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IS THE OXIDATION OF MILACEMIDE BY MONOAMINE OXIDASE A MAJOR FACTOR IN ITS ANTICONVULSANT ACTIONS?

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Abstract—The anticonvulsant drug milacemide (2-*n*-pentylaminoacetamide) is known to be oxidized by monoamine oxidase-B to yield glycinamide which then breaks-down to give glycine. It has been postulated that it is this liberation of glycine in the brain that accounts for the anticonvulsant effects. In order to test this hypothesis, and since amines bearing a methyl-group in the α-position have been shown to be resistant to oxidation by monoamine oxidase, the effects of milacemide were compared with those of α-methyl-milacemide. Although the latter compound was found to be toxic at higher concentrations, it was found to antagonize bicuculline-induced convulsions in mice. When milacemide was administered to mice (0.5 mmol/kg, p.o.) there was a substantial increase in urinary glycinamide excretion. No such increase was observed after the administration of the same dose of α-methyl-milacemide. Furthermore, α-methyl-milacemide was not oxidized by either monoamine oxidase-A or -B *in vitro* to any detectable extent, although it was a competitive inhibitor of both forms of the enzyme. The findings that α-methyl-milacemide has anticonvulsant properties in the bicuculline test but is not a substrate for monoamine oxidase or a source of urinary glycinamide cast doubt on the importance of the oxidation or milacemide to form glycinamide as a major factor in its anticonvulsant action.

Milacemide (2-*n*-pentylaminoacetamide) (Fig. 1) has been reported to have anticonvulsant activity in some animal models of epilepsy [1–4], with antagonism of bicuculline-induced convulsions representing the most sensitive test. However, in many other tests the effective anticonvulsant doses are high and the magnitude of the effects is often rather small. Furthermore, studies on the effects of milacemide on patients suffering various forms of epilepsy have not established a therapeutic effect conclusively (see Ref. 5 for review).

The mechanisms of the anticonvulsant action of milacemide have not been fully elucidated. Janssens de Varebeke *et al.* [6] reported it to be a good substrate for the B-form of monoamine oxidase (EC 1.4.3.4; MAO-B) but to be oxidized poorly by the A-form of that enzyme (MAO-A) with K_m values of 30–90 μM and 1300 μM for MAO-B and -A, respectively. Furthermore, milacemide was shown not to be a substrate for the semicarbazide-sensitive amine oxidase (EC 1.4.3.6) [7]. The acute administration of milacemide to rats was found to result in the urinary elimination of glycinamide, which was partly prevented by pretreatment with *l*-deprenyl, a selective inhibitor of MAO-B, but not by the MAO-A-selective inhibitor clorgyline [6]. Administration of an oral dose of 100 mg/kg milacemide was shown to increase the 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 intraperitoneal administration of the same dose [3]. The 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 confirmed after intraperitoneal administration of the same dose. In

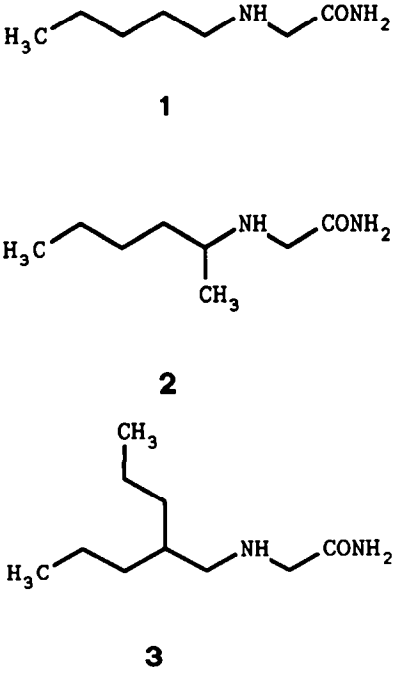

Fig. 1. Structures of milacemide (1), α-methyl-milacemide (2) and β-propyl-milacemide (3).

