JJBS

Volume 5, Number 4, December .2012
ISSN 1995-6673
Pages 269 - 278

Jordan Journal of Biological Sciences

Suicide Inhibition of Monoamine Oxidase from Different Species by Milacemide

Zakia M. Ben Ramadan* and Keith F.Tipton

Department of Biochemistry, Trinity College, Dublin 2, Ireland

Received 19th February 2012; accepted 15th July 2012

Abstract

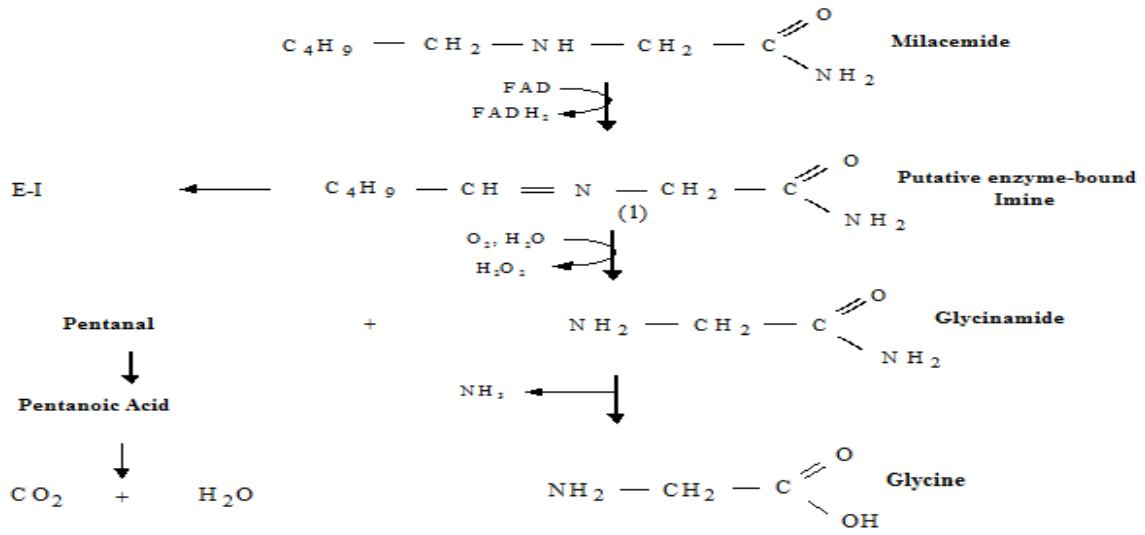
The kinetic parameters for the interactions of the anticonvulsant compound milacemide (2-n-pentylaminoacetamide) with MAO-B from different species have been determined. The data presented here supports the proposed mechanism by which milacemide acts as both a substrate and inhibitor of MAO-B (as a suicide substrate) and show milacemide to be a better substrate than it is an inhibitor for MAO-B from all species, with partition ratios for rat, human and ox liver mitochondrial MAO-B of 12,615; 19,032 and 60,874, mol of product per mol of enzyme inactivated, respectively. In accordance with its higher relative specific activity towards milacemide and the slower rate of inhibition, the partition ratio for ox liver MAO-B is considerably higher than that of the enzyme from rat liver. The partition ratio for human liver mitochondria is close to that of rat liver mitochondria in agreement with the similarity for the half lives ($t_{1/2}$) of these two preparations which were much lower than their respective ($t_{1/2}$) values for the ox preparation. The values obtained for the apparent K_m value (K') (mM) for the inhibition reaction and the inactivation constant (k_{in}) (min^{-1}) for the inhibition were (K') = 0.315, 0.330 and 0.672, (k_{in}) = 0.024 , 0.020 and 0.009 for rat liver mitochondrial MAO-B, rat liver mitochondrial outer-membrane MAO-B and ox liver mitochondrial MAO-B, respectively. At low milacemide concentrations the relative "affinities" of, the rat liver mitochondrial MAO-B and ox liver mitochondrial MAO-B for inhibition by milacemide will be given by their relative values of k_{in} / K' (the "inhibition specificity constant"), this shows milacemide to be a better inhibitor of the rat liver enzyme. The k_{cat}/K_m values confirmed the fact that milacemide is a better substrate for rat liver MAO-B than for ox liver MAO-B and human liver MAO-B. Though the k_{in} values confirmed that milacemide is a much better inhibitor for rat and human liver MAO-B than ox liver MAO-B.

Keywords: Monoamine oxidase (MAO), melacemide, species differences, suicide , inhibition**.**

1. Introduction

Milacemide (2-n-pentylaminoacetamide) is a secondary monoamine-glycine derivative with reported anticonvulsant activity in some, but not all animal models of generalized seizures (Roba *et al.*, 1986). Van Dorsser *et al*. (1983) showed that it was particularly effective in inhibiting the convulsions induced by bicuculline in mice. The effectiveness of milacemide in humans suffering from various forms of epilepsy has not been conclusively established (Roba *et al.*, 1986). Janssens de Varebeke *et al*. (1988) reported that milacemide was a good substrate for MAO-B but that was oxidized only poorly by MAO-A. Oral administration of milacemide to rats resulted in accumulation of glycinamide and glycine in the rat brain (forebrain, cerebellum and medulla) but not in other tissues such as the liver, kidney and lung (Christophe *et al.*, 1983). Intraperitoneal administration of milacemide was reported to increase the glycine levels in the rat cortex, cerebellum and hippocampus significantly (Chapman and Hart, 1988). The acute administration of milacemide to rats led to urinary elimination of glycinamide which was partly prevented by pre-treatment with (-)- deprenyl but not with clorgyline. It appears to affect GABA-ergic mechanisms, since a significant increase in GABA levels in the substantia nigra has been observed (Janssens de Varebeke *et al*., 1983). However, most studies on milacemide have concentrated on the accumulation of the neurotransmitter glycine in the brain. In addition to being a substrate for MAO-B milacemide has been reported to be a time dependent, irreversible inhibitor of MAO-B and a time independent, reversible inhibitor of MAO-A (O'Brien *et al*., 1994a). Janssens de Varebeke *et al*. (1989) have proposed a reaction pathway for milacemide oxidation by MAO-B. , as shown in (Scheme-1) milacemide acts as both a 'suicide' substrate and specific MAO-B inhibitor. E-I represents the irreversibly inhibited species.

