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INTERACTIONS OF SOME ANALOGUES OF THE ANTICONVULSANT MILACEMIDE WITH MONOAMINE OXIDASE

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Abstract—A series of analogues of the anticonvulsant drug milacemide (2-(*n*-pentylamino)-acetamide; Compound I) has been synthesized: 2-(benzylamino)acetamide (Compound II), 2-(phenethylamino)acetamide (Compound III), 2-(2-indol-3-yl)-ethylamino acetamide (Compound IV), 2-(2-(5-methoxyindol-3-yl)ethylamino)-acetamide (Compound V), 2-(2(4-chlorobenzamido)-ethylamino)acetamide (Compound VI), 2-(2-benzamidoethylamino)-acetamide (Compound VII) and 2-(4-(3-chlorobenzyloxy)phenethylamino)acetamide (Compound VIII). These compounds involve retention of the aminoacetamide portion of milacemide but replacement of the pentyl moiety with aromatic residues present in the structures of substrates and inhibitors of the monoamine oxidases. All the compounds tested were substrates for ox liver monoamine oxidase-B (MAO-B), producing an aldehyde that could act as a substrate for ox liver aldehyde dehydrogenase and H_2O_2 as a result of oxidative cleavage which also released glycinamide, although their Michaelis–Menten parameters differed markedly. None showed detectable activity as substrates for rat liver monoamine oxidase-A (MAO-A). Inhibition of the MAO-B by all the compounds except Compounds VIII and IV showed marked time dependence and was at least partly irreversible. There was no apparent change in the inhibition of MAO-A during enzyme-inhibitor preincubation at 37° for 60 min. Compound VIII was a potent reversible inhibitor of both MAO-A and MAO-B ($K_i = 2.8 \pm 0.1$ and 4.1 ± 0.8 μM), respectively. Comparison of the inhibitory potencies and the specificity constants of the series of compounds as substrates for MAO-B revealed no simple correlations with their anticonvulsant activities, as measured by their ability to prevent bicuculline-induced convulsions and death in the mouse. These results suggest that neither inhibition of MAO nor oxidative cleavage by this enzyme to yield glycinamide plays the major role in the anticonvulsant action of these compounds.

Key words: bicuculline; convulsions; 5-hydroxytryptamine; 2-(*n*-pentylamino)acetamide; 2-phenylethylamine; tyramine

Milacemide [2-(*n*-pentylamino)acetamide] (Compound I; Scheme 1) has been reported to have anticonvulsant activity in some, but not all, animal models of epilepsy [1–5]. van Dorsser *et al.* [1], who compared a number of animal models of generalized seizures, showed it to be particularly effective in inhibiting the convulsions induced by bicuculline. The effectiveness of milacemide in humans suffering from various forms of epilepsy has not been conclusively established [6] and its mechanism of action remains unclear. Janssens de Varebeke *et al.* [7] reported that milacemide was a good substrate for the MAO-B‖ (EC 1.4.3.4) but that it was oxidized only poorly by MAO-A. Furthermore, acute administration of milacemide to rats was found to result in the urinary elimination of glycinamide, which was partly prevented by pretreatment with the MAO-B-selective inhibitor *l*-deprenyl but not by the MAO-A-selective inhibitor clorgyline [7]. Oral administration of milacemide (100 mg/kg) resulted in increased concentrations of glycine in rat forebrain, cerebellum and medulla [8]. A significant increase in glycine levels in rat cortex, cerebellum and hippocampus, but not in striatum and substantia nigra, was also reported after i.p. administration of the same dose [3]. Oral administration of milacemide (100 mg/kg) has also been reported to result in a significant increase of the GABA levels in rat substantia nigra [9], but this result was not observed after i.p. administration of the same dose [3].

Thus milacemide acts as a precursor of glycine in the brain. However, it is probable that other factors may contribute to its anticonvulsant activity. In addition to being a substrate for MAO-B milacemide has been reported to be a time-dependent inhibitor of that enzyme [10]. Since MAO inhibitors have, themselves, been reported to have anticonvulsant actions [11, 12] the contribution of the effect on MAO is hard to disentangle from the actions of milacemide on brain glycine levels. The milacemide analogue α-methyl-milacemide [2-((1-methyl)pentyl-amino)acetamide] was shown to be an effective anticonvulsant although it was not a substrate for MAO and did not increase the urinary excretion of glycinamide [13].