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addition to being a substrate for MAO-B, and thus acting as a precursor of glycine in the brain, milacemide has been reported to be a time-dependent irreversible inhibitor of that enzyme [10, 11]. The selectivity of milacemide as a substrate for MAO-B has however been questioned by Truong *et al.* [12], who reported that the acute administration of milacemide potentiated the "wet dog shake" syndrome induced by 5-hydroxytryptophan (5-HTP) in the rat; such a potentiation was observed after pretreatment with clorgyline but not with *l*-deprenyl [13]. A significant decrease in the concentrations of 3,4-dihydroxyphenylacetic acid (DOPAC) in striatum and substantia nigra [14], 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 was found to antagonize the pp'-DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane)-induced myoclonus in mice [15], an effect also displayed by 5-HTP and 5-HT agonists [16]. This myoclonus was found to be antagonized by *l*-deprenyl and by non-selective MAO inhibitors but not by clorgyline [17].

In order to examine the extent to which the formation of glycine by MAO-B might underlie the anticonvulsant effect of milacemide, we have compared its behaviour in antagonizing bicuculline-induced convulsions, and death, in mice with that of the close analogue α-methyl-milacemide (2-[(1-methyl)pentyl] aminoacetamide; Fig. 1). The introduction of a methyl group into this position of milacemide might be expected to prevent it from acting as a substrate for MAO (see Refs 18 and 19). This was assessed by measuring the urinary excretion of glycinamide after administration of milacemide or α-methyl-milacemide (α-MM) and also by determining the interactions of the compounds with MAO *in vitro*.

MATERIALS AND METHODS

Materials. 5-Hydroxytryptamine-[side chain-2-^{14}C]creatine sulphate and 2-phenylethylamine-[ethyl-1-^{14}C] hydrochloride were obtained from Amersham International or New England Nuclear. Clorgyline was a kind gift from May & Baker Ltd (Dagenham, U.K.) and *l*-deprenyl was kindly given by Prof. J. Knoll, Department of Pharmacology, Semmelweis University, Budapest, Hungary. Milacemide and α-methyl-milacemide, both as their hydrochloride salts, were synthesized at Farmitalia Carlo Erba, Milan, Italy. Rat liver mitochondria were prepared and treated as previously described [20].

Metabolic studies. Male mice (CD-1; Charles River, Italy) weighing 22–23 g were used. The animals, fasted overnight, were housed in separate stainless steel metabolism cages in groups of four. Each group was allowed free access to drinking water. Twelve mice (three groups of four) each received 0.5 mmol/kg p.o. of milacemide hydrochloride (M), 12 received the same dose of α-MM hydrochloride and a further 12 were administered water as vehicle. The 0–8 and 8–24 hr urine samples were collected in receivers, that were cooled with dry ice, for the determination of glycinamide (Gly-A).

Analysis. Primary amino acids react with *o*-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol to form intensely-fluorescent derivative complexes [21]. The reaction is complete in less than 1 min at room temperature and the product has an excitation wavelength of 340 nm and an emission wavelength of 455 nm. This procedure was used in conjunction with high-performance liquid chromatography (HPLC) to determine glycinamide [22]. Samples (100 μl) of urine were diluted to 1 mL with water containing DL-homocysteic acid (10 nmol/mL, final concentration) as internal standard. Samples (25 μL) of the diluted urine were reacted with 25 μL of OPA solution (Pierce, Rockford, IL, U.S.A.) using a LABNET modular Spectra-Physics (SP) liquid chromatography system equipped with an automatic system for pre-column primary amino acid derivatization. A 10 μL sample of the reaction mixture was automatically injected onto the analytical column. The HPLC system consisted of a SP 8800 pump, a SP 8780 autosampler and a SP 4270 computing integrator equipped with a LABNET data capture module. A Perkin-Elmer LS-5 fluorescence detector, set at excitation and emission wavelengths of 340 and 455 nm, respectively, was used to analyse the eluted samples. Chromatography was performed with gradient elution from a 5 μm particle-size Hypersil ODS column (250 × 4.6 mm, i.d.) at a flow-rate of 1.5 mL/min. The starting solvent (solvent A) was water: propionate buffer, pH 6.5: acetonitrile (72:20:8, by vol.) and solvent B was water : acetonitrile : methanol : dimethylsulphoxide (42:30:25:3, by vol.). The limit of sensitivity was 20 pmol. A typical chromatogram is shown in Fig. 2.

