Kinetic Studies on the Interactions of Mitochondrial Monoamine Oxidase-B of Human and Pure Ox Liver with 2-(Benzylamino) Acetamide

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ABSTRACT

In an attempts to understand more precisely the role of monoamine oxidase (MAO-B) enzyme in the metabolism of the parent anticonvulsant 2-n-pentylaminoacetamide, the benzylamine derivative 2-benzylamino acetamide, also designated as FCE 25692, which used as a substrate and an inhibitor of MAO-B from human liver mitochondria and pure ox liver. The results obtained indicated that FCE 25692 to act as a suicide substrate with apparent $K_m$ values of 989 and 950 µM and $V_{max}$ values of 0.422 and 93.05 nMol.min$^{-1}$.mg$^{-1}$ for human and the purified ox liver MAO-B, respectively, in a way that can explain that it is a better substrate rather than an inhibitor for MAO-B from both species, with partitions ratios of 1062 and 2733 mol of product per mol of enzyme inactivated, for human and ox preparation, respectively. Moreover, the turnover numbers ($k_{cat}$) and the $k_{cat}/K_m$ values confirmed the fact that FCE 25692 is somewhat a better substrate for MAO-B purified from ox liver than from human liver, with $k_{cat}/K_m$ values of 40.3 and 17.1 min$^{-1}$.mM$^{-1}$, respectively. The progress curves for the inhibition of MAO-B showed that FCE 25692 has an equipotent time-dependent inhibitor of MAO-B in both preparations. It can be confirmed from the close similar inactivation constant $k_{in}$ and half-life values for MAO-B from both preparations; the $t_{1/2}$ for the purified ox liver inhibited by FCE 25692 matched well with its human liver counterpart.

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MAO-B (49.9 min) and for human MAO-B (41.9 min).

Despite the fact that benzyamine is a substrate for the semicarbazide-sensitive amine oxidase (SSAO; EC 1.4.3.6), FCE 25692 was found not to be a substrate or an inhibitor for any of that enzyme from ox plasma or lung.

INTRODUCTION

Monoamine oxidase (MAO) inhibitors are important target of antidepressants in the treatment of psychiatric and neurological disorders. Owing to the risk of the drug’s interaction of MAO inhibitors with diet or other drugs, not used as the first line. Interest in these drugs has also increased markedly in recent years following numerous reports of their neuroprotective actions in vivo and in vitro.  

Hydrazines were the first suicide (mechanism-based) enzyme inhibitors of MAO to be recognized. Those used clinically include phenelzine, phenylhydrazine, nialamide and isocarboxazid. During their catalysis, an activated irreversible inhibitory species is formed through the action of MAO itself (2, 3, 4). These studies stimulated the development of many other inhibitors of this enzyme, which have been used as antidepressants. The anticonvulsant compound milacemide (2-n-pentylaminoacetamide) acts as a pro-drug delivering glycine into the central nervous system (5,6). 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 (7). It has been shown in a previous study by Ben Ramadan (8) that milacemide functions as a suicide substrate and being a better substrate for the enzyme MAO-B than as a mechanism based inhibitor for all the species studied. Milacemide has been found to be readily and completely absorbed, reaching a plasma concentration peak approximately 30 min after administration (9, 10) showed that it was effective in decreasing seizure frequency, well-tolerated and no significant adverse effects were observed. A double blind placebo-controlled study by Saletu (11) indicated that milacemide enhances cognitive functions such as vigilance, learning and memory and also has mood elevating characteristics which benefitted the sociability of the subjects. These findings prompted further investigations into the psychotropic effects and the safety of milacemide in clinical trials. The molecular modification of the parent anticonvulsant compound 2-n-pentylaminoacetamide by varying the R group (pentyl moiety) or alternatively the acetamide moiety could be fruitful and enlightening strategies in drug design. (12) showed the absence of correlations between the anticonvulsant potency and the oxidative metabolism of a series of derivatives, of milacemide (2-n-pentylaminoacetamide) obtained by replacing the pentylamine of milacemide with various MAO substrate residues (tryptamine, 5-methoxytryptamine and 2-phenylethylamine) by MAO-B. Also no correlation between the anticonvulsant activity of these compounds and their inhibitory potency was found. However, the possibility that milacemide exerts at least some of its pharmacological effects as a consequence of MAO-B inhibition cannot be completely excluded and hence further studies aimed at understanding the mechanism of action of this novel compound and some of its analogues were warranted. In the present work,
comparative kinetic studies on the behavior of the analogues, 2-benzylamino acetamide (FCE 25692) as a substrate and an inhibitor of MAO-B from human liver mitochondria and the purified ox liver MAO-B have been done. FCE 25692 was reported by Dostert to be a potent and selective rat liver MAO-B inhibitor and to be deaminated at a slow rate as compared with milacemide and some of the other analogues. Furthermore, it displayed little protection against tonic convulsions and death in mice. It has also been shown by O'Brien to be a time dependent inhibitor of rat liver MAO-B with (IC50 = 490 and 207 µM after 0 and 1h incubation at 37°C, respectively) and a reversible inhibitor of rat liver MAO-A (IC50 = 42.6 and 47.9 µM, after 0 and h incubation at 37°C, respectively) and a time-dependent inhibitor of purified ox liver MAO-B (IC50 = 2150 and 880 µM after 0 and 1h incubation at 37°C, respectively).

