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THE INTERACTIONS OF MILACEMIDE WITH MONOAMINE OXIDASE

EIMEAR M. O'BRIEN,* KEITH F. TIPTON,† JOHN M. MCCRODDEN and MOUSSA B. H. YOUDIM‡

Department of Biochemistry, Trinity College, Dublin 2, Ireland and ‡Department of Pharmacology, Faculty of Medicine, Technion, Haifa, Israel

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Abstract—The interactions of the anticonvulsant drug milacemide (2-*n*-pentylaminoacetamide) with rat liver mitochondrial monoamine oxidases-A and -B have been studied. The compound acts as a substrate for the B-form of the enzyme, with an apparent K_m value of 49 ± 4.7 μM and a V_{max} value of 1.1 ± 0.2 nmol/min/mg. It is also a time-dependent irreversible inhibitor of that enzyme. Any activity of monoamine oxidase-A towards this substrate was too low to allow accurate determinations to be made by either luminometric determination of H_2O_2 formation or spectrophotometric coupling of aldehyde formation to NAD^+ reduction in the presence of aldehyde dehydrogenase. Milacemide was a reversible competitive inhibitor towards monoamine oxidase-A. The inhibitor constant (K_i) was 115 ± 35 μM indicating a higher affinity than that towards monoamine oxidase-B, which was also competitively inhibited in the absence of enzyme–inhibitor preincubation (K_i ≈ 331 ± 185 μM). Determination of the formation of H_2O_2 and the aldehyde product of the oxidative cleavage of milacemide by purified monoamine oxidase-B from ox liver indicated that cleavage resulted solely in the formation of pentanal and glycinamide. There was no evidence for alternative cleavage to pentylamine and oxamaldehyde.

The anticonvulsant drug milacemide (2-*n*-pentylaminoacetamide) [1–5] has been shown to be a good substrate for MAO§-B (EC 1.4.3.4) but to be oxidized only poorly by the A-form of that enzyme (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 [6]. Oral administration of milacemide (100 mg/kg) resulted in increased concentrations of glycine in rat forebrain, cerebellum and medulla [7]. 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].

Thus, milacemide acts as a precursor of glycine in the brain and it has been suggested that this may account for its anticonvulsant actions [see 2, 6, 7], although there is evidence that other factors may also contribute to the anticonvulsant behaviour [see 8]. The formation of glycinamide from milacemide appears to be specific to MAO since it has been shown not to be a substrate for the semicarbazide-sensitive amine oxidase (EC 1.4.3.6) [9]. Although, it has been reported to be a substrate for the flavine-dependent polyamine oxidase (EC 1.5.3.11) [10] in the rat. In that case, the pattern of cleavage is different, since glycinamide is not formed. The likely reaction products are pentylamine and the oxamaldehyde (see Scheme 1). In addition to being a substrate for MAO-B milacemide has been reported to be a time-dependent inhibitor of that enzyme [11, 12]. The inhibitory properties of this compound differed from those of the mechanism-based irreversible inhibitor *l*-deprenyl in that the recovery of enzyme activity following milacemide inhibition was significantly faster, suggesting that the inhibition might be partially reversible [11].

Despite the uncertainties of the mechanisms of action of milacemide, it represents a useful model system for the delivery of substances to the brain. In the present work we report the results of more detailed studies on the actions of milacemide as a substrate and inhibitor MAO and the stoichiometry of the oxidative cleavage reaction.