The selectivity of milacemide as a substrate for MAO-B has been questioned by Truong *et al.* [14],

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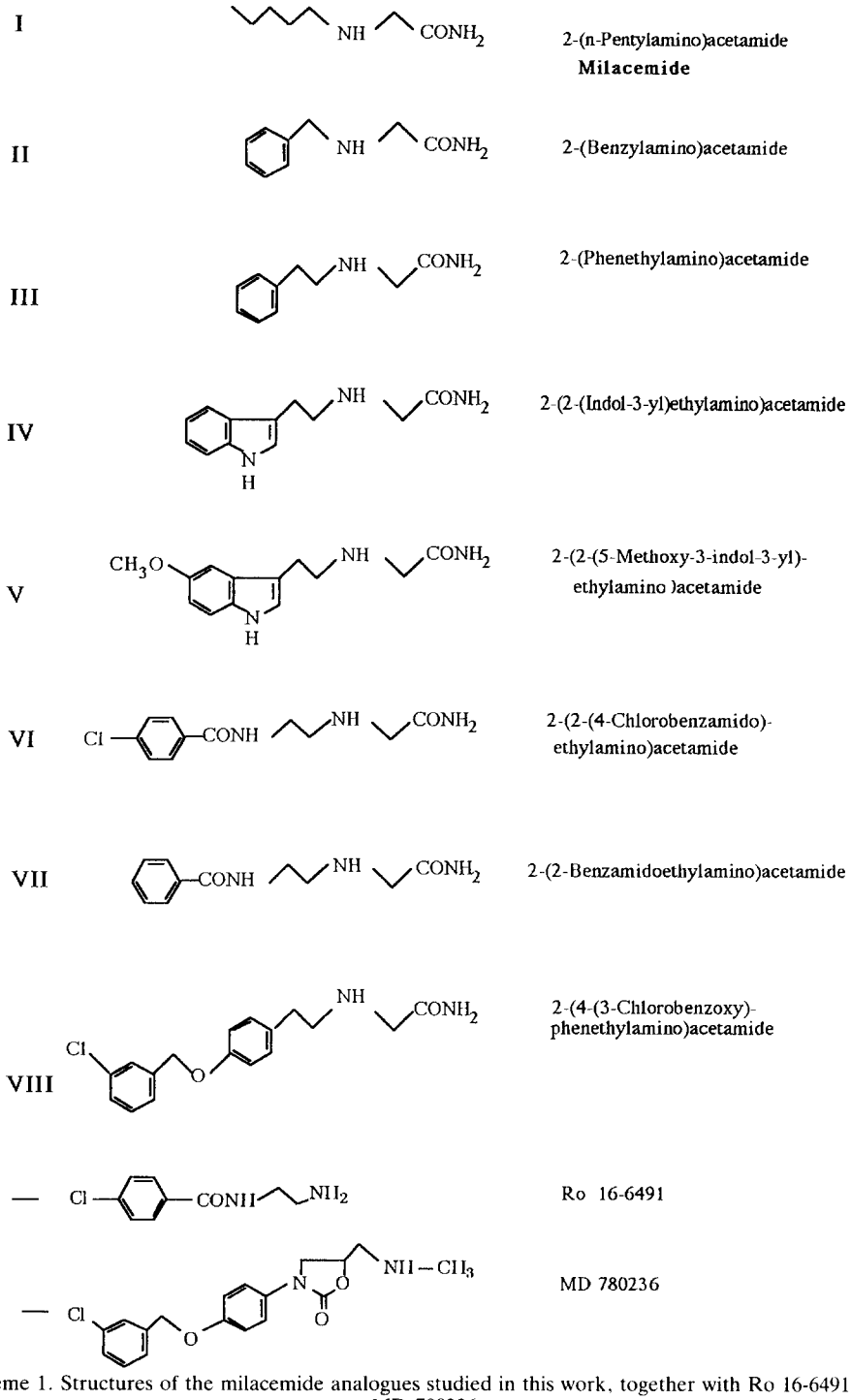
‖ Abbreviations: MAO, monoamine oxidase; MAO-B, monoamine oxidase-B; MAO-A, monoamine oxidase-A; 5-HTP, 5-hydroxytryptophan.



Scheme 1. Structures of the milacemide analogues studied in this work, together with Ro 16-6491 and MD 780236.

who reported that the acute administration of milacemide potentiated the "wet dog shake" syndrome induced by 5-HTP in the rat; such a potentiation was observed after pretreatment with clorgyline but not with *l*-deprenyl [15]. A significant decrease in the concentrations of 3,4-dihydroxyphenylacetic acid (DOPAC) in striatum and substantia nigra [16], as well as significantly increased levels of 5-HT and 5-hydroxyindoleacetic acid in rat frontal cortex and cerebellum have been found after oral administration of 100 mg/kg milacemide (Dostert and Cini, unpublished results). Furthermore, milacemide [17] was found to behave similarly to 5-HTP and 5-HT [18] in antagonizing the *pp'*-

DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane)-induced myoclonus in mice. This myoclonus was also found to be antagonized by *l*-deprenyl and by non-selective MAO inhibitors but not by clorgyline [19].

Despite the uncertainties of the mechanism of action of milacemide as an anticonvulsant agent, it represents a useful model system for the delivery of substances to the brain. Thus, analogues of milacemide might be used to deliver variants of either the glycinamide or the pentylaldehyde portion of the molecule if they were substrates for MAO. In the present study a series of glycinamide derivatives have been examined as substrates for, and inhibitors of, the monoamine oxidases. Their relative potencies as anticonvulsants have also been compared with those of milacemide.