2. Experimental procedures:

2.1. Material:
Benzylamine HCL was obtained from Sigma Co. (2-(benzylamino) acetamide) FCE 25692 was synthesised at Farmitalia Carlo Erba, Milan, Italy. All other chemicals were standard laboratory chemicals and were of analytical reagent grade whenever possible.

2.2. Methods:
Human liver mitochondria were prepared by the method of Kearney. The mitochondrial pellet obtained was suspended in a small volume of 0.1M potassium phosphate buffer, pH 7.2 and stored at -20°C until use for MAO-B. Human liver was obtained within 12 hours of death and transferred to the laboratory on ice. Purified MAO-B from ox liver was prepared by the method of Salach. The semicarbazide-sensitive amine oxidase (EC 1.4.3.6) preparations used were from ox plasma and ox lung which was kindly donated by Dr J.M. Lizcano.

All enzyme assays were performed at 37°C and pH 7.2. MAO-B and the semicarbazide-sensitive amine oxidase (SSAO) activity were determined spectrophotometrically by directly monitoring the formation of benzaldehyde from benzylamine by following the increase in absorbance at 250 nm. 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 (e) of benzaldehyde at 250 nm was taken to be 13.8 x 10^3 M^-1.cm^-1. The oxidation of the suicide substrate FCE 25692 by MAO was examined by the direct spectrophotometric assay for benzaldehyde formation at 250 nm. The Kinetic constants Vmax and Km were determined using the computer program ENZFITTER. The double reciprocal plots are used only for illustrative purposes. The reaction progress curves were analyzed using the computer program MACCURVE-FIT to estimate the values of the maximum product formation at time = ∞ (Amax) and the apparent first-order rate constant for the decline in activity with time (kapp).

The partition ratio (r), which represents the number of mol of product formed per mol of enzyme inhibited (or k3/k4) was calculated by determining the amount of product formed at complete inactivation [P∞] for different enzyme inhibitor ratios, according to the relationship r = (k+3 / k+4) = [P∞] / e₀.
The direct spectrophotometric assay for aldehyde formation was used to examine the reactions of MAO-B (at different concentrations) with FCE 25692 (at different concentrations) while enzyme: FCE 25692 ratio were kept constant. An Uvikon-931 double beam spectrophotometer equipped with a multiccil autochanger, which allow the sequential determination of six samples with the appropriate blanks where the temperature was controlled by the use of a circulating water bath was used. The curves were analyzed by the procedure of Waley (18,19), using the MACCURVE-FIT computer program. Then the apparent K_m value (K') for the inhibition reaction and the inactivation constant (k_in) for the inhibition process were determined according to the following relationships, (18,19)

\[
I_{o} \text{ and } e_{o} \text{ are the initial inhibitor and enzyme concentration, respectively.}
\]

The possible oxidation of FCE 25692 with SSAO was determined by the spectrophotometric procedures under the same conditions as were used for the MAO assays. Inhibition was determined without preincubation between enzyme and inhibitor and after preincubation for 30 min at 37 °C with the inhibitor concentrations up to 10 mM before the addition of substrate.