* Corresponding author. present address: Department of Biochemistry, Faculty of Medicine University of Tripoli-Libya
Email:zakia.ramadan@gmail.com



Scheme 1

Although, it had been assumed in the literature that the formation of the inhibitory neurotransmitter glycine is a prerequisite for the pharmacological activity of milacemide as an anticonvulsant, there is evidence that this is not the case. Studies on the anticonvulsant compound α-methylmilacemide (O'Brien *et al*., 1994a) suggested that oxidation by MAO may terminate, rather than initiate, the pharmacological activity of milacemide. Furthermore studies with some milacemide derivatives (O'Brien *et al*., 1994b) suggested that the metabolism of these compounds by MAO-B or the inhibition of MAO-A or -B by these compounds does not ensure their anticonvulsant effects. O'Brien *et al.* (1991) reported that milacemide was a more powerful time-dependent inhibitor of rat liver mitochondrial MAO-B than of ox liver MAO-B. Such species differences could clearly complicate interpretations of the behavior of milacemide which are based on the use of enzyme preparations from different species.

Species differences in the interactions of the monoamine oxidases with substrates and inhibitors have been previously reported (Ben Ramadan *et al*., 1999). The present work has concentrated on the kinetic of interactions of milacemide and MAO-B from different sources, ox liver mitochondria and ox liver purified MAO-B were used in comparison with each other and with the MAO-B activity from rat liver mitochondrial and rat liver mitochondrial outer-membrane preparations as well as with the enzyme from human liver mitochondria, in attempts to understand more precisely the role of this enzyme in milacemide metabolism and its interactions as a suicide substrate of MAO-B.

2. Experimental Procedures

2.1. Materials

Benzylamine HCL and 5-Hydroxytryptamine creatine sulphate (5-HT) were obtained from Sigma Co. Milacemide hydrochloride WAS synthesised at Farmitalia Carlo Erba, Milan, Italy. [^{3}H]-Pargyline hydrochloride [Phenyl-3, Benzyl-3H] was obtained from New England Nuclear Corp. (Stevenage, U.K.). Pargyline was obtained from Sigma Co. Clorgyline and l-deprenyl were from Research Biochemicals Inc. All other chemicals were standard laboratory chemicals and were of analytical reagent grade whenever possible

2.2. Methods.

Rat liver mitochondria and human liver mitochondria were prepared by the method of Kearney *et al*. (1971). The mitochondrial pellet obtained was suspended in a small volume of 0.1M potassium phosphate buffer, pH 7.2 and stored at -20^{o}C until use for MAO-B or -A activity after pretreatment with clorgyline or deprenyl respectively. Human liver was obtained within 12 hours of death and transferred to the laboratory on ice. Rat liver mitochondrial outer membrane was prepared by the method of (Sottocasa *et al*., 1967) which includes the sub-fractionation of mitochondria by combined swelling, shrinking and sonication followed by discontinuous density-gradient centrifugation. This was followed by ammonium sulphate precipitation as described by (Houslay and Tipton, 1973b). Ox liver mitochondria and Purified MAO-B from ox liver were prepared by the method of (Salach, 1979). the Aldehyde Dehydrogenase (ALDH) was partially purified from ox liver by a modification (Houslay and Tipton, 1973a) of the method of (Deitrich *et al*., 1962).

All enzyme assays were performed at 37^{o}C and pH 7.2. MAO-B activity were determined spectrophotometrically by directly monitoring the formation of benzaldehyde from benzylamine by following the increase in absorbance at 250 nm Tabor *et al*. (1954). The reaction mixture contained 93 mM potassium phosphate buffer, pH 7.2, enzyme preparation at the indicated concentrations and (333 µM) benzylamine. The molar extinction coefficient (ε) of benzaldehyde at 250 nm was taken to be 13.8 x 10^3. $M^{-1}.cm^{-1}$ (Tipton and Youdim, 1983). The activity of MAO-A was examined, using the standard substrate 5-hydroxytryptamine (5-HT), by the coupled assay in which the formation of NADH is followed continuously at 340 nm as the aldehyde product is further oxidized by ALDH (Houslay and Tipton, 1973a).

The oxidation of the suicide substrate milacemide by MAO-B was examined by the coupled spectrophotometric assay using milacemide as the amine substrate. (Houslay and Tipton, 1973a). The assay mixture contained in a total

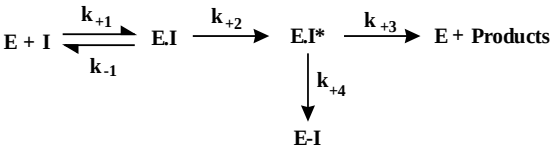
of 2 ml: 80 mM potassium phosphate buffer, pH 7.2, enzyme preparation and amine substrate at the indicated concentrations, 500 µM NAD^+, 0.015 IU of aldehyde dehydrogenase. 1mM pyrazole and 2.5 µM rotenone (in methanol) were included in the reaction mixture to inhibit alcohol dehydrogenase (EC.1.1.1.1) and NADH oxidase (EC.1.6.99.3), respectively. The activity of aldehyde dehydrogenase was not rate limiting under any of the assay conditions. IU of activity is defined as the amount that catalyses the formation of 1µ mol product / min at 37 °C in the presence of 500µM $NAD^+$and 3mM acetaldehyde. The molar extinction coefficient (ε) of NADH at 340 nm was taken to be $6.22x10^3$ $M^{-1}.cm^{-1}$ (Dawson *et al*., 1986).

The Kinetic constants V_{max} and K_m were determined using the computer program ENZFITTER. The reaction progress curves were analyzed using the computer programs ENZFITTER and ULTRAFIT to estimate the values of the maximum product formation (at time -> ∞) (A_{max}) and the apparent the first-order rate constant for the decline in activity with time (k_{app}).