MATERIALS AND METHODS

Materials. 5-Hydroxytryptamine-(side chain-2-^{14}C) creatine sulphate, tyramine-(2-^{14}C) HCl and 2-phenylethylamine-(ethyl-1^{14}C) hydrochloride were obtained from Amersham International or New England Nuclear. *l*-Deprenyl was kindly given by Prof. J. Gaál (Chinoin Pharmaceutical Co. Ltd, Budapest, Hungary). Ox liver mitochondria and purified MAO-B from that source were prepared by the method of Salach [20]. Rat liver mitochondria pretreated with *l*-deprenyl [21] were used as a source of MAO-A activity. Aldehyde dehydrogenase (EC 1.2.1.3) was prepared from ox liver as previously described [22] and 1 U of activity is defined as the amount that catalyses the formation of 1 μmol product/min at 37° in the presence of 500 μM NAD^+ and 3 mM acetaldehyde. The molar extinction coefficient of NADH at 340 nm was taken to be 6220/M/cm [23]. Horseradish peroxidase (EC 1.11.1.7) Type II was obtained from Sigma.

Synthesis of glycinamide derivatives. The structures of the compounds examined in these studies are shown in Scheme 1. In addition to milacemide (Compound I), the acetamide derivatives prepared were: 2-(benzylamino)acetamide (Compound II), 2-(phenethylamino)acetamide (Compound III), 2-(2-indol-3-yl)-ethylaminoacetamide (Compound IV), 2-(2-(5-methoxyindol-3-yl)ethylamino)acetamide (Compound V), 2-(2-(4-chlorobenzamido)-ethylamino)-acetamide (Compound VI), 2-(2-benzamidoethylamino)acetamide (Compound VII), and 2-(4-(3-chlorobenzyloxy)phenethylamino)acetamide (Compound VIII).

Compounds I–III were prepared as reported by Roncucci *et al.* [24]; Compounds IV–VII were synthesized analogously by condensation of the corresponding primary amine (e.g. tryptamine for compound IV) with 2-chloroacetamide in ethanol at reflux in the presence of an excess of potassium carbonate. The primary amines were either purchased from Aldrich (for Compounds IV and V) or prepared by condensation of the appropriate benzoic acid ethylester with ethylenediamine according to the method of Hall [25]. Compound VIII was synthesized as described elsewhere [26].

Stock solutions of Compound VIII were made up in water and heated to 37° to dissolve. Solutions of the other milacemide analogues at the required concentrations were prepared in water and could be stored at −20° until used.

Methods. All enzyme assays were performed at 37° and pH 7.2. MAO activity towards 5-HT (100 μM), 2-phenylethylamine (10 μM) as substrates for MAO-A and MAO-B, respectively, or 150 μM tyramine was determined by the radiochemical procedure previously described [27].

The activity of MAO-B towards the milacemide analogues, with the exception of 2-(benzylamino)-acetamide (compound II), was examined by the coupled spectrophotometric assay, in which the formation of NADH is followed continuously at 340 nm as the aldehyde product is further oxidized by aldehyde dehydrogenase [22]. The assay mixture contained 80 mM potassium phosphate buffer, pH 7.2, 500 μM NAD^+, 0.015 U of aldehyde dehydrogenase, the enzyme preparation and the milacemide analogue at the concentrations indicated. The activity of aldehyde dehydrogenase was not rate limiting under any of the assay conditions used.

The ability of the milacemide analogues to act as a substrate for MAO was also assessed by the coupled spectrophotometric assay and by determining the rate of hydrogen peroxide formation by the luminometric procedure [28]. In the latter case the reaction mixture contained in a final volume of 3 mL, 93.3 mM potassium phosphate buffer, pH 7.2, 3.1 mM sodium azide and an appropriate concentration of mitochondria or MAO-B. The assay mixture was incubated in a cuvette for 5–7 min at 37°. The reaction was initiated by the addition of substrate. At specific time intervals, 50–100 μL aliquots were withdrawn and injected into vials containing 67 mM Tris–HCl buffer, pH 8.0, 750 nM horseradish peroxidase and 25 μM luminol. The instantaneous luminescent signal was recorded and the absolute concentration of hydrogen peroxide present in the MAO reaction at that time of the assay could be calculated by reference to a standard curve.

The oxidation of 2-(benzylamino)acetamide (Compound II; 333 μM) was determined spectrophotometrically by following the increase in absorbance at 250 nm by a procedure based on the direct spectrophotometric procedure of Tabor *et al.* [29]. The reaction mixture contained 93 mM potassium phosphate buffer, pH 7.2, enzyme and the milacemide analogue. The molar extinction coefficient of the benzaldehyde formed was taken to be 13,800/M/cm [30].

