA.* Groups of four male Swiss white mice were given the MAO inhibitors i.p. in saline followed, after 1 hr, by an intraventricular injection of 0·1 μg of [2-^{14}C]L-DOPA (c. 10 nCi) in 0·02 ml of saline. After 20 min, the mice were killed, their brains were removed, homogenised and the total homogenate from each group fractionated according to the method described by Gey and Pletscher [9]. Brains from saline-injected controls were put through the same procedure.

RESULTS

Preliminary experiments established that the unsubstituted phenyloxadiazole compound, PCO (I, R = H) inhibited MAO from various sources, the inhibition being greater when enzyme and inhibitor were pre-incubated in the absence of substrate (Table 1). The presence of oxygen during the pre-incubation

Table 1. Inhibition of rat MAO by PCO: pre-incubation requirement

Tissue source	Substrate	% Inhibition: Pre-incubation without substrate	% Inhibition: Inhibitor added with substrate
Liver	Tyramine	32	6
	5-HT	50	22
Brain	Tyramine	34	10
	5-HT	68	1·5
Heart	Tyramine	74	54
	5-HT	74	32
Kidney	Tyramine	77	42
	5-HT	74	26

Manometric assay. Pre-incubation time without substrate 30 min. Other conditions as in text.

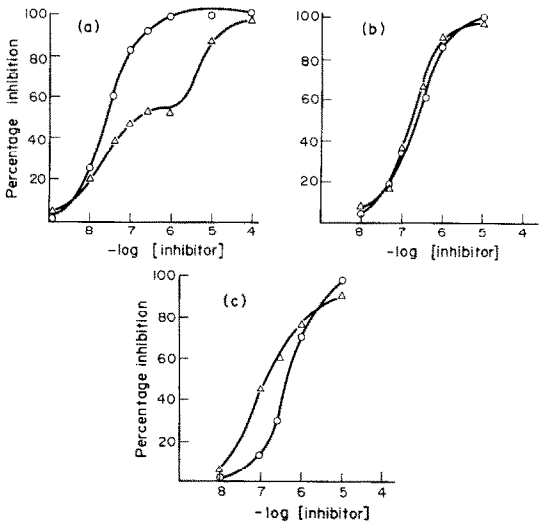


Fig. 1. Inhibition of rat liver mitochondrial MAO by (a) 2-chloro PCO, (b) 3-chloro PCO and (c) 3-nitro PCO. The enzyme was pre-incubated with the concentrations of inhibitor indicated for 30 min and enzyme activity measured using radioactive substrate as described in the text. Percentage inhibitions were calculated from the activity of the enzyme pre-incubated in the absence of inhibitor.

period was not necessary for inhibition to occur and once inhibited, enzyme activity could not be restored by washing or dialysis. The degree of inhibition was found to depend on the duration of pre-incubation, reaching a maximum of 80 per cent after 20 min incubation with 5×10^{-6} M PCO using 5-HT as substrate.

In all subsequent experiments with inhibitors, a pre-incubation time of 30 min was used. The inhibition produced by various initial concentrations of inhibitors was measured and from plots such as those in Fig. 1, I_{25}, I_{50} and I_{75} values could be obtained. These parameters are dependent on the conditions used, particularly on enzyme concentration and comparison of results from different experiments must be made with caution. However, they provide a useful first indication of the relative potencies of different compounds examined under identical conditions and can also be used to indicate clearly, selective inhibition of the oxidation of certain substrates in a multi-substrate enzyme preparation.

The substrate selectivity observed with PCO is shown in Table 2 which gives I_{50} values for MAO from 4 rat tissues with various amines. It can be seen that very different relative I_{50} values were found in the preparations from different tissues.

The selective inhibition observed with PCO was not dependent on the integrity of the membrane in the membrane bound MAO preparations used. Essentially similar results were obtained with a solubilised preparation using radiometric assay (Table 3). I_{50} values obtained corresponded with those found for the membrane bound enzyme using the radiometric assay and were c. 1/100 of those found using manometric assay. This emphasises the arbitrary nature of an I_{50} value. The manometric assay requires enzyme concentrations 50–100 times greater than the radiometric assay and for potent irreversible inhibitors

Table 2. Inhibition of rat MAO preparations by PCO: substrate variation

Substrate	I_{50} values			
	Liver	Kidney	Heart	Brain
Tyramine	4×10^{-5} M	5×10^{-7} M	4×10^{-7} M	3×10^{-6} M
Serotonin	10^{-6}	3×10^{-7}	$2{\cdot}5 \times 10^{-7}$	4×10^{-7}
Tryptamine	3×10^{-5}	5×10^{-7}	3×10^{-7}	5×10^{-6}
Dopamine	$2{\cdot}5 \times 10^{-5}$	6×10^{-7}	4×10^{-7}	3×10^{-6}

Enzyme assays were carried out by the standard manometric procedure described in the text.