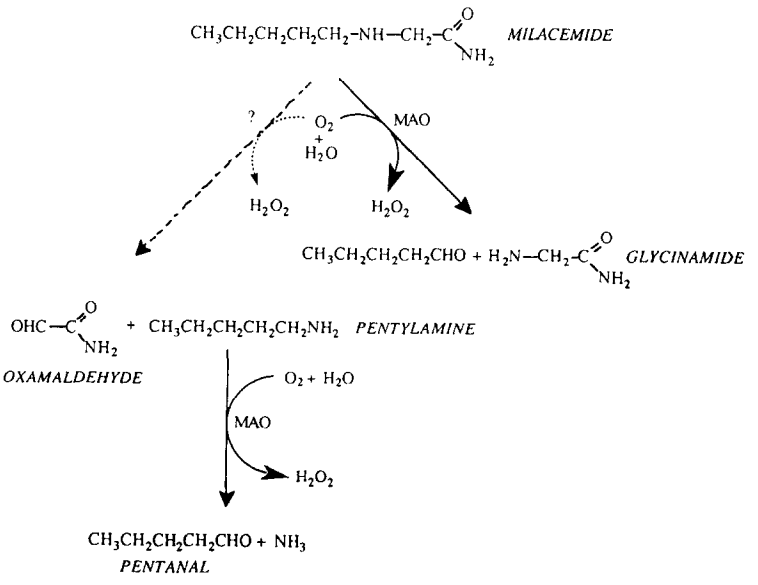
MATERIALS AND METHODS

Materials. 5-HT-[*side chain*-2-^{14}C]creatinine sulphate and 2-phenylethylamine-[ethyl-1-^{14}C]hydrochloride were obtained from Amersham International (Amersham, U.K.) or New England Nuclear Corp. (Stevenage, U.K.). Clorgyline was a kind gift from May & Baker Pty Ltd (Dagenham, U.K.) and *l*-deprenyl was kindly given by Prof. J Gaál, Chinoin Pharmaceutical Co. Ltd (Budapest, Hungary). Milacemide hydrochloride was from Continental Pharma-Searle (Mont-Saint-Guilbert, Belgium). Rat liver mitochondria were prepared by the method of Kearney *et al.* [13]. Purified MAO-B from ox liver was prepared by the method of Salach [14]. Aldehyde dehydrogenase (EC 1.2.1.3) was prepared from ox

* Present address: Xenova Ltd, 240 Bath Road, Slough, Berkshire SL1 4EF, U.K.

† Corresponding author.

§ Abbreviations: 5-HT, 5-hydroxytryptamine; MAO, monoamine oxidase; milacemide, 2-*n*-pentylaminoacetamide.



Scheme 1. Pathways of milacemide oxidation. The solid line shows the oxidation of milacemide to pentanal and glycinamide first proposed by Janssens de Varebeke *et al.* [6, 11]. The broken line indicates the possible alternative cleavage to give pentylamine and oxamaldehyde, which may be catalysed by polyamine oxidase [10]. Any pentylamine formed would be readily oxidized to pentanal by MAO-B.

liver as described previously [15] and one unit of activity is defined as the amount that catalyses the formation of 1 μmol/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^{-1} cm^{-1} [16]. Horseradish peroxidase (EC 1.11.1.7) Type II was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Methods. All enzyme assays were performed at 37° and pH 7.2. MAO activity towards 5-HT (100 μM) and 2-phenylethylamine (10 μM) as substrates for MAO-A and -B, respectively was determined by the radiochemical procedure described previously [17]. Determinations were performed in triplicate and each experiment was repeated at least twice; statistical data refer to the number of replicate experiments in each case. In studies of the dependence of inhibition on the milacemide concentration and incubation time, the enzyme preparation and inhibitor were incubated in 80 mM phosphate buffer, pH 7.2, for 0 or 60 min before the reaction was started. Time-courses of inhibition were also performed over longer periods of enzyme–inhibitor preincubation. In all cases control incubations were used in which the inhibitor solution was replaced by an equivalent volume of water. IC_{50} values (concentrations of inhibitor giving 50% inhibition) were determined using the commercially available computer program 'KaleidaGraph' (version 2.1 for Apple Macintosh). In studies of the kinetics of inhibition the substrate concentrations were varied at a series of fixed concentrations of milacemide. Data were analysed by non-linear regression analysis to allow the determination of the type of inhibition and the inhibitor constants.

The reversibility of inhibition was determined by repeated centrifugation and resuspension [18]. This procedure involved incubation of enzyme samples with either milacemide (1 and 2 mM for studies with MAO-A and -B, respectively) or 800 μM *d*-amphetamine 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.