The reversibility of inhibition was determined, with mitochondria as the enzyme source, by repeated centrifugation and resuspension [31] as detailed in [13]. The reversibility of inhibition of purified MAO-B by milacemide was also studied by dilution [32]. Enzyme samples (10 times the final concentration) were incubated for 1 hr at 37° with different concentrations of milacemide related to its IC_{50} value (0.25–100 IC_{50}; where IC_{50} is the inhibitor concentration giving 50% inhibition under the stated conditions). The samples were then diluted 10 times into the assay mixture. A parallel experiment was performed where the enzyme (10 times the final concentration) was incubated for 1 hr at 37° with an

Table 1. Kinetic parameters for milacemide and its analogues by purified ox liver MAO-B

Compound	K_m (N) (μM)	k_{cat} (N) (min)	k_{cat}/K_m (min/μM)
I	231 ± 38 (7)	920 ± 148 (4)	3.98
II	940 ± 127 (4)	95 ± 21 (4)	0.10
III	326 ± 84 (4)	320 ± 63 (4)	0.98
IV	484 ± 24 (2)	30 ± 2 (2)	0.06
V	—	—	0.004
VI	259 ± 45 (3)	263 ± 125 (3)	1.01
VII	755 ± 212 (5)	268 ± 61 (5)	0.35
VIII	Insolubility precluded determination of parameters		

The coupled spectrophotometric assay with aldehyde dehydrogenase was used in all cases except for Compound II, where the direct spectrophotometric assay was used. Values quoted are the means ± range or SEM from two or more separate determinations (N), as indicated. The catalytic constant k_{cat} (maximum velocity/enzyme concentration) values were calculated from the enzyme active site concentrations determined by the radioactive pargyline binding procedure, as described in the text.

equivalent amount of water. The samples were then diluted 10 times into an assay mixture containing the same final concentration of milacemide as would have been obtained after dilution of the first group of samples. Both sets were assayed for activity using radiolabelled substrate. The curves of percentage inhibition against the final inhibitor concentration would be identical for the samples that had been pretreated with inhibitor and those that had not if the inhibition was reversible. A significant divergence of the curves reflecting a greater inhibition of the samples that had been pre-incubated with milacemide would be indicative of irreversible inhibition.

Michaelis–Menten kinetic parameters were determined by direct fitting of data for the dependence of initial velocity on substrate concentration by non-linear regression. The double-reciprocal plot is used only for illustrative purposes. The MAO-B concentrations were determined by the radioactive pargyline binding procedure [33] adapted for use with soluble enzyme preparations [34]. Although there was some variation between the final specific activities of different MAO-B preparations from ox liver, it was found that these depended solely on the molar enzyme concentration, as determined by pargyline binding.

Anticonvulsant potencies were assessed in the mouse by determining the effective dose for antagonizing bicuculline-induced convulsions and death in 50% of the animals (ED_{50}), as previously described [13].

RESULTS

Behaviour as substrates

The ability of the compounds to act as substrates for MAO-B was assessed and the kinetic constants were determined from the dependence of initial rate on substrate concentration. The Michaelis–Menten parameters determined for each substrate are shown in Table 1 and representative plots of the behaviour are shown in Fig. 1. It was not possible to determine K_m and V_{max} values for Compound V since saturation was not achieved; see Fig. 1A. However, the value of k_{cat}/K_m, determined from the slope of that graph, is shown in Table 1. Insolubility at higher concentrations also prevented the determination of Michaelis–Menten parameters for Compound VIII. A concentration of 300 μM, which was the maximum concentration for solubility, gave an initial rate that was less than 1.7% of that observed with milacemide under the same conditions. The kinetic parameters for the oxidation of *n*-pentylamine, the "parent amine" of milacemide, were also determined for comparative purposes. A K_m of 56 ± 5 μM (mean ± SEM of seven separate determinations) was determined, and the V_{max} value was approximately 3.5 times greater that obtained with milacemide.

None of the compounds gave detectable activity, assessed by the luminometric assay, as substrates for rat liver MAO-A when assayed at concentrations corresponding to their zero-time IC_{50} values as inhibitors of that enzyme (see Table 2). Under the same conditions tyramine (150 μM) was oxidized by the rat liver mitochondrial MAO-A preparation with a specific activity of 2 ± 0.2 nmol/min/mg (mean ± SEM of four separate determinations).

Inhibition studies

Insoluble preparations of MAO are required to assess reversibility of inhibition using the procedure of repeated centrifugation and resuspension [31], and for comparability, ox liver mitochondria were used as a source of MAO-B in these studies and to assess the inhibitory behaviour of these compounds. Milacemide, itself, was a time-dependent inhibitor of the enzyme (see Table 2). All of the other compounds, with the exception of Compounds IV and VIII, were found to be time-dependent inhibitors of the enzyme. The IC_{50} values determined in the absence of preincubation with the enzyme and after preincubation for 60 min with the enzyme are given in Table 2. Prolonged incubation of the MAO-B preparation at 37° for up to 240 min with Compound