The absolute concentrations of MAO and MAO-B in rat and ox mitochondrial preparations were determined by [^{3}H]-pargyline binding using a modification (Gomez *et al.*, 1986) of the procedure of (Parkinson and Callingham, 1980) (Figs 10,11). The enzyme concentrations (e_o) in human liver mitochondria were calculated from the values reported by (O'Carroll, 1984) for human platelets using 2-phenylethylamine (PEA) as a substrate, by relating these values to the expected values for human liver mitochondria using benzylamine as a substrate and assuming the molecular activity of human platelet MAO-B to be equal to that of human liver MAO-B. This value was calculated to be 19.9 pmol.enzyme/ mg.protein^{-1}.

The absolute concentrations of purified ox liver MAO-B (Fig 12) were determined using the procedure of (Anderson and Tipton, 1994) developed from that used by (Rehavi *et al.*, 1982).

The partition ratio (r) was calculated by determining the amount of product formed at complete inactivation [P_∞] for different enzyme inhibitor ratios, according to the relationship (Waley, 1980) $r = (k_{+3} / k_{+4}) = [P_\infty] / e_0$



(I) Milacemide, (E) the Enzyme, (E.I) a non-covalent compound, (EI^*) an activated intermediate. (E-I) the irreversibly inhibited species. The choice in this reaction is governed by the partition ratio (r) which represents the number of mol of product formed per mol of enzyme inhibited (or k_3/k_4).

The apparent K_m value ($K^{'}$) for the inhibition reaction and the inactivation constant (k_{in}) for the inhibition process were determined by analyzing the curves of the reactions of MAO-B with milacemide at different concentrations of each while enzyme milacemide ratios kept fixed, using the ULTRAFIT computer program. The data obtained were then analyzed by the procedure of (Waley, 1980 ; 1985), using the MacCurve-Fit computer program. In this analysis the half-time of inhibition reaction (t1/2) was determined at various milacemide concentrations and a plot of Io.t1/2 versus the initial milacemide concentration (Io) was constructed.

3. Results

3.1. The oxidation of milacemide by liver mitochondrial MAO from different species

The time course for the oxidation of milacemide by human liver mitochondrial MAO-B was similar to those for rat liver mitochondrial and rat liver mitochondrial outer-membrane MAO-B and were linear only for the first few minutes. After the reaction had ceased completely (70-90 min) the activity could not be restored by the addition of a further aliquot of milacemide or benzylamine. However, the reaction could be restored by the addition of a further sample of mitochondrial MAO-B (Fig.1, representative). indicating that the reaction had not ceased because of substrate depletion or the establishment of the equilibrium of a reversible reaction.

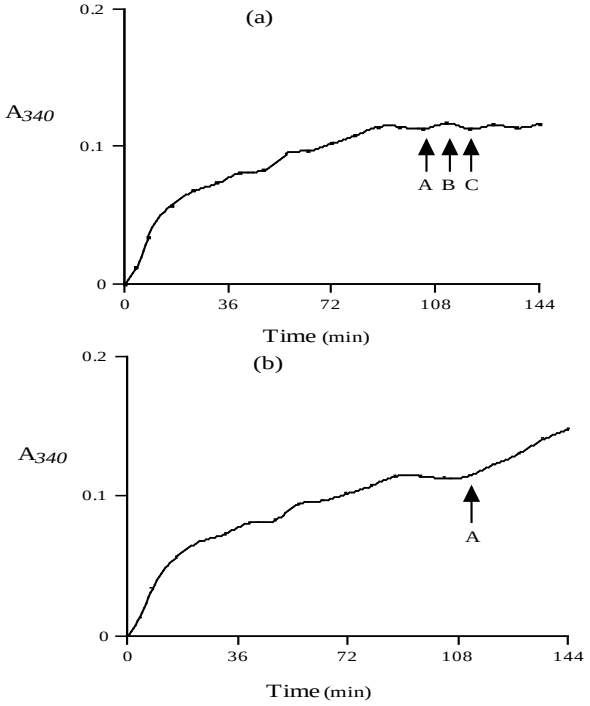


Figure 1. Time-course of Oxidation of Milacemide by Human Liver Mitochondrial monoamine Oxidase-B.
The reaction between human liver mitochondria (473 µg) and Milacemide (2 mM) was followed spectrophotometrically at 340 nm using the coupled assay. Upon completion of initial reaction, (a) At the point indicated by A, a further sample of milacemide was added to raise the final concentration by 2 mM. In a second parallel experiment, at the point indicated by B (333µM) benzylamine was added. In a third experiment, at the point marked C, a further aliquots of ALDH and NAD+ were added. (b) At the point indicated by A more human liver mitochondria was added to the reaction mixture and the reaction was followed further.

The time course for oxidation of 333µM 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 (BenRamadan *et al.*, 2007). The time-courses for the oxidation of milacemide by ox liver mitochondrial MAO-B and purified ox liver MAO-B (Figs 4a and 4b) clearly deviated from linearity over 1 h. After the reaction had ceased completely (7 to 11 h depend on the preparation.

Figures 2, 3, 4a and b show a series of progress curves for the inhibition of rat liver mitochondrial outer membrane MAO-B and human and ox liver mitochondrial MAO-B, respectively, by different concentrations of milacemide. Analysis of these curves showed the dependence of the initial rate of the reaction to obey Michaelis-Menten kinetics in all enzyme preparations used. For both the rat and human liver preparations the rate of the reaction and final absorbance values did not show significant increase with increased Milacemide concentration above 2 mM (Figs 2 and3).