3. Results:
3.1. Time-courses of Oxidation of FCE 25692 by liver mitochondrial MAO-B from both species:
The initial rates of FCE 25692 oxidation, were found to be linear function of the enzyme concentration. The time-courses for the oxidation of FCE 25692 by MAO-B from both species used deviated from linearity after few minutes of starting the reactions. After the reaction had ceased almost completely (75 and 100 min) for human and ox liver MAO-B, respectively, it could not be restored by the addition of more substrate indicating that the reaction had not ceased due to substrate depletion or the establishment of an equilibrium of a reversible reaction. Neither could any activity be detected when (333µM) benzylamine was added. However, the addition of more enzyme was found to restore the activity as shown in Fig 1 (it is taken as a representative for the enzyme from both preparations).

Figs 2 and 3 show series reaction progress curves of, human and pure ox liver mitochondrial MAO-B with different concentrations of FCE 25692, respectively. These reactions obeyed the Michaelis-Menten kinetics. The K_m and the V_max for the oxidation of FCE 25692 by MAO-B from both preparations were determined and compared with their respective values for the parent amine benzylamine. The values obtained are shown in (Table 1).

Figs. 4 and 5 illustrate the determination of the Michaelis constants for the oxidation of FCE 25692 and the parent amine benzylamine by human liver MAO-B, respectively and are taken as being representative of the other enzyme form used. The progress curves for the inhibition of monoamine oxidase-B from both species used by FCE 25692 would be consistent with this compound acting as both a substrate and as an irreversible mechanism-based inhibitor of the enzyme (suicide substrate inhibitor) according to the mechanism shown in Scheme-1. The
partition ratio (r) was calculated by determining the amount of product formed at complete inactivation \([P_\infty]\) for different enzyme inhibitor ratios. The absolute enzyme concentrations (\(e_0\)) were determined as described in (Ben Ramadan 8). The values obtained for the partition ratio of human and pure ox liver mitochondrial MAO-B were 1062 and 2733, respectively (Table 2).

3.2. The Kinetic Parameters for the Mechanism Based Interactions of MAO-B from both species with FCE 25692:

Fig.6a show a series of reaction-progress curves for pure ox liver mitochondrial MAO-B, with different concentrations of FCE 25692 but with the enzyme: FCE 25692 ratio kept constant. These curves were analyzed by the procedure of Waley (18,19), and a plot of \(I_o^{t_1/2}\) versus the initial FCE 25692 concentration \(I_o\) was constructed (Fig. 6b). From this the values of the apparent \(K_m\) (\(K'\)) and the inactivation constants \((k_{in})\) obtained and they were \((K') 1.16 \text{ mM}\) and \((k_{in}) 0.014 \text{ min}^{-1}\) for pure ox liver mitochondrial MAO-B, as shown in Table 2. The value of the half-life \((t_1/2)\) for human liver mitochondria obtained in this study is close to its respective \((t_1/2)\) value for the pure ox MAO-B. The value of \((k_{in})\) for human liver mitochondria was calculated from the value of the half-life \((t_1/2)\) and was found to be 0.016, which is closer to the values obtained for MAO-B from ox liver preparation.

3.3. Interactions of FCE 25692 with the Semicarbazide-Sensitive Amine Oxidases (SSAO):

There was no significant oxidation of FCE 25692 by any of these SSAO preparations. Furthermore the compound was ineffective as an inhibitor of any of the SSAO preparations (no data shown). The possibility that either of these enzymes might cleave this compound to form benzylamine plus oxamaldehyde can be discounted since the former product would be further oxidized by either of these SSAO preparations to form benzaldehyde.