Table 3. Inhibition of soluble rat liver MAO by PCO

Inhibitor concentration	% Inhibition	
	5-HT	Tyramine
10^{-8} M	11	0
5×10^{-8}	13	0
10^{-7}	58	8
5×10^{-7}	80	44
10^{-6}	98	55
5×10^{-6}	100	61
10^{-5}	100	75
10^{-4}	100	98

Soluble MAO was prepared as described in the text and assayed by the standard radiometric procedure.

which react fairly specifically with the enzyme active centre, this makes a considerable difference to the I_{50} values observed.

In order to compare the behaviour of a number of compounds of the series, experiments were carried out with rat liver MAO using a range of concentrations of the inhibitors with tyramine and serotonin as substrates. When the curves obtained by plotting the negative logarithm of the inhibitor concentrations against percentage inhibition were examined, the compounds fell into one of three groups. The first, exemplified by the 2-chloro compound (Fig. 1a), were serotonin selective. A second group was non-selective, e.g. the 3-chloro compound (Fig. 1b). The third group was tyramine-selective. The results obtained with 3-nitro PCO are shown (Fig. 1c). The results obtained with the whole series of compounds are expressed in the form of I_{25} and I_{75} values in Table 4. Serotonin selective inhibitors give values of

$$\frac{I_{75}\ \text{tyramine}}{I_{75}\ \text{serotonin}}$$

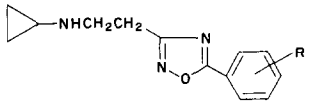
which are greater than 1 and tyramine selective inhibitors, values of

$$\frac{I_{25}\ \text{tyramine}}{I_{25}\ \text{serotonin}}$$

which are much smaller than 1. Otherwise these ratios approximate to unity.

It can be seen that PCO, its 2-chloro, 4-chloro and 4-methyl derivatives, and the 3-pyridyl compound are serotonin selective, the 3-chloro and 3,4-dichloro compounds non-selective, whereas the 3-nitro and 3,4-dimethoxy compounds are tyramine selective. The

Table 4. MAO inhibition *in vitro*



Ro No.	Structure R =	I_{75} (molar concn)			I_{25} (molar concn)		
		A 5-HT	B Tyramine	Ratio B:A	C 5-HT	D Tyramine	Ratio D:C
03–4155	H	5×10^{-6}	3×10^{-4}	60	10^{-7}	2×10^{-7}	2·0
03–4751	2-Cl	2×10^{-6}	3×10^{-5}	15	6×10^{-8}	10^{-7}	1·67
03–4336	3-Cl	10^{-5}	10^{-5}	1·0	5×10^{-7}	5×10^{-7}	1·0
03–4298	4-Cl	4×10^{-6}	6×10^{-5}	15	5×10^{-7}	10^{-6}	2·0
03–4693	3,4-diCl	5×10^{-6}	5×10^{-6}	1·0	8×10^{-7}	5×10^{-7}	0·63
03–4272	3-NO_2	5×10^{-5}	4×10^{-5}	0·8	2×10^{-5}	10^{-6}	0·05
03–4571	4-CH_3	6×10^{-7}	8×10^{-6}	13·3	4×10^{-7}	6×10^{-7}	1·5
03–4755	3,4-diOCH_3	4×10^{-5}	3×10^{-5}	0·75	10^{-5}	10^{-6}	0·1
03–4771	3-pyridyl (replaces phenyl ring)	10^{-4}	$> 10^{-3}$	>10	10^{-5}	10^{-5}	1·0
	Tranylcypromine	5×10^{-6}	5×10^{-6}	1·0	10^{-6}	2×10^{-6}	2·0
	Deprenil	10^{-4}	10^{-4}	1·0	4×10^{-5}	8×10^{-7}	0·02

Enzyme inhibition was measured manometrically as described in the text and I_{25} and I_{75} values obtained from plots of inhibitor concentration against percentage inhibition.