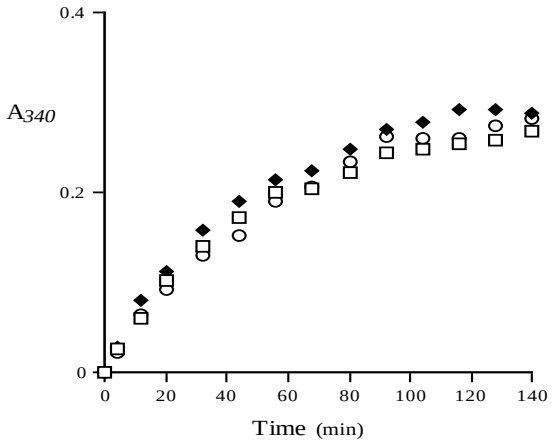


Figure 2(a). Time Courses of Oxidation of Milacemide at a Series of Different Concentrations by Rat Liver Mitochondrial Outer-membrane MAO-B.The reactions between rat liver mitochondrial outer-membrane MAO-B (330 µg) and Milacemide, (□) 2, (■) 3, and (O) 5 mM, were followed spectrophotometrically at 340 nm using the coupled assay. The points shown are the results from representative experiments.

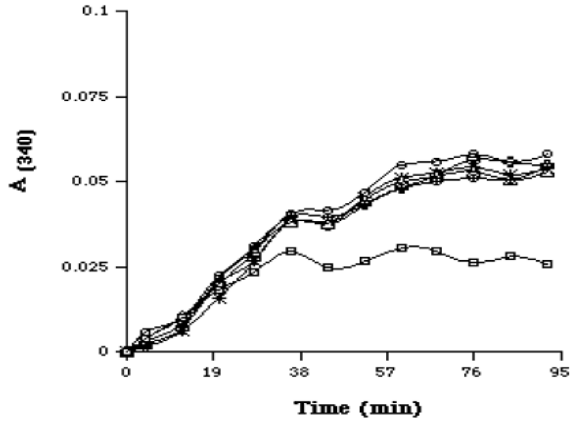


Figure 2(b). Time Courses of Oxidation of Milacemide at a Series of Different Concentrations by Rat Liver Mitochondrial MAO-B.The reactions between rat liver mitochondrial MAO-B (300 µg) and Milacemide, (□) 1, (□) 2, (✳) 3, (O) 4 and (⊕) 5 mM, were followed spectrophotometrically at 340 nm using the coupled assay. The points shown are the results from representative experiments.

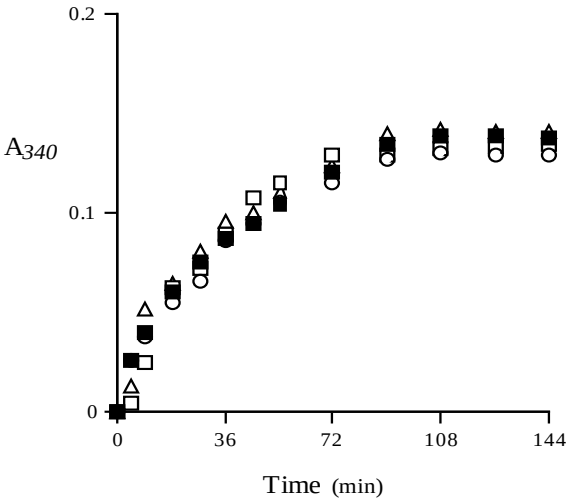


Figure 3. Time Courses of Oxidation of Milacemide at a Series of Different Concentrations by Human Liver Mitochondrial MAO-B.The reactions between human liver mitochondrial MAO-B (473 µg) and milacemide (■) 2, (O) 4, (□) 6, and (Δ) 8 mM, were followed spectrophotometrically at 340 nm using the coupled assay. The points shown are the results from representative experiments.

However as expected the increase for ox liver preparations was up to 6mM milacemide (Figs 4a and b). The Michaelis constants (K_m) and the maximum velocities (V_{max}) for the oxidation of milacemide by MAO-B from all the enzyme preparations used were determined using the coupled spectrophotometric assay. For comparison, the values of K_m and V_{max} for the parent amine, n-pentylamine with the different preparations were also determined (Table 1). (Fig.5) illustrates the determination of the Michaelis constants for the oxidation of milacemide and the parent amine n-pentylamine by human liver MAO-B and is taken as being representative of the other enzyme forms used.

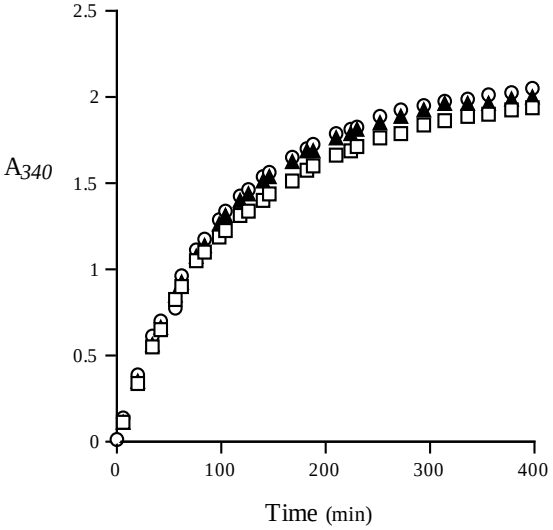


Figure 4(a). Time Courses of Oxidation of Milacemide at a Series of Different Concentrations by Ox Liver Mitochondrial MAO-B. The reactions between ox liver mitochondria (340 µg) and Milacemide, (□) 6, (▲) 9, and (O) 10 mM, were followed spectrophotometrically at 340 nm using the coupled assay. NAD^+ was at a final concentration of 1.5 mM. The points shown are the results from representative experiments.

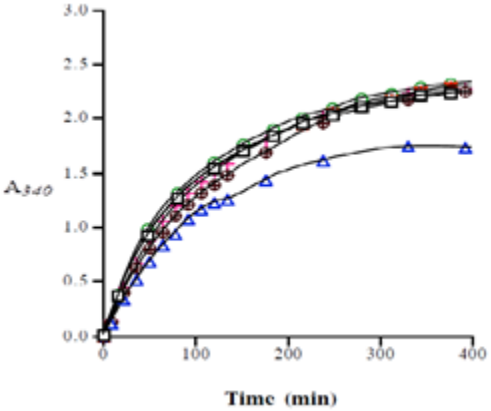


Figure 4(b). Time Courses of Oxidation of Milacemide at a Series of Different Concentrations by Purified Ox Liver Mitochondrial MAO-B.
The reactions between purified ox liver mitochondrial MAOB (20 µg) and Milacemide (□) 4, (□) 6, (⊕) 8, (+) 9 and (□) 10 mM, were followed spectrophotometrically at 340 nm using the coupled assay. NAD+ was at a final concentration of 1.5 mM. The points shown are the results from representative experiments.

