Biochemical Pharmacology, Vol. 30, No. 19, pp. 2728–2731, 1981.
Printed in Great Britain.

0006-2952/81/192728-04 $02.00/0

Studies on the mechanism of action of caroxazone, a new antidepressant drug

(*Received* 27 *October* 1980; *accepted* 15 *April* 1981)

Caroxazone [2-oxo-2H-1,3-benzoxazine-3(4H)-acetamide] is a clinically-active antidepressant drug [1, 2] with a novel structure. It has strong anti-reserpine activity in a number of animal species and potentiates the effects of noradrenaline and indolalkylamines [3, 4]. Previous work showed that *in vitro* caroxazone inhibited monoamine oxidase (MAO) (EC 1.4.3.4.) activity in rat brain mitochondria [4] and raised the concentrations of various monoamines in the rat brain [5]. This pharmacological and biochemical profile prompted us to examine in detail its mechanism of action with regard to its effects on MAO and on amine uptake.

Male Sprague–Dawley rats, weighing 180–250 g, were decapitated; the brain (without cerebellum) was quickly removed and homogenized with a Potter–Elvehjem homogenizer with Teflon pestle (0.15 mm clearance) for 30 sec in 10 volumes of 0.3 M mannitol and 0.1 mM EDTA, pH 7.4. A mitochondrial fraction was obtained [6]. The crude mitochondrial pellet was washed three times (10,000 *g*, 10 min) by resuspending in 0.1 M phosphate buffer, pH 7.4; the final pellet was resuspended in the same buffer to a protein content of 1 mg · ml^{-1} and this suspension was used as enzyme source. All operations were performed at 0–2°. MAO activity was assayed by a radiometric method derived from that of Wurtman and Axelrod [7] using the following substrates, each at three concentrations (ranges in parentheses): [^{14}C] serotonin (5-HT) (1–10 μM); [^{14}C]phenylethylamine (PEA) (0.1–1 μM); [^{3}H]dopamine (DA) (10–100 μM). The final volume was 1.5 ml.

The incubation was performed at 37° for 10–30 min with shaking and the reaction was stopped by adding 0.15 ml of 37 per cent HCl (in the case of 5-HT and DA) or 70 per cent perchloric acid (for PEA).

The deaminated products were extracted twice with 3 ml of appropriate solvent: a mixture of ethyl acetate and toluene 1:1 (v/v) for DA; toluene for PEA; peroxide-free ethyl ether for 5-HT [8]. After centrifugation, the radioactivity of 1.5 ml portions of the organic phase was measured. Protein content was determined by the biuret method using bovine serum albumin as standard. MAO activity was expressed as nmoles of product formed · mg protein^{-1}·hr^{-1}. The *in vitro* inhibitory effect of drugs on MAO was assayed by preincubating the mitochondrial fraction with four to six concentrations of each drug covering a 100–1000 fold range for each concentration of the substrate.

Reversibility of *in vitro* MAO inhibition by drugs was assessed by dialysis and washing. The rat brain mitochondrial fraction was incubated with the drug concentration that gave maximal MAO inhibition. A portion was taken to measure the enzymatic activity as previously described. The remaining fraction was either dialyzed for 48 hr at 4° against six changes (5 l. each) of 0.1 M phosphate buffer, pH 7.4, or washed four times with this buffer. MAO activity was again assayed, and the inhibition before and after dialysis or washing was compared. Reversibility of MAO inhibition by caroxazone was also studied by the method of Ackermann [9]. Moreover to study the prevention of irreversible MAO inhibition by tranylcypromine, rats were pretreated with either caroxazone (100 mg · kg^{-1} p.o.) or harmine (10 mg · kg^{-1} i.p.). After 1 or 23 hr they received tranylcypromine (6.25 mg · kg^{-1} p.o.) and 1 hr later were killed. MAO activity was assayed in brain homogenates as described, except that final concentrations of 25 μM [^{14}C]5-HT or 1 μM [^{14}C]PEA were used. Inhibition induced by tranylcypromine in rats pretreated with caroxazone or harmine was compared with that obtained in non-pretreated animals.