Two different assay procedures were used to determine the activity of MAO-B towards milacemide. 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, was performed as described previously [15]. 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 milacemide at the concentrations indicated. When mitochondria were used as the enzyme source, 1 mM pyrazole and 2.5 μM rotenone (in methanol) were included in the reaction mixture to inhibit alcohol dehydrogenase (EC 1.1.1.1) and NADH oxidase (EC 1.6.99.3), respectively. The activity of aldehyde dehydrogenase was not rate limiting under any of the assay conditions used.

The ability of milacemide to act as a substrate for MAO was also assessed by determining the rate of H_2O_2 formation by the luminometric procedure [19]. 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 milacemide. 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 H_2O_2 present in the MAO reaction at that time of the assay was calculated by reference to the standard curve.

For 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 [20]. 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. The oxidation of benzylamine (333 μM) was determined at 250 nm by the direct spectrophotometric procedure [21].

RESULTS

The inhibition of rat liver mitochondrial MAO by milacemide

In the absence of preincubation milacemide was found to inhibit rat liver mitochondrial MAO-A and -B (Fig. 1), with IC_{50} values of 163 ± 67 μM (mean ± range of two separate determinations) and 455 ± 22 μM (mean ± SEM of four separate determinations), respectively.

Preincubation of rat liver mitochondria with milacemide for 1 hr at 37° was found to induce a time-dependent inactivation of MAO-B whereas no such time-dependent inhibition was observed with MAO-A. The IC_{50} values for the inhibition of PEA and 5-HT oxidation after preincubation were 75.3 ± 5.5 μM (mean ± SEM of four separate determinations) and 152 ± 56 μM (mean ± range of two separate determinations), respectively.

Extended time-courses of the inhibition of MAO-A and -B by milacemide were determined (Fig. 2). Control experiments in which milacemide was incubated under identical conditions but in the absence of enzyme were included. It was confirmed that milacemide was a time-dependent inhibitor of MAO-B, with inhibition by 170 μM milacemide rising from 26 ± 4% at 0 time to 91 ± 2% after 4 hr (mean ± range of two separate determinations in each case). Experiments with MAO-A confirmed that there was no time-dependent increase in the extent of inhibition for periods of up to 4 hr with sufficient milacemide to give initial inhibition of 32.0 ± 0.5% (mean ± range of two separate determinations).

The reversibility of the inhibition of MAO-A and -B by milacemide was assessed (Fig. 3) by repeated centrifugation and resuspension. The reversibility of inhibition of MAO-A by *d*-amphetamine is also shown for comparison. Milacemide, like amphetamine [22], was shown to be a reversible inhibitor of MAO-A, whereas it proved to be an irreversible inhibitor of MAO-B.

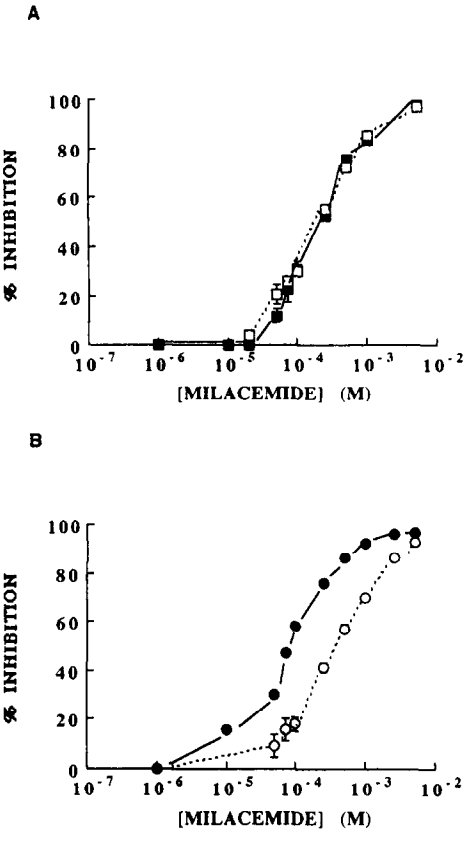


Fig. 1. Inhibition of rat liver mitochondrial monoamine oxidases by milacemide. The mitochondrial preparation (0.2 mg protein/mL) was incubated at 37° with the indicated concentrations of milacemide for 0 (open symbols) or 60 min (closed symbols) before the MAO-A activity was determined towards 100 μM 5-HT (A; ■, □) or the MAO-B activity was determined towards 10 μM 2-phenylethylamine (B; ●, ○). Each point is the mean value ± standard error of ratio (SER) from triplicate determinations in a single experiment. Where error-bars are not visible they were smaller than the plotting symbols used.