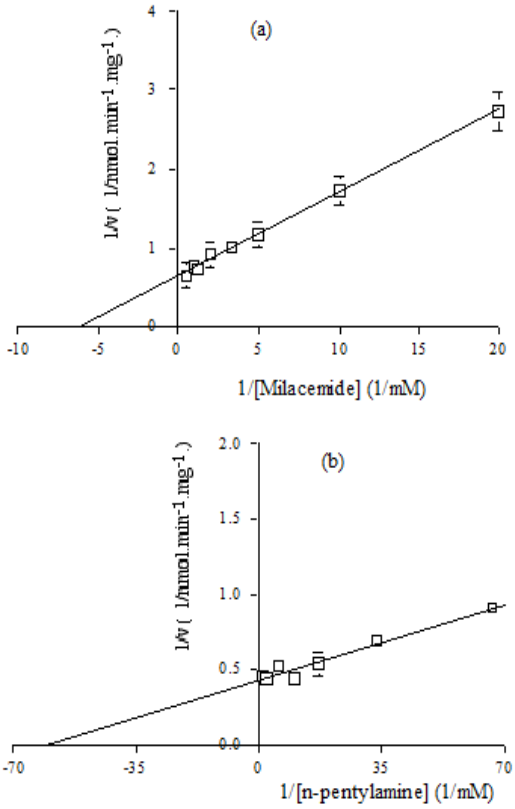


Figure 5. Determination of the Michaelis Constant for the Oxidation of (a) Milacemide and (b) n-pentylamine by Human Liver Mitochondrial MAO-B
The initial rates of oxidation of (a) milacemide and (b) n-pentylamine by human liver mitochondrial MAO-B were measured spectrophotometrically at 340 nm using the coupled assay, over the indicated range of concentrations. The results are presented as a double reciprocal Lineweaver-Burk plot. Each point represents the mean value from two separate experiments.

Table 1. Kinetic Parameters for the Oxidation of Milacemide and n-Pentylamine by Different Preparations of Monoamine Oxidase-B.

Enzyme preparation	Substrate	K_m (μM)	V_{max} (nmol.min^{-1}.mg^{-1}.)
Rat Liver Mitochondria	Milacemide	50 ± 4.7 (3)	1.2 ± 0.09
	n-Pentylamine	39 ± 8.3 (2)	4.15 ± 0.05
Outer membrane	Milacemide	45 ± 4.1 (3)	2.2 ± 0.2
Ox Liver Mitochondria	Milacemide	274 ± 26 (3)	2.832± 0.23
	n-Pentylamine	25.8± 7.2 (3)	4.94 ± 0.06
Purified MAO-B	Milacemide	240 ± 5.1 (3)	380 ± 83
Human Liver Mitochondria	Milacemide	194.8 ± 6.7 (3)	1.718 ± 0.23
	n-Pentylamine	16.35 ± 3.24 (2)	2.359 ± 0.12

The progress curves for the inhibition of mitochondrial monoamine oxidase-B from the different species used, by milacemide are consistent with this compound acting as both a substrate and as an irreversible mechanism-based inhibitor of the enzyme (suicide substrate) according to the mechanism shown above in Scheme1. The partition ratios (r) for all preparations used were determined as described above and shown in (Table 2). The values obtained for the partition ratio of rat, human and ox liver mitochondrial MAO-B were 12,615; 19,032 and 60,874 mol of product per mol of enzyme inactivated, respectively.

3.2. Determination of the kinetic parameters for the mechanism based interactions of MAO-B from different species with milacemide.

(Figs.6 and 8) show a series of reaction-progress curves for different concentrations of, rat liver mitochondrial, outer-membrane MAO-B and ox liver mitochondrial MAO-B, respectively with different concentrations of milacemide but with the enzyme: milacemide ratio kept constant. These curves were analyzed by the procedure of (Waley, 1980 ;1985), as described earlier and according to the following relationships.

$$I_o t_{1/2} = \left(\frac{\ln (2-M)}{(1-M)}\right) \frac{K'}{k_{in}} + \frac{\ln 2}{k_{in}} I_o$$

$$k_{in} = \frac{k_{+2}\ k_{+4}}{k_{+2} + k_{+3} + k_{+4}} = \frac{\ln 2}{\text{slope}}$$

$$K' = (\text{ordinate intercept}).k \quad \frac{(1-M)}{\ln (2-M)}$$

$$\text{where:} \quad M = (1+r).e/I_o$$

I_o and e_o are the initial inhibitor and enzyme concentration, respectively.

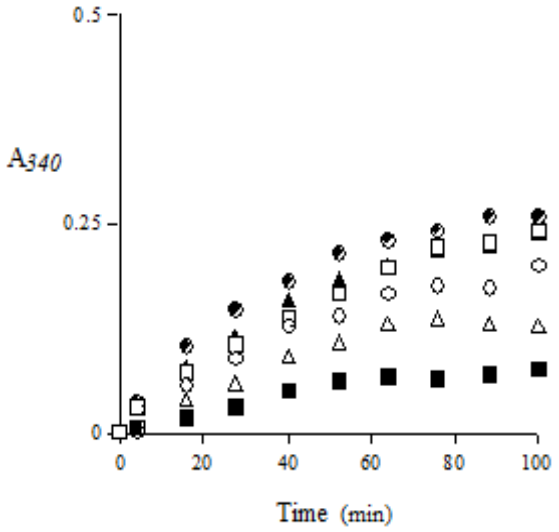


Figure 6. Time Courses of Oxidation of Milacemide by Rat Liver Mitochondrial Outer-membrane MAO-B at Different Concentrations of EachThe reactions of rat liver mitochondrial outer-membrane MAO-B and milacemide, (■) 2 (Δ) 3, (□) 4, (□) 4.5, ( ) 5, and ( ) 6 mM, were determined using the coupled assay. The ratio between the enzyme concentration and milacemide concentration was kept fixed. The points shown are the results from representative experiments.