Enzyme assays. Monoamine oxidase activity was assayed, at 37° and pH 7.2, by the radiochemical procedure previously described with 100 μM 5-HT and 10 μM 2-phenylethylamine as substrates for MAO-A and -B, respectively [23]. In studies of the kinetics of inhibition the concentrations of these substrates were varied at a series of fixed concentrations of α-methyl-milacemide. Data were analysed by non-linear regression analysis to allow the determination of the type of inhibition and the inhibitor constants. In studies on the behaviour of MAO-A the B-form was inhibited by preincubation of the mitochondrial sample, at a protein concentration of 2 mg/mL in 80 mM phosphate buffer, pH 7.2, with 0.3 μM *l*-deprenyl for 60 min at 37°. Similar preincubations with 0.3 μM clorgyline were used to inhibit MAO-A prior to studies with the B-form. Assays with either 100 μM 5-HT or 10 μM 2-phenylethylamine were used to confirm that these pretreatments resulted in complete inhibition of the sensitive form of MAO. Control experiments were performed to ensure that the initial rate of the reaction was measured under all conditions used here.

In studies of the dependence of inhibition on the α-methyl-milacemide concentration and incubation time the enzyme and inhibitor were incubated in 80 mM phosphate buffer, pH 7.2, for 0 or 1 hr before the reaction was started by the addition of substrate.

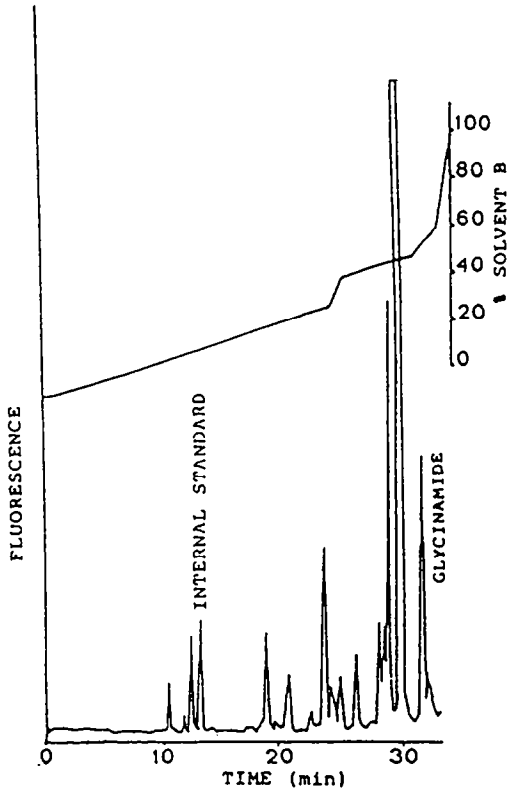

Fig. 2. Typical chromatogram of OPA-treated urines from mice administered with milacemide (0.5 mmol/kg p.o.). Glycinamide was identified by retention time.

Time-courses of inhibition were also performed over longer periods of enzyme–inhibitor preincubation. In all cases control incubations were performed in which the inhibitor solution was replaced by an equal volume of water. The percentage inhibition was calculated with respect to the appropriate control. In all studies determinations were performed at least in triplicate and mean values are presented ± SE as appropriate.