The kinetic behaviour of milacemide as a reversible inhibitor, without enzyme–inhibitor preincubation, is shown in Fig. 4A and B. Inhibition was linearly competitive towards the amine substrates for both forms of the enzyme. The K_i values were estimated to be 115 ± 35 and 331 ± 185 μM for MAO-A and -B, respectively (mean ± range of two separate determinations in each case).

The oxidation of milacemide by rat liver mitochondrial MAO

The time-course for the oxidation of milacemide by rat liver mitochondrial MAO-B was found to be

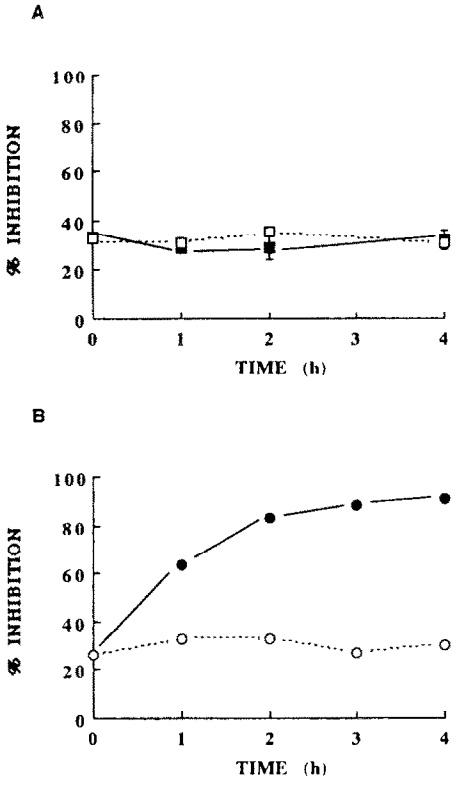


Fig. 2. Time-courses of inhibition of rat liver mitochondrial MAO-A and -B by milacemide. (A) Milacemide (200 μM) was incubated with the mitochondria (■) or with buffer (□; broken line) for the times indicated before the activity of MAO-A was determined. (B) Milacemide (170 μM) was incubated with the mitochondria (●) or with buffer (○; broken line) for the times indicated before the activity of MAO-B was determined. Each point is the mean value ± range from two separate experiments each involving triplicate determinations. Where error-bars are not visible they were smaller than the plotting symbols used. Other details were as described in Fig. 1.