4. Discussion:

The progress curves for the inhibition of MAO-B by FCE 25692 would be consistent with the compound acting as both a substrate and time-dependent irreversible inhibitor of the enzyme. This can explain that the mechanism of action of FCE 25692 met the experimental criteria indicative of an enzyme-activated, irreversible inhibitor and is similar to the mechanism of action of milacemide, which acts as both a substrate and inhibitor of MAO-B and to that described for MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 3-[(3-methylamino)methyl]-2-oxazolidinone methanesulphonate (MD 780236). This work supports the expected reaction pathway of FCE 25692 oxidation by MAO-B as shown in Scheme-1. FCE 25692 is oxidized by MAO-B to form an imine intermediate. This intermediate can react with MAO-B to form a covalently bound intermediate that inactivates the enzyme or is hydrolyzed to liberate glycaminde and benzaldehyde. The latter product is then oxidized to benzoic acid. Benzoic acid is readily oxidized to \(\text{CO}_2\) and \(H_2O\), whereas, glycaminde is broken down by amidase activities in brain microsomes to glycine. The detailed reaction mechanism may be more complex than this with intermediate radical intermediates being the actual inhibitory species.

No large species differences can be recorded in this study between ox and human liver MAO-B and the progress curves of the reactions, as monitored using the direct spectrophotometric assay, were
seen to depart from linearity after few minutes with both forms of MAO-B studied. The values of the half-lives (Table 2) confirmed this; the t_{1/2} values for the liver mitochondrial MAO-B of pure ox and human were nearly close. The high values of the partition ratios Table (2) for both preparations indicate that FCE 25692 function as a better substrate for the enzyme MAO-B than as a mechanism-based inhibitor, in accordance with the behavior of milacemide as recorded by Ben Ramadan. The partition ratio for pure ox liver MAO-B (2733) is considerably higher than that of the enzyme from human liver mitochondria (1062).

The progress curves for the inhibition of MAO-B from both preparations showed that FCE 25692 has an equipotent time-dependent inhibitor of MAO-B in the ox and human preparations, and this is in agreement with the IC_{50} values for ox and rat mitochondrial enzymes previously reported by O'Brien. FCE 25692 was shown to have close K_{m} values as a substrate for the ox and human liver enzymes, used in these studies (950 and 989 µM, respectively). The k_{cat} value (Table 3) obtained for the purified enzyme from ox liver (38.3 min^{-1}) was somewhat higher than that for the human liver mitochondrial MAO-B (16.9 min^{-1}). These differences are reflected in the specificity constants (k_{cat}/K_{m} values) which are the most useful indicators of the substrate specificity of an enzyme. 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 FCE 25692 is considerably a better substrate for MAO-B from ox than human preparation with k_{cat}/K_{m} values of (40.3 and 17.1 min^{-1}.mM^{-1}, respectively) while The inactivation constant k_{in} and the half-lives values confirmed that FCE 25692 was an equipotent time-dependent inhibitor of MAO-B in ox and human preparations. The selectivity of FCE 25692 and milacemide as substrates for MAO-B may be related to the lipophilicity of these compounds, which is a common property of several MAO-B substrates. It is clear that milacemide is a much better substrate for MAO-B from ox and human preparations than its benzylamine derivative, FCE 25692 in present studies. This could reflect the behavior of the precursor amines from which they were derived, since n-pentylamine the parent amine of milacemide has a much lower K_{m} towards MAO-B than benzylamine, the parent amine of FCE 25692. This difference may be an effect of the electronegative benzene ring of benzylamine.

Despite the fact that benzylamine is a substrate for the semicarbazide-sensitive amine oxidase (SSAO; EC 1.4.3.6), FCE 25692 was found not to be a substrate or an inhibitor for any of that enzyme from ox plasma or lung.

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References
Oxidase (1-methyl-2-phenylethyl) hydrazine and its Potentiation by Cyanide


20. Tipton KF, Fowler CJ, McCrodden


Table 1: The Kinetic Parameters for the Oxidation of FCE 25692 and Benzylamine by Monoamine Oxidase-B from the two Preparations

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Human Liver Mitochondria</th>
<th>Pure Ox Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Benzylamine</td>
<td>FCE 25692A</td>
</tr>
<tr>
<td>(K_m) (µM)</td>
<td>180 ± 80 (2)</td>
<td>989 ± 120 (2)</td>
</tr>
<tr>
<td>(V_{max}) (nMol.min(^{-1}).mg(^{-1}))</td>
<td>5.900 ± 1.3</td>
<td>0.422± 0.06</td>
</tr>
</tbody>
</table>