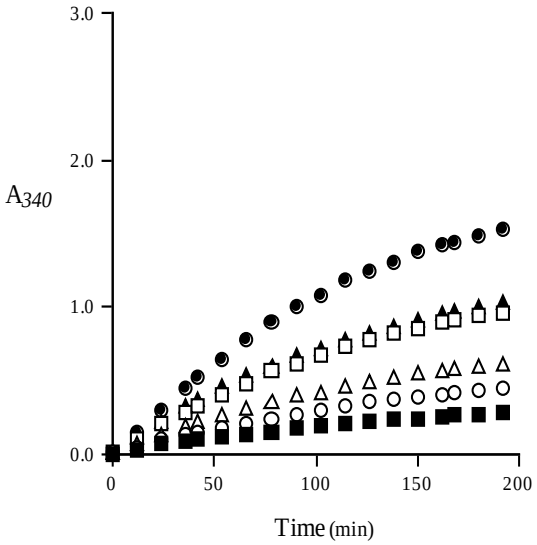


Figure 8. Time Courses of Oxidation of Milacemide by Ox Liver Mitochondrial MAO-B at Different Concentrations of Each

The reactions between ox liver mitochondria, (47-330 ug) and Milacemide, (■) 2, (O) 3, (Δ) 4, (□) 5.5, ( ) 6, and ( ) 9 mM, were followed spectrophotometrically at 340 nm using the coupled assay. NAD^+ was at a final concentration of 1.5 mM. The ratio between the enzyme concentration and milacemide concentration was kept fixed. The points shown are the results from representative experiments.

The half-time of inhibition reaction ($t_{1/2}$) was determined at various milacemide concentrations and a plot of $I_o.t_{1/2}$ versus the initial milacemide concentration (I_o) were constructed for each (Figs.7 and 9, respectively).

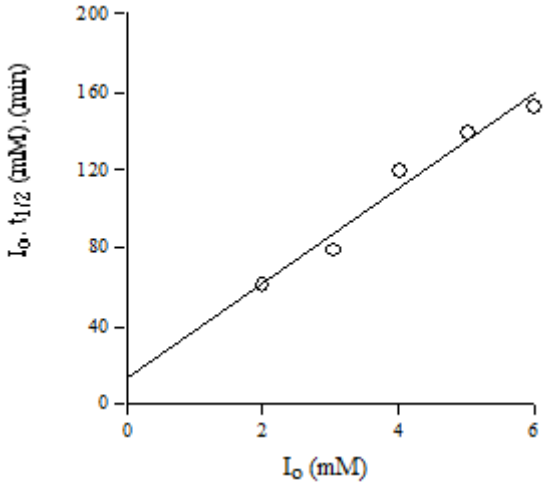


Figure 7. Half-time Plot for the Mechanism Based Inhibition of Rat liver Mitochondrial Outer-membrane MAO-B by Milacemide The plot of Io.t 1/2 against io for a series of experiments in which Io was varied from 2 mM to 6 mM and eo / i Io was kept fixed where eo is the initial concentration of rat liver mitochondrial outer-membranes MAO-B and Io .0is the initial concentration of milacemide. Data from Fig.6.

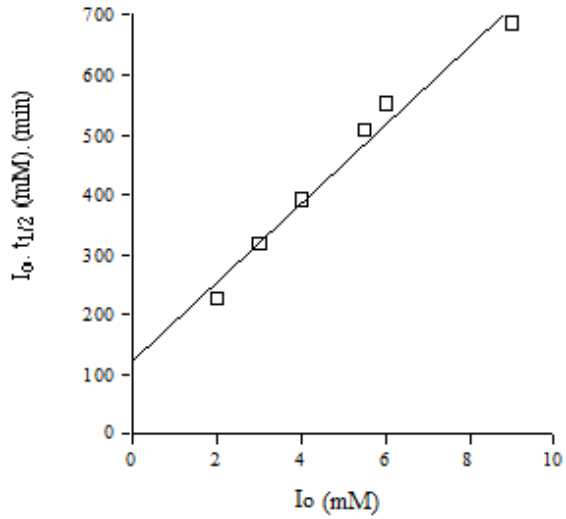


Figure 9. Half-time Plot for the Mechanism Based Inhibitor of Ox liver Mitochondrial MAO-B by Milacemide

The plot of Io.t 1/2 against Io for a series of experiments in which Io was varied from 2 mM to 9 mM and eo / I Io was kept fixed where eo is the initial concentration of ox liver mitochondrial MAO-B and Io is the initial concentration of milacemide. Data from Fig. 8 were used

Then the apparent K_m value (K') (mM) for the inhibition reaction and the inactivation constant (k_{in}) (min^{-1}) for the inhibition were determined. The values obtained were (K') = 0.315 ± 0.04, 0.330 ± 0.05 and 0.672 ± 0.35, (k_{in}) = 0.024 ± 0.004, 0.020 ± 0.011 and 0.009 ± 0.001 for rat liver mitochondrial MAO-B, rat liver mitochondrial outer-membrane MAO-B and ox liver mitochondrial MAO-B, respectively, as shown in (Table 2)

Table 2. Kinetic Parameters for the Inhibition of Different Preparations of Monoamine Oxidase-B with Milacemide.

Enzyme Preparation	$t_{1/2}$ (min)	r ---	K' (mM)	k_{in} (min^{-1})
A. RAT LIVER				
Mitochondria	28.8 ± 3.3	12,615 ± 1727	0.315 ± 0.04	0.024 ± 0.004
Outer membrane	34.0 ± 2.6	13,942 ± 9840	0.330 ± 0.05	0.020 ± 0.011
B. OX` LIVER				
Mitochondrial	74.5 ± 2.4	60,874 ± 2160	0.672 ± 0.35	0.009 ± 0.001
Purified MAO-B	100 ± 15	66,912 ± 5283	ND	0.007 ± 0.002
C. Human Liver				
Mitochondria	27.8 ± 3.6	19,032 ± 1711	ND	0.025 ± 0.002

4. Discussion

The progress curves for the inhibition of rat and human liver MAO-B by milacemide would be consistent with the compound acting as both a substrate and time-dependent irreversible inhibitor of the enzyme (Tipton, 2001). Milacemide met the experimental criteria indicative of an enzyme-activated, irreversible inhibitor (Walsh, 1982) and this work supports the proposed reaction pathway of milacemide oxidation put forward by (Janssens de Varebeke *et al.*, 1989) and shown in Scheme-1.