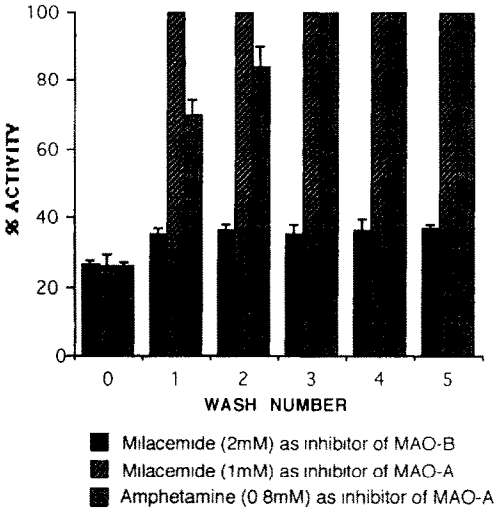


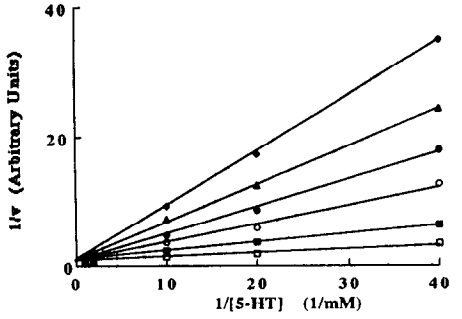
Fig. 3. Assessment of the reversibility of the inhibition of rat liver mitochondrial MAO-A and -B by milacemide. The mitochondrial preparation (2 mg protein/mL) was incubated at 37° with the indicated concentrations of milacemide for 60 min followed by repeated centrifugation and resuspension, as described in the text. Activities of MAO-A and -B were assayed immediately (0) and after each of five consecutive centrifugation–resuspension cycles. The inhibition of MAO-A by *d*-amphetamine was used as a control, since it is known to be a reversible inhibitor [22]. Percentage activity is calculated with respect to samples treated in the same way except that the inhibitor solution was replaced by an equal volume of buffer. The inhibition of MAO-A by milacemide and amphetamine was not significant after the first and third washes, respectively. Each point is the mean value ± range from two separate experiments each involving triplicate determinations.

linear for a very short period. After the change in absorbance had ceased completely (55 min) the activity could not be restored by the addition of more milacemide. However, the activity could be restored by the addition of a further aliquot of rat liver mitochondrial MAO-B (Fig. 5). The time-course for the oxidation of 333 μM benzylamine under the same conditions was found to be linear for 1 hr indicating that the enzyme was not becoming inactivated under the experimental conditions. Furthermore, control experiments (data not shown) indicated that milacemide was not a time-dependent inhibitor of the coupling enzyme, aldehyde dehydrogenase, over a 50 min preincubation period.

A Michaelis constant was determined for the oxidation of milacemide by rat liver mitochondrial MAO-B using the coupled spectrophotometric assay (Fig. 6). An apparent K_m value of 49 ± 4.7 μM and a V_{max} value of 1.1 ± 0.2 nmol/min/mg were determined (mean ± range of four separate determinations). The oxidation of milacemide by rat liver mitochondrial MAO-A was studied using the luminometric and coupled spectrophotometric assays. Luminometrically, no activity was detected using a final milacemide concentration of 1.2 mM and final protein concentrations in the range 0.06–0.4 mg/ml. Attempts to determine kinetic parameters with the coupled spectrophotometric assay were unsuccessful. High concentrations of protein were necessary and the very low rates of activity detected were too close to the detection limits of the assay procedure to allow accurate estimations.

Stoichiometry of milacemide oxidation by MAO-B

The enzyme source used in the studies described above was rat liver mitochondria since the rodent has been used for animal studies on the anticonvulsant behaviour of milacemide and there appear to be no significant differences in behaviour between the rat liver and brain enzymes. However, for the purposes of determining the stoichiometry of the reaction a purified preparation of MAO-B from ox liver [14] was used to avoid possible complications from the presence of other activities. Figure 7 shows a comparison between the initial rates of production of the aldehyde product and H_2O_2 as a function of the concentration of MAO-B. Similar determinations



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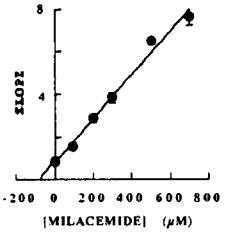

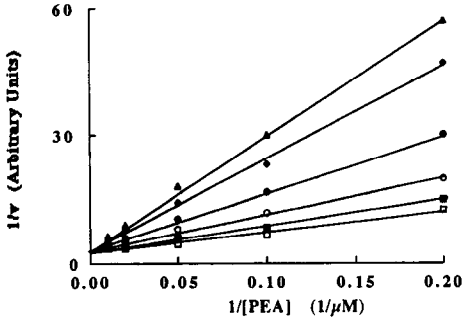

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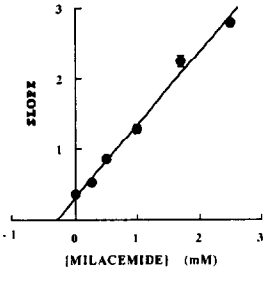

Fig. 4. Kinetics of the inhibition of rat liver monoamine oxidase by milacemide. Initial rates with MAO-A (A) were measured with 5-HT as substrate and in the presence of 0 (□), 90 (■), 200 (○), 300 (●), 500 (▲) and 700 μM (◆) milacemide. Initial rates with MAO-B (B) were measured with 2-phenylethylamine as substrate and in the presence of 0 (□), 0.25 (■), 0.5 (○), 1.0 (●), 1.7 (◆) and 2.5 mM (▲) milacemide. Each point is the mean of triplicate determinations. In all cases the SEM values were no more than 5% of the mean values. Insets: Dependence of the slopes of the lines from the main graphs on the concentration of milacemide. Each point is the mean ± SE determined by non-linear regression. The intercept on the milacemide concentration axis gives the value of $-K_i$.