Table 5. Tryptamine excretion by rats treated with PCO and analogues

Drug treatment i.p.	Tryptamine excretion μg/24 hr
Saline	1·9 ± 0·3
PCO 10 mg/kg	12·5 ± 2·4
4-Cl-PCO 10 mg/kg	14·2 ± 1·9
3-NO_2 PCO 10 mg/kg	6·3 ± 1·7
3-NO_2 PCO 25 mg/kg	14·7 ± 3·4

Groups of 6 animals were treated with drugs as indicated and tryptamine determined fluorimetrically in urine collected during the following 24 hr.

known inhibitors Tranylcypromine(*N*-phenylethylcyclopropylamine) and Deprenil ((-)-(R)-*N*,α-dimethyl-*N*-2 (propynylphenethylamine)) are included for comparison, the former being a non-selective and the latter a tyramine selective inhibitor.

Inhibition of MAO in rats and mice following administration of some of these inhibitors was assessed in various ways. Urinary tryptamine excretion during 24 hr following administration of the compounds is given in Table 5. It can be seen that in all cases increased amounts of tryptamine were excreted by the treated animals. It was also found that the inhibitors modified the metabolism of [^{14}C]DOPA, injected intracerebrally into mice, in the same way as other monoamine oxidase inhibitors (Table 6), increasing the proportion of radioactivity recovered in the amine and amino acid fractions.

It could be thus inferred that these inhibitors were acting on MAO *in vivo* and this was confirmed by assaying enzyme activity in preparations of the enzyme from treated animals. The results following single drug administrations are given in Table 7. In both liver and brain clear evidence for selective inhibition of serotonin oxidation was obtained for PCO and its 4-Cl analogue, whereas tranylcypromine and 3-nitro-PCO administration gave enzyme preparations which were equally inhibited with both substrates.

On longer term administration of PCO, the selectivity largely disappeared. The results obtained by examining enzyme preparations from animals dosed twice daily for three days and killed after one further dose on the fourth day are given in Table 8. It can be seen that there is some difference between the inhibitions found for the brain enzyme using serotonin and tyramine as substrates, but this is less clear cut than after single administration of the drugs.

DISCUSSION

This series of cyclopropylamines resembles other arylalkyl substituted cyclopropylamines in inhibiting mitochondrial MAO. As with tranylcypromine (2-phenylcyclopropylamine), inhibition is essentially irreversible and shows time dependence. Unlike hydrazine inhibitors such as iproniazid, PCO and its analogues do not require O_2 to be present during incubation with the enzyme for inhibition to occur.

Various inhibitors of MAO have been reported to show a selective action against the oxidation of certain substrates [4]. Clorgyline, like PCO, shows a selective action against serotonin oxidation [1] and Deprenil, a selective action against tyramine oxidation similar to, but more marked than that found for the 3-nitro and 3,4-dimethoxy analogues of PCO [10]. The selectivity of these inhibitors has been accounted for by postulating that they react at different rates with two types of MAO, the A enzyme, characterised by its ability to oxidise serotonin but not benzylamine, and the B enzyme, which oxidises benzylamine but not serotonin [11]. Some evidence has been obtained that presynaptic neuronal tissue contains only the A enzyme [12] and consistent with

Table 7. MAO activity in rat tissues after single doses of PCO and analogues

Drug treatment mg/kg i.p.	% inhibition compared to saline treated controls			
	Liver		Brain	
	5-HT	Tyramine	5-HT	Tyramine
PCO 1	27	0	35	10
10	68	2	53	18
4-Cl-PCO 1	50	21	80	28
10	86	56	88	50
3-NO_2-PCO 1	4	8	7	5
10	36	46	37	35
Tranylcypromine 1	72	57	82	83
10	95	89	100	100

Groups of 4 female rats were used for each treatment, killed 2 hr after drug administration and organs pooled for enzyme preparation. Enzymes were assayed manometrically.