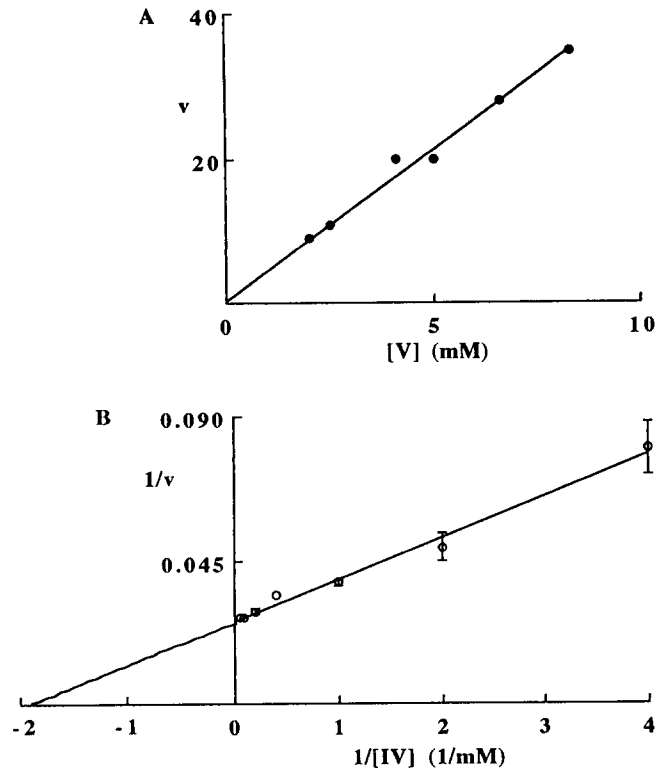


Fig. 1. Kinetics of the oxidation of Compounds V (A), and IV (B) by purified ox liver MAO-B. The results in B are presented as double-reciprocal plots and each point is the mean ± range of duplicate determinations. Initial velocities (v) are expressed as mol product/min/mol enzyme. The data in A are a direct plot of initial velocity versus substrate concentration, since the dependence did not approach saturation over the concentration range used.

Table 2. IC_{50} values for the inhibition of MAO-B from ox liver mitochondria and MAO-A from rat liver mitochondria by milacemide (I) and its analogues

Compound	Enzyme	IC_{50} (μM) Preincubation time (min) 0	60
I	MAO-B	985 ± 15	283 ± 26
	MAO-A	163 ± 67	152 ± 56
II	MAO-B	2150 ± 50	880 ± 100
	MAO-A	42.6 ± 11	47.9 ± 6.3
III	MAO-B	613 ± 63	190 ± 20
	MAO-A	205 ± 75	237 ± 64
IV	MAO-B	447 ± 67	441 ± 61
	MAO-A	13.9 ± 2.3	13.6 ± 0.9
V	MAO-B	2600 ± 10	1410 ± 20
	MAO-A	144 ± 24	112 ± 35
VI	MAO-B	1200 ± 100	249 ± 20
	MAO-A	64.3 ± 22	56.6 ± 22
VII	MAO-B	3900 ± 100	373 ± 97
	MAO-A	94.6 ± 3.4	86.5 ± 4.6
VIII	MAO-B	4.1 ± 0.8	3.5 ± 0.4
	MAO-A	2.8 ± 0.1	2.9 ± 0.3

Each value represents the mean ± range of two separate experiments, except in the case of compound V (MAO-B) where the values shown are the means ± standard error of ratio (SER) from single IC_{50} determinations where independent inhibition estimations were made in triplicate. Tyramine and 5-HT were used as the substrates for MAO-B and MAO-A, respectively, and activities were determined radiochemically.

IV resulted in a small increase in the degree of inhibition (Fig. 2A), whereas there was no change in the inhibition elicited by Compound VIII. The other compounds showed a more pronounced increase in inhibitory potency, as shown for Compound II in Fig. 2B. In all cases, control experiments in which the inhibitor was incubated under identical conditions, but in the absence of enzyme, before the reaction was started by the addition of enzyme and substrate, showed no change in inhibitory potencies over these extended incubation times, indicating that there was no significant breakdown of these compounds to products that were either more or less inhibitory under these conditions.

Although the significance of IC_{50} values will depend on the type of inhibition involved and, in the case of irreversible inhibitors, on the enzyme concentration itself (see Refs. 35, 36 for reviews), they nevertheless provide a useful guide to potency and time dependence under any set of defined conditions. Time-dependent inhibition does not necessarily indicate that an irreversible process is involved [37]. Reversibility was assessed by the repeated washing by centrifugation and resuspension after preincubation of the enzyme and inhibitor for 60 min at 37°. Under these conditions Compound III was found to be an irreversible inhibitor of MAO-B, with very little activity being regained after five centrifugation–resuspension cycles (Fig. 3A). The

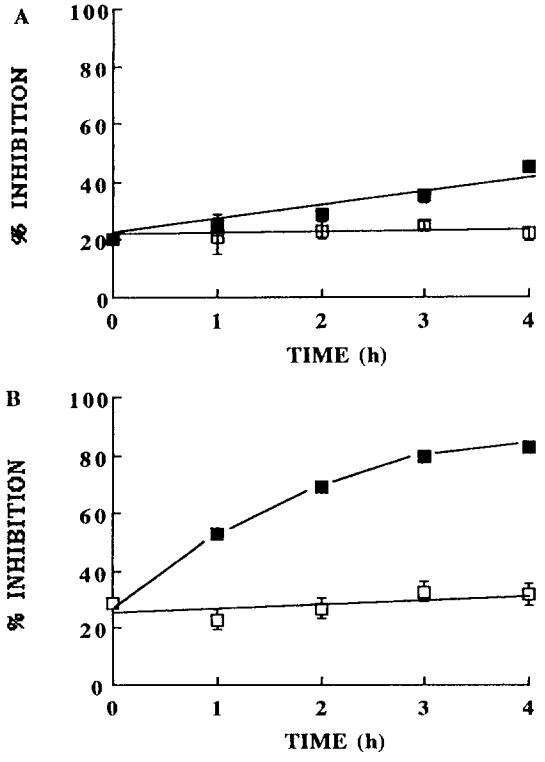


Fig. 2. Time-courses of the inhibition of ox liver mitochondrial MAO-B by 200 μM Compound IV (A) and 750 μM Compound II (B). The enzyme preparation, at a concentration of 0.2 mg protein/mL, was incubated at 37° with the inhibitor (■) or with the equivalent volume of buffer (□) for the indicated time before the MAO-B activity was determined towards 150 μM tyramine. Each point is the mean ± standard error of ratio (SER) of a triplicate determination.