To study the drug effect on the levels of monoamines and their acid metabolites in the brain, rats were treated orally with increasing doses of either caroxazone (3.12–25 mg · kg^{-1}) or tranylcypromine (0.78–3.12 mg · kg^{-1}) and killed 2 hr later. Striatal HVA (homovanillic acid) and DOPAC (3,4-dihydroxyphenylacetic acid) were isolated on columns of Sephadex G-10 [10] and assayed fluorometrically [11]. 5-HT and 5-HIAA (5-hydroxyindolacetic acid) were determined in the rest of the brain [12].

The present data show that caroxazone is a MAO inhibitor. *In vitro*, with the rat brain mitochondrial fraction, the IC_{50}s for deamination of 5-HT and DA are 32–39 μM (Table 1). The deamination of PEA (IC_{50} = 4.5 μM) is 7 times

Table 1. *In vitro* effect of caroxazone and other inhibitors on MAO-A and MAO-B activities in the rat brain mitochondrial fraction

	[^{14}C] 5-HT	[^{3}H] DA	[^{14}C] PEA
Drug		IC_{50}, μM	
Caroxazone	32	39	4.5
Tranylcypromine	0.35	0.14	0.23
Clorgyline	0.004	0.003	3.1
Harmine	0.003	0.009	7.0
Deprenil	1.7	1.3	0.019

Drugs were preincubated with the enzyme preparation for 30 min at 37° before addition of substrates. Incubation was continued after addition of the substrates (three concentrations of each). Drugs were tested in a range of 4–6 concentrations for each concentration of substrate. Mean IC_{50}s are reported.

Table 2. Effect of caroxazone and tranylcypromine on concentrations of dopamine metabolites HVA and DOPAC and 5-hydroxyindoles in the rat brain

Experimental group	Dose ($mg \cdot kg^{-1}$)	HVA		DOPAC		5-HT		5-HIAA	
		$\mu g \cdot g^{-1}$	ED_{50} ($mg \cdot kg^{-1}$)	$\mu g \cdot g^{-1}$	ED_{50} ($mg \cdot kg^{-1}$)	$\mu g \cdot g^{-1}$	ED_{50} ($mg \cdot kg^{-1}$)	$\mu g \cdot g^{-1}$	ED_{50} ($mg \cdot kg^{-1}$)
(1) Control	–	0.46 ± 0.03		0.80 ± 0.04		0.45 ± 0.02		0.55 ± 0.02	
caroxazone	3.12	0.33 ± 0.02		0.50 ± 0.02		–		–	
	6.25	0.28 ± 0.01	10.6	0.30 ± 0.03	4.95	0.56 ± 0.03	>25	0.47 ± 0.02	N.E.
	12.50	0.23 ± 0.01		0.29 ± 0.02		0.60 ± 0.03		0.46 ± 0.02	
	25.00	0.15 ± 0.01		0.23 ± 0.02		0.63 ± 0.02		0.44 ± 0.02	
(2) Control	–	0.48 ± 0.03		0.85 ± 0.02		0.50 ± 0.03		0.61 ± 0.02	
tranylcypromine	0.78	0.39 ± 0.02		0.54 ± 0.02		0.58 ± 0.03		0.55 ± 0.02*	
	1.56	0.11 ± 0.01	1.13	0.21 ± 0.03	0.99	0.73 ± 0.04	1.86	0.46 ± 0.02	3.78
	2.34	N.D.		0.14 ± 0.02		–		–	
	3.12	N.D.		0.09 ± 0.008		0.87 ± 0.05		0.32 ± 0.01	

The drug effect on HVA and DOPAC was studied on groups of 7 rats; for 5-HT and 5-HIAA, 13 animals were treated with caroxazone and 23 with tranylcypromine. Animals were killed 2 hr after treatment. HVA and DOPAC were assayed in the striatum. 5-HT and 5-HIAA in the rest of the brain. Mean concentrations ($\mu g/g$) ± S.E.M. and ED_{50} ($mg \cdot kg^{-1}$) are shown. The latter were calculated by the least squares method. All values are statistically different from controls (Dunnett's test: $P < 0.01$) except when indicated (*); N.D. = not detectable; N.E. = not evaluable.