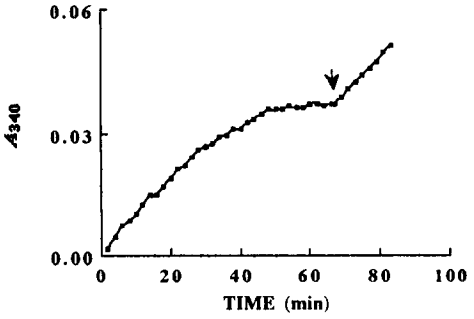

Fig. 5. Time-course of the oxidation of milacemide by rat liver mitochondrial MAO-B. The oxidation of milacemide (2 mM) was followed continuously at 340 nm using the coupled spectrophotometric assay in the presence of 500 μM NAD^+ and 0.6 mg mitochondrial protein. The points shown are the results of a representative experiment. At the point indicated by the arrow a further aliquot of MAO-B (0.6 mg) was added.

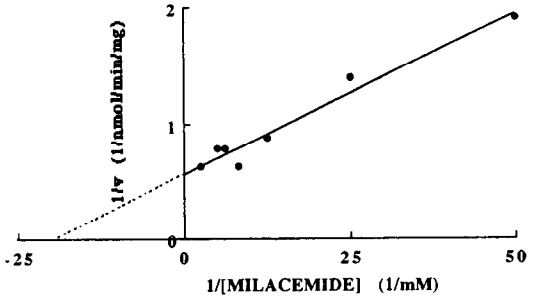

Fig. 6. Determination of the Michaelis constant for the oxidation of milacemide by rat liver mitochondrial MAO-B. Initial rates were determined by the coupled spectrophotometric assay, as described in the text. The Michaelis parameters were determined by non-linear regression and the data are presented as a double-reciprocal plot for illustrative purposes.

with the rat liver mitochondrial preparation showed no significant difference between the maximum velocity of aldehyde production (V_{max} = 1.1 ± 0.2 nmol/min/mg protein, mean ± range of four separate determinations) and the initial rate of H_2O_2 formation [19] at saturating milacemide concentrations (833 μM; 1.2 ± 0.1, mean ± range of four determinations).

DISCUSSION

Milacemide was shown to be a potent inhibitor of MAO-A and -B from rat liver mitochondrial preparations. The IC_{50} values determined without enzyme–inhibitor preincubation were approximately three times lower towards MAO-A than towards the B form of the enzyme (IC_{50} MAO-B/IC_{50} MAO-A = 2.79). However, while the degree of inhibition of MAO-A did not change after 1 hr enzyme–inhibitor

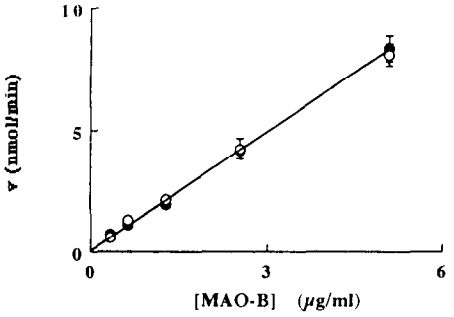

Fig. 7. Effects of the concentration of purified ox liver MAO-B on the initial rate of milacemide oxidation. The initial concentration of milacemide was 1.3 mM. Activities were determined using the luminometric procedure for measuring H_2O_2 formation (○) or the coupled spectrophotometric assay for aldehyde production (●), as described in the text. Each point is the mean ± range from duplicate determinations.