inhibition by Compound II showed partial recovery of activity but an irreversible component was evident with about 30% inhibition still remaining after four centrifugation–resuspension cycles (Fig. 3B). Similarly, there was an apparently irreversible component to the inhibition by Compounds V, VI and VII, with approximately 22%, 44% and 38% inhibition, respectively, remaining after four centrifugation–resuspension cycles (data not shown). In contrast, the inhibition by Compound IV (Fig. 3C) and Compound VIII (data not shown), which had shown no significant time-dependent increase in inhibition over the 60 min preincubation period, behaved as reversible inhibitors with recovery of activity being essentially complete after three and four centrifugation–resuspension cycles, respectively. The reversibility of inhibition of purified MAO-B by milacemide was assessed by the dilution procedure [32]. As shown in Fig. 4, the time-dependent inhibition was irreversible, since dilution of the enzyme–milacemide mixture after pre-incubation failed to restore the activity.

Rat liver mitochondria which had been pretreated with *l*-deprenyl were used as the source of MAO-A

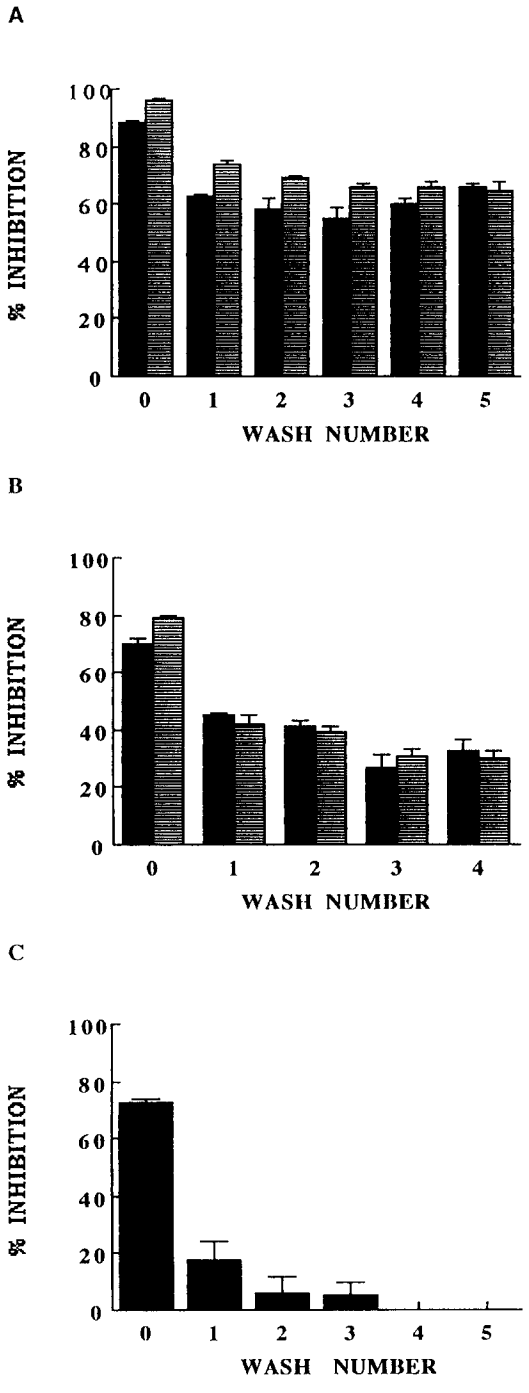


Fig. 3. Assessment of the reversibility of the inhibition of ox liver mitochondrial MAO-B by Compounds III (A), II (B) and IV (C). The enzyme preparation, at a concentration of 2 mg protein/mL, was incubated at 37° with a sufficient concentration of the inhibitor to give between 70 and 95% inhibition after 60 min. After that time the samples were subjected to repeated centrifugation and resuspension as described in the text. The activities were assayed before centrifugation (0) and after each of four or five centrifugation–resuspension cycles (1–5). For A and B each column is the mean ± SER of a triplicate determination, and the black and striped bars represent the results of separate, triplicate experiments. For C each column is the mean ± range of two separate triplicate determinations.