The present work has confirmed the species differences between rat and ox liver MAO-B reported by (O'Brien *et al.*, 1991). Milacemide was shown to be a poorer substrate of the ox liver preparations and the progress curves of the reactions, as monitored using the coupled spectrophotometric assay, were seen to depart from linearity much more slowly with ox liver mitochondria and purified MAO-B from this source than was the case with the rat liver preparations. Milacemide was more effective as a time-dependent inhibitor of the rat liver mitochondrial preparations. The possibility that the differences might have resulted from the purification of the enzyme from its membrane environment can be discounted, since there was no significant difference between the reactions obtained with purified ox liver MAO-B and that enzyme in mitochondrial preparations. Hence, the solubilisation and delipidation procedures, as well as the subsequent purification steps, used in the preparation of MAO-B cannot account for the discrepancies observed between rat and ox tissues. Similarly the sonication procedure used to prepare rat outer membranes from rat liver mitochondria did not greatly affect the interactions of the MAO-B from this source with milacemide. The progress curves for human liver mitochondria MAO-B were more similar to those from the rat preparations.

Milacemide was shown to have a lower K_m value as a substrate for rat liver MAO-B than for the human and ox liver enzyme. Moreover the K_m value for human liver preparation was about half its value for the ox liver preparation. Not, surprisingly in view of these differences in K_m value, the saturating concentration (i.e. the concentration at which the enzyme is saturated and further increase in the substrate concentration will not result in any detectable increase in the rate of the reaction or the maximum product formed) was lower for the rat and human liver preparations than for those from ox liver. Though the K_m value of n-pentylamine obtained in this study for human liver mitochondria was lower than that obtained for ox and rat liver mitochondria, the K_m value for milacemide with rat liver mitochondria was lower than that obtained by human and ox liver mitochondria.

(Janssens de Varebeke *et al.*, 1989) reported milacemide to be a poor substrate for MAO-A. They reported a V_{max} value for rat liver MAO-A of 0.6 n mol.min^{-1}.mg $protein^{-1}$. However, in the present study with rat liver MAO-A using the coupled spectrophotometric assay, a milacemide concentration range of 1mM to 18 mM was used, but no activity could be detected (no data shown). Similarly, (O'Brien *et al.*, 1994a), who used a luminometric assay for hydrogen peroxide formation, could detect no MAO-A activity towards milacemide. It is, of course possible that MAO-A does oxidize milacemide, but with an activity which is so low that it is below the detection limits of the assay procedures employed. However, the sensitivity of the coupled spectrophotometric assay is such that activities of the level reported by (Janssens de Varebeke *et al.*, 1989) would not have gone undetected.

The values for the absolute enzyme concentrations (number of pmol /mg protein) for MAO-A and MAO-B in rat liver mitochondria and for MAO-B in the ox liver mitochondria (Figs 10,11,12) obtained in this study were about twice the values reported by (Gomez *et al.*, 1986).

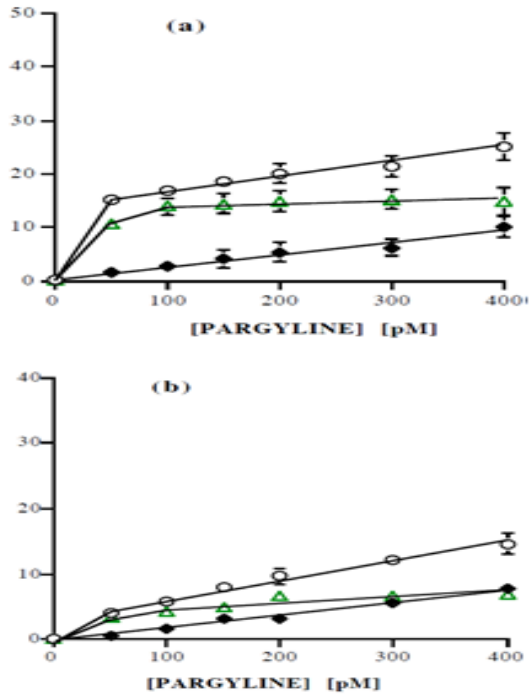


Figure 10. The Binding of [^{3}H]-pargyline to Rat Liver Mitochondrial Monoamine Oxidase and Monoamine Oxidase B
The binding of [^{3}H]-pargyline to rat Liver mitochondrial (a) total Monoamine Oxidase and (b)Monoamine Oxidase B were determined as described in methods. Specific binding (Δ) to rat liver mitochondrial enzyme is defined as the differences between the total binding (O) and the nonspecific binding(■). Each point is the mean range of triplicate determinations in a single experiment.
The vertical axis represent (pM [^{3}H]Parg.Bound/Mg.Protein)

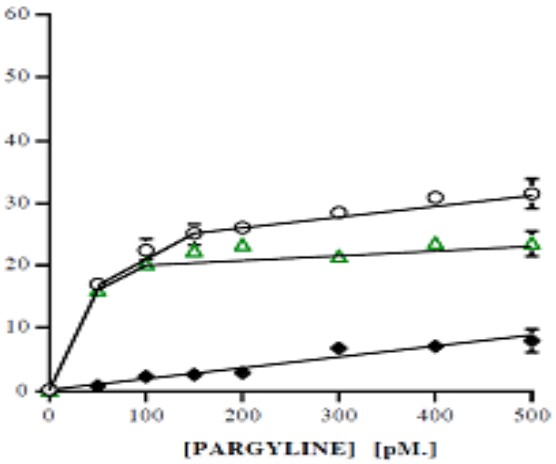


Figure 11. The Binding of [^{3}H]-pargyline to Ox Liver Mitochondrial Monoamine Oxidase B
The binding of [^{3}H]-pargyline to Ox Liver Mitochondrial Monoamine Oxidase B were determined as described in methods. Specific binding (Δ) to ox liver mitochondrial enzyme is defined as the differences between the total binding (O) and the nonspecific binding(■). Each point is the mean range of triplicate determinations in a single experiment. The vertical axis represent (pM [^{3}H]Parg.Bound/Mg.Protein)