preincubation, the degree of MAO-B inhibition increased significantly. The IC_{50} values after 1 hr enzyme–inhibitor preincubation were 2-fold lower towards MAO-B than towards the A form of the enzyme. Centrifugation–resuspension studies showed that milacemide was an irreversible inhibitor of MAO-B, whereas, it proved to be a reversible inhibitor of MAO-A. These results were substantiated by preincubation of milacemide with rat liver mitochondrial MAO for periods of up to 4 hr. This preincubation induced a time-dependent inactivation of MAO-B consistent with milacemide being an irreversible inhibitor of that form of the enzyme, whereas, no time-dependent inhibition was observed with MAO-A, consistent with milacemide being a freely reversible inhibitor of this form of the enzyme.

The inhibition was evaluated further and K_i values were determined towards each form of MAO. In the absence of preincubation the inhibitor of both forms of MAO, with respect to the amine substrate, was shown to be competitive with milacemide being more potent as an inhibitor of MAO-A. Calculation of the approximate K_i values using the IC_{50} values obtained in the absence of preincubation and the K_m values for the relevant substrates [23] yielded values that were comparable to those determined experimentally.

The competitive nature of this inhibition is consistent with milacemide binding to the active site for MAO. In the case of MAO-B, the coupled spectrophotometric assay showed that the oxidation of milacemide by MAO-B results in an aldehyde product which subsequently is a substrate for aldehyde dehydrogenase. Moreover, the number of moles of aldehyde formed, as determined by the coupled spectrophotometric assay, was in close agreement with the number of moles of H_2O_2 formed as assessed by the luminometric assay. This direct 1:1 stoichiometry between aldehyde and H_2O_2 formation during milacemide oxidation indicates that oxidative cleavage within this compound occurs to form pentanal, glycinamide and H_2O_2. Thus, the oxidative cleavage of milacemide occurs entirely at the pentyl side of the substituted amine (see Scheme 1). If there had been any cleavage at the acetamido side, the products would have been oxamaldehyde and pentylamine. Since the latter compound is a good substrate for MAO-B [24–26], its further oxidation to the corresponding aldehyde would give rise to further molecule of H_2O_2: resulting in a stoichiometry of 2 mol of H_2O_2 for each mol of aldehyde formed (see Scheme 1). Janssens de Varebeke *et al.* [6] reported that milacemide was a substrate for MAO-A with a maximum velocity that was about 10% of that of MAO-B. The results obtained with MAO-A in the present studies were not inconsistent with it oxidizing milacemide, but the very low activities precluded accurate determination of the kinetic parameters for this process.

The progress curve for the inhibition of MAO-B by milacemide would be consistent with the compound acting as both a substrate and time-dependent irreversible inhibitor of the enzyme. After the change in absorbance had ceased using rat liver mitochondrial MAO-B (55 min) the activity could not be restarted by the addition of a further aliquot of milacemide indicating that the reaction had not ceased because of substrate depletion or the establishment of the equilibrium of a reversible reaction. However, the activity could be restored by the addition of a further aliquot of MAO-B. Hence, milacemide behaves both as a substrate and an enzyme-activated, irreversible inhibitor of MAO-B [27]. Such behaviour is similar to that described for 3-{4-[(3-chlorophenyl)methoxy]phenyl}-5-[methylamino)methyl]-2-oxazolidinone methanesulphonate (MD 780236) [28] and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [29]. The substrate 2-phenylethylamine also acts as a time-dependent inhibitor of MAO-B [30], but in that case the inhibition differs from that observed here in being reversible. The inhibition by milacemide has been suggested to be partially reversible [11], but no such reversibility could be demonstrated under the conditions used in the present work.

The work described above confirms and extends the results from studies with rat brain homogenates reported by Janssens de Varebeke *et al.* [6, 11] indicating that milacemide was both a substrate and inhibitor of MAO-B and that the oxidative cleavage proceeds to yield glycinamide and the aldehyde product. Although the significance of this process for the anticonvulsant action of milacemide, rather than for the termination of its actions, remains to be established the compound represents a useful prototype for compounds that may act as systems for delivering specific products to the brain.

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