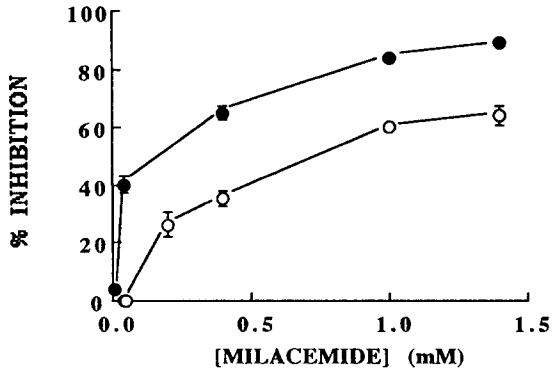


Fig. 4. Assessment of the reversibility of the inhibition of purified ox liver MAO-B by milacemide. The enzyme preparation, at a concentration of 50 μg protein/mL, was incubated for 60 min at 37° with different concentrations of milacemide (in the range of 0.17–50 times the IC_{50} value) before the samples were diluted 10 times into the assay mixture and the activity was determined towards 10 μM 2-phenethylamine (●). In parallel experiments (○) the enzyme was incubated for 60 min at 37° with the equivalent volume of water before being diluted 10 times into assay mixture containing the same final concentration of milacemide as would have been obtained in the first group of samples. For both sets of data the percentage inhibition, determined with respect to control samples treated in the same way but in the absence of inhibitor, is plotted against the final milacemide concentration. Each point is the mean ± SER of a triplicate determination.

Table 3. Anticonvulsant activities of the aminoacetamide derivatives

Compound	ED_{50} (mg/kg) Pretreatment time (hr, p.o.) 1	3	6
I	301 (238–381)	391 (314–489)	343 (276–426)
II	>400	>400	>400
III	281 (202–530)	>400	>400
IV	>400	>400	>400
V	>400	>400	>400
VI	>400	>400	>400
VII	>400	>400	>400
VIII	205 (162–262)	252 (196–360)	234 (107–305)

The compounds were administered (p.o.) to mice and the effective dose for inhibiting bicuculline-induced convulsions was determined as described previously [13]. Each value is the mean of two or three determinations. Values in parentheses are the 95% confidence limits.

activity. There was no detectable time dependence of the inhibition by any of the compounds tested.

Anticonvulsant activities

The anticonvulsant potencies of milacemide and its analogues were assessed in the mouse antibicuculline model, as previously described [13]. The results are summarized in Table 3. The effective doses of the different compounds differed widely and showed no obvious relationship to their abilities to act as either substrates or inhibitors of MAO-B.

DISCUSSION

The compounds studied in the present work have concentrated on replacement of the pentylamino group of milacemide while keeping the acetamide moiety intact. Thus, their oxidative cleavage by MAO would be expected to deliver glycine to the brain. The derivatives were based on structural relationships to MAO substrates and inhibitors. Compounds II and III were acetamido derivatives of the MAO-B selective substrates benzylamine and 2-phenethylamine, Compound IV was the corresponding derivative of the tryptamine, which is a substrate for both forms of MAO, and compound V was based on the 5-methyl derivative of the MAO-A selective substrate 5-HT (see Ref. 38 for an account of the specificities of the monoamine oxidases). Compounds VI and VII were based on the structure of the potent selective MAO-B inhibitor Ro 16-6491 [39] (see Scheme 1). It has been reported that substitutions to the aromatic ring of Ro 16-6491 have little effect on its potency and selectivity [39]. Hence, the analogue (Compound VII) lacking the 4-chloro substitution on the aromatic ring was also investigated. Benzyloxyphenyl derivatives of the oxazolidinones are potent inhibitors of both MAO-A and MAO-B [40, 41] and at least some members of the series (see the structure of MD 780236 in Scheme 1) are also substrates for the enzyme [42]. With these compounds it was found that the nature of the substituent in the aromatic ring is important for providing selectivity, and a chlorine substituent affords effective MAO-B inhibition [43]. Compound VIII was structurally related to this series of inhibitors, and the chlorine substitution on the benzene ring was retained.

All of the milacemide analogues studied were oxidized by purified MAO-B from ox liver to an aldehyde product. This product could be metabolized further to an acid derivative by NAD^+-dependent aldehyde dehydrogenase. The effectiveness of several of the compounds studied here as substrates for MAO-B suggests that they may be useful models for delivering their metabolites to the brain.

Under the conditions used in this study, none of the analogues was a substrate for rat liver mitochondrial MAO-A, as tested by the coupled and luminometric assays. Thus, any activity with this form of the enzyme was below the detection limits of these assay procedures. This is in accord with the results obtained with the parent compound milacemide [44].

Youdim *et al.* [45], in an independent study, synthesized Compound III. The K_m value reported in that work for Compound III (12 μM) as a substrate for MAO-B was not in accord with the value obtained in the present study. However, the enzyme preparation, guinea-pig mitochondria as opposed to

purified MAO-B from ox liver, may account for this discrepancy.

The specificity constant k_{cat}/K_m is an indication of the ability of substrates, at low concentrations, to compete with one another [46] and therefore represents a useful measure of the substrate specificity of an enzyme. In all the cases studied it was found that replacement of the aliphatic chain of milacemide by an arylalkyl moiety decreased the value of k_{cat}/K_m considerably. Thus, milacemide was found to be the most effective substrate, being about 4-fold more efficient than the Ro 16-6491 derivative (Compound VI) and the 2-phenethylamine derivative (Compound III), which were the next best substrates. The most poorly oxidized substrate was the benzyloxyphenethyl derivative (Compound VIII), followed by the 5-methoxytryptamine (Compound V) and the tryptamine (compound IV) derivatives, which were 995-fold and 66-fold, respectively, less efficient substrates than milacemide.