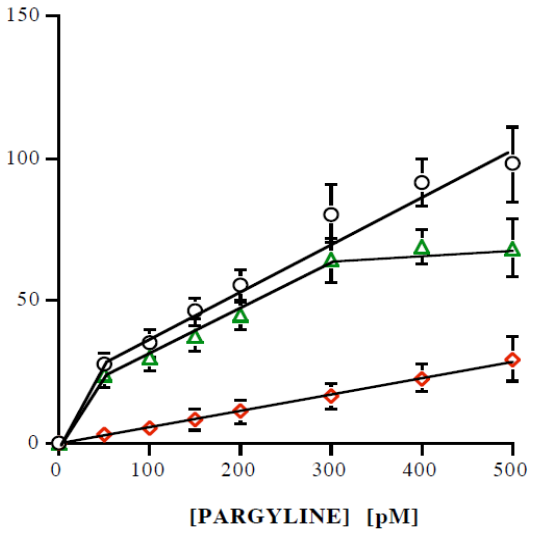


Figure 12. The Binding of [3H]-pargyline to Purified Ox Liver Mitochondrial Monoamine Oxidase B
The binding of [3H]-pargyline to Purified Ox Liver Mitochondrial Monoamine Oxidase B were determined as described in methods. Specific binding (☐) to purified ox liver mitochondrial enzyme is defined as the differences between the total binding (☐) and the nonspecific binding( ). Each point is the mean range of triplicate determinations in a single experiment.
The vertical axis represent (pM [3H]Parg.Bound/Mg.Protein)

The reason for this discrepancy is that the earlier values, as well as those from several other studies, were underestimated because the loss in protein during the stages that the protein samples were washed was not taken into account (Anderson, M.C. unpublished work).

In the present work control samples for protein estimation were carried through the identical procedure as for the binding study and the calculated recoveries were used to correct for protein loss in the pargyline-bound samples . The recoveries of protein were much higher for the rat liver mitochondrial outer membrane preparation and the values obtained for MAO-A and -B in the present work are in closer agreement with those obtained by (Gomez *et al*. 1986) for this preparation.

The high values for the partition ratios (Table 2) for all the preparations used indicate that milacemide function as a better substrate for the enzyme MAO-B than as a mechanism based inhibitor. In accordance with its higher relative specific activity towards milacemide and the slower rate of inhibition, the partition ratio for ox liver MAO-B is considerably higher than that of the enzyme from rat liver. This is in consistent with the inhibition curves for ox liver preparations which took such a long time to reach the completion. The partition ratio for human liver mitochondria is close to that of rat liver mitochondria in agreement with the similarity for the half lives ($t_{1/2}$) of these two preparations (Table 2) which were much lower than their respective ($t_{1/2}$) values for the ox preparation. Furthermore the value of k_{in} for rat liver MAO-B is about three times that for the ox liver enzyme, whereas the apparent K_m value for the inhibitory process is about 2 times higher for ox MAO-B than for the rat enzyme. The inhibition specificity constant, k_{in} / K' for rat liver MAO-B is about 6 times higher than that for ox liver MAO-B. Since the partition ratio of rat liver MAO-B is lower than that of the ox liver enzyme it is possible to achieve complete inhibition of the former enzyme at lower milacemide concentrations than are necessary to inhibit the latter. Comparison of the data for the two species shows milacemide to be a poorer substrate but a better inhibitor of the rat liver enzyme. This would lead one to expect considerable differences in the pharmacokinetics of milacemide in the two species. Thus, under such conditions, milacemide might be expected to be a much more efficient deliverer of glycine to the ox brain. The differences in the values of K_m and K' between the enzymes from these two sources will further complicate the behavior at lower administered milacemide concentrations where the dose may be sufficient to inhibit the rat enzyme but not that from ox. The turnover numbers (k_{cat}) and the k_{cat}/K_m values are compared with the inhibition specificity constants (K_{in} /K') in (Table 3) These values confirmed the fact that milacemide is a better substrate for rat liver MAO-B (6055&6196 $min^{-1}.mM^{-1}$) than for ox liver MAO-B (1826 &1952 $min^{-1}.mM^{-1}$) and human liver MAO-B (2453 $min^{-1}.mM^{-1}$). Though the k_{in} values confirmed that milacemide is a much better inhibitor for rat and human liver MAO-B than ox liver MAO-B. It is clear that the rat may not necessarily be an appropriate model for predicting the behavior of compounds of this type in the human.

Table 3. Comparison of the Kinetic Parameters Milacemide as a Substrate and an Inhibitor of the Monoamine Oxidase-B Preparations.

Enzyme Preparation	K_m (mM)	k_{cat}[a] (min^{-1})	k_{cat}/K_m ($min^{-1}.mM^{-1}$)	K' (mM)	k_{in} (min^{-1})	k_{in}/K' ($min^{-1}.M^{-1}$)
A. RAT LIVER						
Mitochondria	0.050	302.76	6055	0.315	0.024	76.2
Outer membranes	0.045	278.84	6196	0.330	0.020	60.6
B. OX` LIVER						
Mitochondria	0.300	547.86	1826	0.672	0.009	13.4
Purified MAO-B	0.240	468.38	1952	ND	0.007	ND
C. Human Liver						
Mitochondria	0.194	475.8	2453	ND	0.025	ND

Acknowledgements

We are grateful to Dr Andrew Mc Donald for his help with computing skills and to the Health Research Board and Forbairt Ireland, for support of this work.

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