Table 6. Metabolism of [2-^{14}C]L-DOPA in mice after treatment with PCO and analogues

Metabolite fraction	% of total counts in supernatant recovered in each fraction			
	Saline treated	Ro 4-1340	PCO	4-Cl-PCO
Amines	8·5	26·6	24·3	30·6
Phenol carbonic acids + phenol alcohols	13·1	11·5	8·6	5·2
Amino acids	25·8	34·2	29·4	26·3

Drugs were administered at 20 mg/kg i.p. to groups of 4 male mice followed after 1 hr by [2-^{14}C]L-DOPA intraventricularly. Mice killed 20 min later and pooled brains homogenised and supernatant fractionated.

Ro 4–1340 is L-α-alanyl-N_2-isopropylhydrazide monohydrochloride.

Table 8. MAO activity in rat tissues after multiple doses of PCO and analogues

Drug	Substrate	% inhibition compared to saline treated controls			
		Liver	Brain	Heart	Kidney
PCO	5-HT	28	86	83	41
	Tyramine	35	60	76	43
	Dopamine	43	92	83	37
4-Cl-PCO	5-HT	38	90	92	56
	Tyramine	40	71	88	51
	Dopamine	41	90	85	48
3-NO_2-PCO	5-HT	42	80	90	32
	Tyramine	56	71	70	38
	Dopamine	60	83	92	41
Tranylcypromine	5-HT	90	100	86	87
	Tyramine	89	100	80	89
	Dopamine	86	92	75	85

Groups of 4 rats were treated with drugs 1 mg/kg i.p. twice daily for 3 days (9.00 and 18.00 hr) and once (9.00 hr) on day 4. Animals were killed 4 hr after the final administration and organs pooled for enzyme preparation. Enzymes were assayed manometrically.

this, it has been found that noradrenaline and normetanephrine in addition to serotonin are specific substrates for this form. Only the B enzyme oxidises phenylethylamine whereas other endogenous substrates are oxidised by both enzymes [13]. The properties found for the inhibitors studied here are consistent with the concept of A and B enzymes, the inhibitor curves for tyramine oxidation (Figs. 1a and 1c) being biphasic because it is oxidised by both forms of the enzyme. Small modifications to the inhibitor molecule have a marked effect on the selectivity between the two sites.

From the small number of compounds examined it is possible to draw only a few conclusions concerning the structural determinants of selectivity. Preferential reaction with the A-site seems to depend on the absence of a substituent in the 3-position of the phenyl ring. Compounds with a 3-substituent are either non-selective or react more readily with the B-site. Since both 3-nitro-PCO and 3,4-dimethoxy-PCO show a preference for the B-site, the effect would seem to be steric rather than electronic, a conclusion also supported by the behaviour of the 3-pyridyl compound which, although similar in electron distribution to the 3-nitro compound, shows marked A-site selectivity. It is of interest that Fuller *et al.* [15], when discussing structure activity relationships in their series of phenoxyethylcyclopropylamines, had to give special weighting to steric effects of a substituent in the 3-position. Comparison of models shows that the phenyl ring of PCO is capable of assuming a configuration in which its position relative to the cyclopropylamine N atom is similar to that of the phenyl ring in phenoxyethylcyclopropylamine.

The functional significance of the A and B enzymes remains obscure. Despite the evidence already referred to, suggesting that intraneuronal MAO is of the A form, it is clear that this form is not restricted to that location. Nor does the total absence of the A form from pig brain encourage a belief that this form of MAO is of special significance in neurones. The substrate specificity of the forms is not complete. For example, blood platelets metabolise serotonin to 5-hydroxyindole acetic acid and 5-hydroxy tryptophol [14] despite their reported lack of A-type enzyme. This complicates attempts to assign specific roles to the two forms. More information on the behaviour of monoamine oxidase in its functional state is required.

Highly specific inhibitors for A and B enzymes *in vivo* would assist this work but the evidence from the studies with PCO and its analogues reported here suggests that these compounds do not exhibit *in vivo* such a clear cut selectivity as they show *in vitro*. It may partly be because the reaction rates with the two centres are insufficiently different, but the very marked changes produced by altered substitution in the phenyl ring also means that metabolites may show different specificities to those of the parent drug. However, the results after single administration of PCO and 4-Cl-PCO (Table 7) support previous findings [2] with A-selective inhibitors in showing that this type of selective inhibition can occur *in vivo*, although much less evident when multiple doses of the drugs were given (Table 8).

The compounds have been used *in vitro* to produce MAO with the A-site blocked so that kinetic studies of the B-site can be made [7].

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