more sensitive to caroxazone than that of 5-HT, suggesting greater specificity of this drug towards the form B of MAO. The IC_{50}s of tranylcypromine are between 0.1 and 0.3 μM with no great difference between the three substrates, a finding in accordance with the designation of this drug as a non-specific MAO inhibitor. Clorgyline and harmine (approximately 800–2000 times more active on 5-HT than on PEA) and L-deprenyl (90 times more effective on PEA than on 5-HT) show the expected specificity towards forms A and B of MAO, respectively [15]. The present data confirm that in the rat brain DA is a preferred substrate of MAO A [16]; in fact its deamination is 430 times more sensitive to clorgyline than to L-deprenyl.

The effect on monoamine metabolism in brain demonstrates that caroxazone also inhibits MAO *in vivo* (Table 2). The level of both dopamine metabolites (HVA and DOPAC) in the striatum is reduced after administration of 3.12 mg · kg^{-1} caroxazone and this effect is dose-dependent with ED_{50}s of 4.95–10.6 mg · kg^{-1}. 5-HT metabolism is less affected by caroxazone; the ED_{50} for its increase is well above 25 mg · kg^{-1} reaching 70 mg · kg^{-1} (extrapolated), whereas the reduction of its metabolite 5-HIAA is only slight and not related to the dose. Tranylcypromine is more active than caroxazone on the metabolism of both amines; again, the metabolites of DA are affected more than that of 5-HT as shown by comparison of the ED_{50}s (1 vs 3.8 mg · kg^{-1}).

The least active of the tested doses of caroxazone are 6.25 mg · kg^{-1} for 5-HT and 3.12 mg · kg^{-1} for HVA and DOPAC. It is pertinent to recall that the doses of caroxazone with antireserpine activity are in the range of 5–10 mg · kg^{-1} p.o. These results seem to indicate that the pharmacological activities of caroxazone are related to its MAO-inhibiting effect.

The present findings also show that caroxazone is a reversible inhibitor of MAO. This conclusion is supported by the concomitant results of two different *in vitro* procedures, i.e. washing and dialysis, which completely remove caroxazone from brain mitochondria. Results shown in the white columns of Fig. 1 confirm that the addition of caroxazone (50 μM), tranylcypromine (1 μM) or harmine (either 1 or 100 μM) almost completely inhibits MAO with 5-HT or PEA as substrates. It is also further confirmed that the oxidation of PEA is less sensitive to harmine than that of 5-HT. The black columns show that inhibition by caroxazone and harmine is completely abolished by washing or dialysis of mitochondria preincubated with drugs. MAO inhibition by tranylcypromine is not affected at all by these two procedures.

Reversibility of caroxazone effect is confirmed by the lack of difference between inhibition of MAO with and without preincubation and by the results obtained using the Ackermann method in which MAO activity in the presence of inhibitor was plotted against protein concentration, resulting in a straight line passing through the origin with a less steep slope than in the absence of inhibitor (results not shown).

As reversible inhibitors, caroxazone and harmine should protect MAO from the effects of covalently bound drugs which therefore irreversibly inhibit the enzyme. This is indeed the case as shown in Fig. 2. Tranylcypromine induces almost complete inhibition with both 5-HT and PEA at 2 and 24 hr. Pretreatment with caroxazone significantly reduces the MAO inhibitory activity of tranylcypromine with both substrates at 2 hr and, to a lesser extent, also at 24 hr. Harmine is effective only with 5-HT at 2 hr, thus confirming both the reversibility of its MAO inhibition and the specificity towards MAO A.