The reversibility of inhibition was determined by repeated centrifugation and resuspension [24]. This procedure involved incubation of enzyme samples with α-methyl-milacemide for 1 hr at 37°. Small aliquots were taken for assay and the remainder of the sample was centrifuged at approximately 14,000 *g* for 10 min. The sediment was resuspended in buffer to the original volume and assayed. This procedure was repeated a further four times. Control samples were preincubated in the absence of inhibitor but otherwise treated in the same way. The degree of inhibition at each stage is expressed as a percentage of the activity of the corresponding control samples.

The ability of α-methyl-milacemide to act as a substrate for MAO was assessed by determining the rate of hydrogen peroxide formation by a luminometric procedure. Full details of the performance and characterization of this assay are to be published elsewhere (O'Brien and Tipton, manuscript in preparation). Briefly: each assay mixture contained, in a total volume of 3 mL, 85 mM potassium phosphate buffer, pH 7.2, 3.1 mM sodium azide, enzyme and α-MM. After incubation at 37° for fixed times 100-μL samples were withdrawn and immediately injected into 1.0 mL of a mixture, at 37°, containing 68 mM Tris–HCl buffer, pH 8.0, 20 μg horseradish peroxidase and 132 μM luminol in an LKB model 1250 luminometer. The light emission was measured and related to the hydrogen peroxide concentration by means of a standard curve produced by adding known amounts of this compound to similar luminescence mixtures. Since no activity was detected in the studies with α-MM, control experiments were performed with benzylamine, tyramine and milacemide to confirm the validity and determine the sensitivity of this procedure.

Anticonvulsant activity. Antibicuculline activity was determined in mice essentially as described by van Dorsser *et al.* [1]. Three graded doses of either M or α-MM were administered orally and after 1, 3 and 6 hr bicuculline was injected intravenously at a dose of 0.6 mg/kg. Twenty animals were used per dose and the effects of each dose were determined three times at each of the treatment times. The activities of M and α-MM were each expressed, by pooling the results of the three experiments, as the dose calculated by regression analysis which protected 50% of the animals from convulsions and death for 1 hr after injection of bicuculline (ED_{50} and

Table 1. Urinary excretion of glycinamide by the mouse.

Treatment	Number of animals	Glycinamide excreted (nmol/8 hr)
Milacemide	12	6464 ± 1771*
α-Methyl-milacemide	12	767 ± 375^{NS}
Control	12	619 ± 139

Total urinary excretion, identified by retention time, was determined over the 8-hr period following administration of 0.5 mmol/kg (p.o.) milacemide, α-MM or vehicle. Values are means ± SD (N = 3). Significance was determined by the Student's *t*-test for unpaired data.

NS, not significantly different from control; *$P < 0.01$.

Table 2. Anticonvulsant activities of milacemide (M) and α-methyl-milacemide (α-MM) in the mouse

Compound	Dose (mg/kg p.o.)	Time (hr)*	Deaths before bicuculline	Number protected	ED_{50} (range)†
M	200	1	0	21/60	
M	400	1	0	37/60	301
M	800	1	0	48/60	(238–381)
M	200	3	0	17/60	
M	400	3	0	30/60	391
M	800	3	0	44/60	(314–489)
M	200	6	0	19/60	
M	400	6	0	33/60	343
M	800	6	0	47/60	(276–426)
α-MM	200	1	0	21/60	
α-MM	400	1	0	28/60	ND
α-MM	800	1	28/60	9/34‡	
α-MM	200	3	0	18/60	
α-MM	400	3	1	23/59	736
α-MM	800	3	6/60	28/54	(374–1449)
α-MM	200	6	0	21/60	
α-MM	400	6	0	27/60	532
α-MM	800	6	17/60	24/43	(298–1023)

The ability of the compounds to protect against bicuculline-induced convulsions and death was determined as described in the text.

* Time between administration of M or α-MM and that of bicuculline.

† Values expressed as mg/kg p.o. with 95% confidence limits.

‡ The animals in this group appeared severely distressed.

ND, not determined.

95% confidence limits). To facilitate comparison with published results on the anticonvulsant activity of milacemide the doses used are expressed in mg/kg. Expressed in terms of their hydrochloride salt, a dose of 200 mg would correspond to 1.11 mmol of M and 1.03 mmol of α-MM.