The large variations in the values of k_{cat}/K_m constant within this series is particularly noteworthy. The effects of subtle structural differences are particularly evident with the tryptamine (Compound IV) and the 5-methoxytryptamine (Compound V) derivatives, where the 5-methoxy substitution on the indole ring decreased substrate specificity 15-fold. Similarly, elimination of the chlorine substitution on the Ro 16-6491 derivative (Compound VI), producing Compound VII, decreased substrate specificity 3-fold, and shortening of the chain between the nitrogen atom and the benzene ring in the 2-phenethylamine derivative (Compound III) to produce the benzylamine derivative (Compound II) decreased the specificity constant 10-fold.

The IC_{50} values for the inhibition of ox liver mitochondrial MAO-B by the milacemide analogues (Table 2) gives a quantitative comparison and evaluation of the inhibitory potencies of these compounds in relation to each other. All of the compounds studied, with the exception of the benzyloxyphenethyl derivative (Compound VIII), were time-dependent inhibitors of the enzyme, although inhibition by the tryptamine derivative (Compound IV) developed only slowly with time. The replacement of the pentyl moiety by a phenethyl residue (Compound III) slightly increased the MAO-B inhibitory effect at both 0 and 1 hr before the incubation of the enzyme and inhibitor. However, shortening the chain between the nitrogen atom and the benzene ring (Compound II) reduced the inhibition, by about 2-fold and 3-fold, respectively, after 0 and 1 hr preincubation at 37°, as compared to the efficiency of milacemide. Replacement of the phenethyl moiety by the indolylethyl residue (Compound IV) gave an inhibitor that was somewhat more potent than milacemide, but showed only slight time dependence. However, its 5-methoxy-derivative (Compound V) was considerably less potent as an inhibitor but showed more marked time dependence. The derivative showing structural similarities to the potent MAO-B inhibitor Ro 16-6491 (Compound VI) displayed very similar potencies to milacemide after both 0 and 1 hr preincubation at 37°. However, when the chlorine substitution on the aromatic moiety was removed (Compound VII), a marked decrease in inhibitory potency as compared to milacemide and to the *para* chloro compound was observed. The benzyloxyphenethyl derivative (Compound VIII) had the most marked MAO-B inhibitory properties of all the compounds studied, but the inhibition did not increase on preincubation with the enzyme.

The inhibition by those compounds that gave time-dependent inhibition of MAO-B proved to be at least partly irreversible. In contrast, none of the compounds examined gave significant changes in inhibitory potency when incubated with the rat liver mitochondrial MAO-A. In the absence of preincubation all the compounds examined were more potent as reversible inhibitors of MAO-A. Again, Compound VIII was the most effective inhibitor. Since reversible MAO-A inhibitors appear to be safe and effective antidepressants [47] and it has been suggested that at least some MAO-B inhibitors may be "neuroprotective" [48], it would be interesting to examine the pharmacological profile of Compound VIII, which possess both these properties.

Milacemide has an unusual spectrum of pharmacological activities. The extent to which its anticonvulsant activity is associated with the slow delivery of its metabolites to the brain, the effects of the parent compound and the inhibition of MAO, remain to be determined. There is no correlation between the activities of MAO-B towards the compounds studied here and their potencies as anticonvulsants in the mouse model. The 2-phenethyl derivative (Compound III) had similar K_m and V_{max} values to the Ro 16-6491 derivative (Compound VI), yet the latter compound did not show anticonvulsant effects *in vivo*. Furthermore, Compound VIII was the most effective anticonvulsant *in vivo* yet it is an extremely poor substrate for MAO-B. These results suggest that the formation of glycine in the brain through the action of MAO is not the major factor in the anticonvulsant actions. Although it is possible that the actions of other enzyme systems may be involved in the formation of glycine or that differences in bioavailability may contribute to the observed lack of correlation, these results would be consistent with the previous observation that α-methylmilacemide, which does not act as a glycine precursor, is also an effective anticonvulsant [13].

There is some evidence that inhibitors of MAO have anticonvulsant activity [11, 12]. The lack of any simple correlation between the potencies of the compounds studied in the present work as MAO inhibitors and their effectiveness as anticonvulsants might suggest that inhibition of MAO is not the major factor in their actions. Indeed, the present *in vitro* studies show that compounds based on Ro 16-6491 (i.e. Compounds VI and VII) and the tryptamine derivative (Compound IV), although not effective anticonvulsants, were just as potent inhibitors of MAO-B as Compound III, which was an effective anticonvulsant. However, since the bioavailability and pharmacokinetics of these compounds are not known, further studies on the effectiveness of the milacemide analogues as MAO inhibitors *in vivo* would be necessary to confirm the

absence of correlation between the inhibition of MAO and the anticonvulsant activity.

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