On account of its reversible binding, the inhibitory and protecting effects of caroxazone on MAO (and its pharmacological and therapeutic activities) depend on the drug concentration at the enzyme level, and therefore on its pharmacokinetics which is correlated with the dose. Conversely, for irreversible inhibitors, once the enzyme is blocked, restoration of its activity depends on the rate of *de novo* protein synthesis which is characteristic for each organ, but in any case is a lengthy process [17]. The latter observation elucidates the mechanism of the cumulative effect of irreversible MAO inhibitors. Such different mechanisms explain why there is a good correlation between the caroxazone dose, plasma concentration and potentiating effect on the pressure response to tyramine [18], while the correlation is lacking for irreversible MAO inhibitors [19].

Caroxazone up to the dose of 100 mg · kg^{-1} i.p. or p.o.

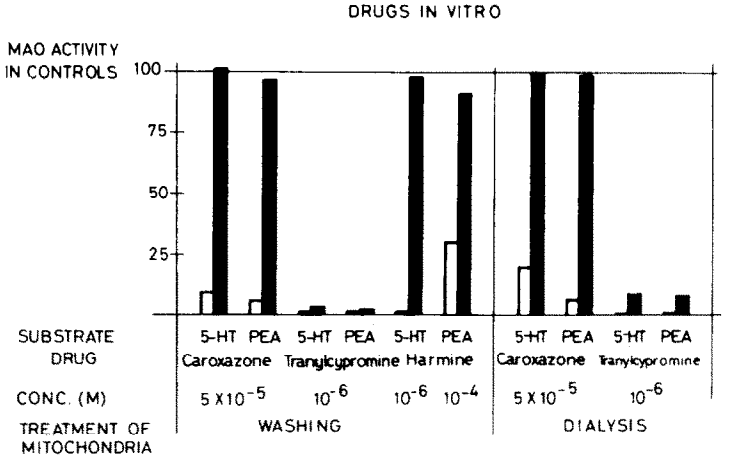

Fig. 1. Testing the reversibility of MAO inhibition by caroxazone and other drugs. The brain mitochondrial fraction was incubated with drugs at 37° for 30 min and MAO activity was determined before (white columns) and after (black columns) washing or dialysis. Results are expressed as per cent changes of MAO activity in comparison with controls.

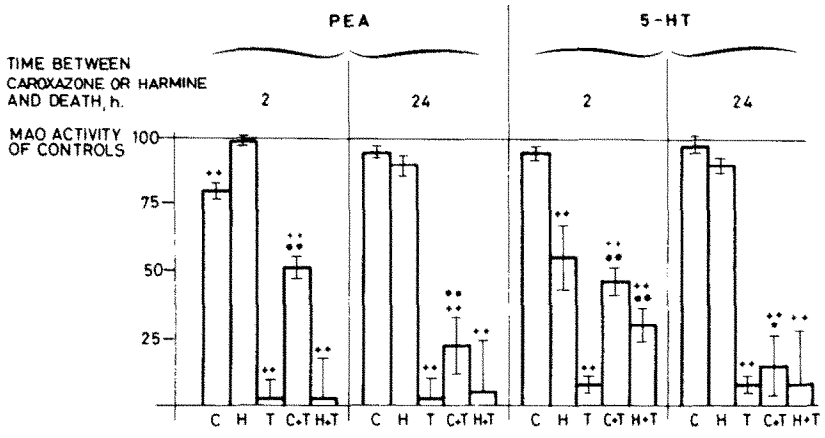

Fig. 2. Protective effect of caroxazone (C) and harmine (H) against irreversible MAO inhibition induced by tranylcypromine (T). Groups of 8 rats were treated with caroxazone (100 mg · kg^{-1} p.o.) or harmine (10 mg · kg^{-1} i.p.) and killed after either 2 or 24 hr. Other groups were treated with caroxazone or harmine as before but also received tranylcypromine (6.25 mg · kg^{-1} p.o.) 1 hr before death. MAO activity was assayed in brain homogenates either with 1 μM [^{14}C]PEA or 25 μM[^{14}C]5-HT. Results are expressed as per cent changes of MAO activity in comparison with controls (mean ± S.E.M.). ++P < 0.01 compared to control value; *P < 0.05 or **P < 0.01 compared to tranylcypromine alone (Statistical analysis by Dunnett's test).

does not inhibit the uptake of NA and 5-HT by rat brain slices. Imipramine is more active by the i.p. route (ED_{50} = 5.1 mg · kg^{-1}) than p.o. (ED_{50} = 17.3 mg · kg^{-1}) on NA uptake. Chlorimipramine inhibits 5-HT uptake with an ED_{50} of 36.3 mg · kg^{-1} i.p. In other *in vitro* experiments caroxazone was found to have no effect on 5-HT uptake and release by rat platelets (G. de Gaetano, personal communication) and on α_2 adrenergic receptors in rat brain (results not shown).