RESULTS

In vivo *studies*

The urinary excretion of glycinamide was determined over an 8-hr period following the administration of either milacemide or α-methyl-milacemide (0.5 mmol/kg p.o.). The glycinamide excretion was also measured during the 8–24 hr period, but it was found that the majority of the excretion of this compound had occurred within the initial 8 hr. Similar results have been reported from studies in the rat [7]. As shown in Table 1, administration of milacemide resulted in a substantial increase in the quantity of glycinamide excreted over the 8-hr period. In contrast the excretion of glycinamide by the animals that had been administered α-MM was not significantly different from the control value.

Assessment of the anticonvulsant activity of α-methyl-milacemide was hampered by a degree of toxicity of this compound which was not observed with milacemide at the same doses (see Table 2). Nevertheless the data obtained with the surviving animals were adequate to allow the dose that was effective in preventing bicuculline-induced convulsions and death in half the surviving animals (the ED_{50} value) to be estimated. Comparison of the ED_{50} values shown in Table 2 indicates that α-MM was effective as an anticonvulsant in the anti-bicuculline test, although it was somewhat less potent than the parent compound milacemide.

In vitro *studies*

α-Methyl-milacemide was found to be an inhibitor of both forms of MAO, but the inhibition curves, shown in Fig. 3, indicate that the extent of inhibition was not altered when the enzyme was preincubated with α-methyl-milacemide for 1 hr at 37° before the addition of substrate to start the assay. IC_{50} values of 200 ± 5.7 μM and 69 ± 3.6 μM (mean values ± SEM) were determined for MAO-A and -B, respectively. Further experiments (data not shown) indicated that there was no time-dependent increase in the extent of inhibition of either MAO-A or -B when samples were incubated for periods of up to 4 hr with sufficient α-MM to give initial inhibition in the range 20 to 30%. The lack of any time-dependence in the inhibition would be consistent with α-MM acting as a reversible inhibitor of the enzyme. This was confirmed by the results shown in Table 3 where the procedure of repeated washing, by centrifugation and resuspension, can be seen to

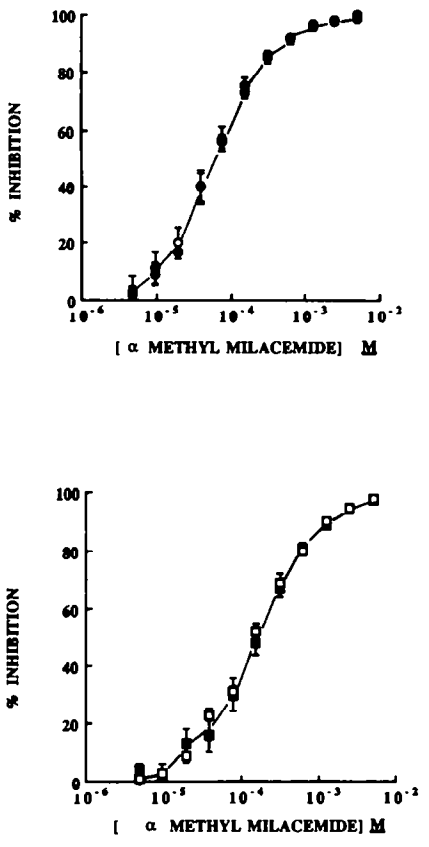

Fig. 3. The effects of α-methyl-milacemide concentration on the activity of rat liver mitochondrial MAO. The enzyme preparation was incubated for 0 (open symbols) or 1 hr (closed symbols) with the indicated concentration of α-MM before activity was determined towards 100 μM 5-HT (■) or 10 μM 2-phenylethylamine (●). Percentage inhibition was calculated with respect to samples preincubated for the same periods in the absence of inhibitor. Each point is the mean value ± SER from triplicate determinations in a single experiment.