In conclusion, caroxazone has been shown to be a MAO inhibitor and probably such inhibition could account for its pharmacological activities. Available evidence gives no support to an alternative mechanism of action. The reversible nature of its activity clearly differentiates caroxazone from the other MAO inhibitors used in clinical practice which produce irreversible, and therefore long-lasting, inhibition. On the other hand, among the other reversible MAO inhibitors, none has yet been proved to be of clinical value.

Ricerca e Sviluppo Biologico Biochimica Farmacologica e Ricerca e Sviluppo Chimico Centro Ricerche Farmitalia Carlo Erba 20014 Nerviano, Italy

ANTONIO MORETTI*
CARLA CACCIA
GABRIELLA CALDERINI
MILENA MENOZZI
ANGELO AMICO

* Author to whom correspondence should be addressed.

REFERENCES

1. S. Cecchini, P. Petri, R. Ardito, S. R. Bareggi and A. Torriti, *J. Int. Med. Res.* **6**, 388 (1978).
2. L. Conti, L. Bonollo, A. Martini, P. Fornaro, A. Guerrini, C. Romerio, E. Scillieri, S. Soverini, M. Stramba-Badiale and G. B. Cassano, *Curr. Ther. Res.* **27**, 458 (1980).
3. G. K. Suchowsky and L. Pegrassi, *Arzneim.-Forsch., Drug Res.* **19**, 643 (1969).
4. G. K. Suchowsky, L. Pegrassi, A. Moretti and A. Bonsignori *Arch. int. Pharmacodyn.* **182**, 332 (1969).
5. A. Moretti, L. Pegrassi, A. H. Glässer and G. K. Suchowsky, *Boll. Chim. Farm.* **113**, 36 (1974).
6. K. Ozawa, K. Seta, H. Takeda, K. Ando, H. Handa and C. Araki, *J. Biochem. Tokyo* **59**, 501 (1966).
7. R. J. Wurtman and J. Axelrod, *Biochem. Pharmac.* **12**, 1439 (1963).
8. M. Jain, F. Sands and R. W. von Korff, *Analyt. Biochem.* **52**, 542 (1973).
9. W. W. Ackermann and V. R. Potter, *Proc. Soc. exp. Biol. Med.* **72**, 1 (1949).
10. B. H. C. Westerink and J. Korf, *Eur. J. Pharmacol.* **38**, 281 (1976).
11. P. F. Spano and N. H. Neff, *Analyt. Biochem.* **42**, 113 (1971).
12. G. Curzon and A. R. Green, *Br. J. Pharmac.* **39**, 653 (1971).
13. S. B. Ross and A. L. Renyi, *Eur. J. Pharmac.* **2**, 181 (1967).
14. S. B. Ross and A. L. Renyi, *Eur J. Pharmac.* **7**, 270 (1969).
15. N. H. Neff and H.-Y. T. Yang, *Life Sci.* **14**, 2061 (1974).
16. P. C. Waldmeier, A. Delini-Stula and L. Maître, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **292**, 9 (1976).
17. G. Planz, K. Quiring and D. Palm, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **273**, 27 (1972).
18. A. Martini, L. Bonollo, R. Sega, A. Palermo, E. Braibanti and F. B. Nicolis, *Br. J. Clin. Pharmacol.* (in press).
19. D. Palm, H.-J. Fengler, H.-G. Güllner, G. Planz. K. Quiring, B. May, D. Helmstaedt, B. Lemmer, H. K. Moon and Ch. Holler, *Eur. J. Clin. Pharmacol.* **3**, 82 (1971).