Table 3. Assessment of the reversibility of the inhibition of rat liver mitochondrial MAO-A and MAO-B by α-methyl-milacemide.

Wash number	% Inhibition MAO-A	MAO-B
0	75.5 ± 0.5	83.5 ± 0.5
1	0	29.5 ± 3.5
2	0	0

Preincubation of enzyme and inhibitor followed by centrifugation and resuspension was performed as described in the text.

Activities of MAO-A (0.2 mg protein/mL) and MAO-B (0.4 mg protein/mL) were assayed immediately (0) and after each of five washes (1–5). There was no significant inhibition of either form after washes 3, 4 and 5.

Each value is the mean ± SD of triplicate determinations in two separate experiments.

cause essentially complete recovery of the activities of samples of enzyme which had been preincubated with α-MM for 1 hr at 37°.

The kinetic behaviour of α-MM as an inhibitor is shown in Fig. 4. Inhibition was linearly competitive towards the amine substrate with both forms of the enzyme. The K_i values were determined to be 80 and 67 μM for MAO-A and -B, respectively (see Table 4). Assay of enzyme activity by determining the production of H_2O_2 gave no detectable activity of either form of MAO towards α-MM concentrations of up to 0.7 mM. The specific activity of MAO-A under these conditions was determined to be 2.56 ± 0.06 nmol product/min/mg protein with 150 μM tyramine as substrate (mean ± SD from two separate experiments). The amount of protein used in this assay was 0.2 mg. Corresponding values determined for MAO-B were 1.9 ± 0.1 and 1.13 ± 0.13 nmol/min/mg protein with 333 benzylamine and 163 μM milacemide, respectively, as substrates (mean ± SD from two separate experiments). The amount of protein used in these assays was 0.2 mg for the benzylamine assay and 0.6 mg for the milacemide assay. In attempts to detect activity towards α-MM assay protein concentrations in the range 0.06 to 1 mg were used. The sensitivity of the luminometric assay was such that activities of less than 2.2 ± 0.03% of that observed with milacemide would have been detected.

DISCUSSION

Direct studies on the interactions of α-methyl-milacemide with MAO *in vitro* showed the compound to be a reversible inhibitor of both forms of the enzyme. The competitive nature of the inhibition would be consistent with it binding to the active site. However, there was no detectable activity of either form of MAO towards α-MM as a substrate. Similar behaviour has been reported with primary amine substrates of MAO where substitution of a methyl-group for one of the hydrogens at the α-carbon of benzylamine, 2-phenylethylamine and tryptamine results in the formation of reversible inhibitors with no significant activities as substrates [18, 19]. We have also shown that similar substitutions of a methyl-group into the MAO-A selective mechanism-based irreversible inhibitor clorgyline results in the modified compounds behaving as simple reversible competitive inhibitors of MAO [25].

The failure of either form of MAO to show detectable activity towards α-methyl-milacemide is in agreement with the results of the *in vivo* metabolic studies which showed that administration of this compound did not result in any significant elevation of urinary glycinamide excretion whereas there was a substantial increase after administration of a similar quantity of milacemide. It has been shown recently that in the rat milacemide may also be oxidized by the flavine-dependent polyamine oxidase [26]. It is not clear to what extent such oxidation may also occur in the mouse. However, the site of cleavage in the polyamine oxidase catalysed reaction is different from that of MAO and thus would not lead to glycinamide formation.

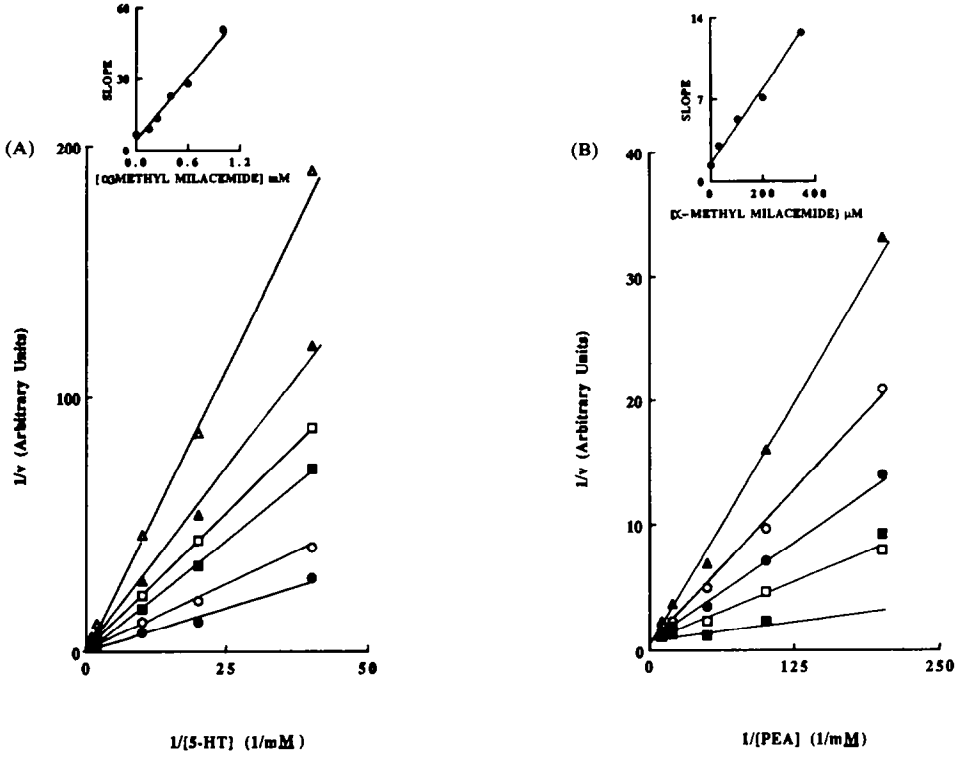

Fig. 4. Kinetics of the inhibition of MAO by α-methyl-milacemide. Initial rates were measured in the presence of the indicated concentrations of 5-HT (A) or 2-phenylethylamine (B) and in the presence of A: 0 (●), 0.15 mM (○), 0.25 mM (■), 0.4 mM (□), 0.6 mM (▲), and 1 mM (△) or B: 0 (■), 32 μM (□), 100 μM (●), 200 μM (○) and 350 μM (▲) α-MM. Each point is the mean of triplicate determinations in a single experiment. The insets show the dependencies of the slopes on the inhibitor concentration.

Table 4. K_i values for the inhibition of MAO-A and MAO-B by α-methyl-milacemide

	K_i (μM)*	Type of inhibition
MAO-A	80 ± 4.0	Competitive
MAO-B	67 ± 4.8	Competitive

* Each value represents the mean ± SD from two separate experiments in each of which independent determinations were made in triplicate.

The results presented here show that α-methyl-milacemide is active as an anticonvulsant in the bicuculline test, albeit somewhat less effective than milacemide itself. Although it is possible that this simple change to the milacemide molecule might result in α-MM exerting its effects by a completely different mechanism, a more straightforward explanation would suggest that the generation of glycinamide, and hence glycine, is not essential to the anticonvulsant action of either compound. Another reasonable approach to study whether the anticonvulsant activity of milacemide depends on the formation of glycine would have been to determine its effect after the inhibition of MAO-B. However, when *l*-deprenyl was given at a dose known to selectively inhibit MAO-B (5 mg/kg p.o.) [27], about 45% of the mice were protected from bicuculline-induced lethality. This agreed with previous results, showing a moderate anticonvulsant activity of (±)deprenyl after intravenous bicuculline at the dose of 0.7 mg/kg [28].

Finally, it is worth noting that another alkyl-branched derivative of milacemide, β-propyl-milacemide (2-[(2-propyl)pentyl]aminoacetamide; see Fig. 1), although being readily deaminated by MAO-B, was shown to produce a strong proconvulsant effect in mice and rats [29].

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