The Direct spectrophotometric assay at 250 nm was used in all cases. Values quoted are the means ± S.E. from the curve fits obtained from two or more separate determinations (as shown in brackets).
Table 2: The Kinetic Parameters for the interactions of the two Preparations of Monoamine Oxidase-B with FCE 25692

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Human Liver Mitochondria</th>
<th>Pure Ox Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>t1/2 (min)</td>
<td>41.9 ± 4.7</td>
<td>49.9 ± 3</td>
</tr>
<tr>
<td>r</td>
<td>1062 ± 145.6</td>
<td>2733 ± 291</td>
</tr>
<tr>
<td>K' (mM)</td>
<td>ND</td>
<td>1.161 ± 0.09</td>
</tr>
<tr>
<td>k_{in} (min^{-1})</td>
<td>0.016 ± 0.005</td>
<td>0.014 ± 0.004</td>
</tr>
</tbody>
</table>

The values were determined by the procedure of Waley^{(18,19)} as described in the text. Each value represents the mean ± S.E. For at least 3 replicates.

Table 3: A Comparison of the kinetic Parameters for FCE 25692 as a Substrate and an Inhibitor of the Monoamine Oxidase-B Preparations

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Human Liver Mitochondria</th>
<th>Pure Ox Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_m (mM)</td>
<td>0.989</td>
<td>0.950</td>
</tr>
<tr>
<td>K_{cat} (min^{-1})</td>
<td>16.9</td>
<td>38.3</td>
</tr>
<tr>
<td>k_{cat}/K_m (min^{-1}.mM^{-1})</td>
<td>17.1</td>
<td>40.3</td>
</tr>
<tr>
<td>K' (mM)</td>
<td>ND</td>
<td>1.161</td>
</tr>
<tr>
<td>k_{in} (min^{-1})</td>
<td>0.016</td>
<td>0.014</td>
</tr>
<tr>
<td>k_{in}/K' (min^{-1}.M^{-1})</td>
<td>ND</td>
<td>12.0</td>
</tr>
</tbody>
</table>

The catalytic constant $k_{cat}$ (maximum velocity / enzyme concentration) = K_{in}.r.

Figure 1. Time Course of Oxidation of FCE 25692 by Human Liver Mitochondrial Monoamine Oxidase-B
The reaction between human liver mitochondria (550 µg) and FCE 25692 (4 mM) was followed spectrophotometrically at 250 nm using the direct assay. Upon completion of initial reaction, (a) at the point indicated by A, a further sample of FCE 25692 was added to raise the final concentration by 4 mM. In a second parallel experiment, at the point indicated by B (333 µM) benzylamine was added. (b) At the point indicated by A more human liver mitochondria was added to the reaction mixture and the reaction was followed further.

Figure 2. Time Courses of Oxidation of FCE 25692 at a Series of Different Concentrations by Human Liver Mitochondrial MAO-B

The reactions between human liver mitochondria (550 µg) and FCE 25692, (▲) 3, (○) 4, (■) 6, and (△) 8 mM, were followed spectrophotometrically at 250 nm using the direct assay. The points shown are the results from four representative experiments.

Figure 3. Time Courses of Oxidation of FCE 25692 at a Series of Different Concentrations by Pure Ox Liver Mitochondrial MAO-B
The reactions between pure ox liver mitochondrial MAO-B (200 μg) and FCE 25692, (△) 3, (○) 4, (⊗) 5, and (□) 6 mM, were followed spectrophotometrically at 250 nm using the direct assay. The points shown are the results from four representative experiments.

Figure 4. Determination of the Michaelis Constant for the Oxidation of FCE 25692 by Human Liver Mitochondrial MAO-B:

The initial rates of oxidation of FCE 25692 by human liver mitochondria were measured, over the indicated range of concentrations. The results are presented as a double reciprocal Lineweaver-Burk plot. Each point represents the mean value from three separate experiments.
Figure 5. Determination of the Michaelis Constant for the Oxidation of Benzylamine by Human Liver Mitochondrial MAO-B:

The initial rates of oxidation of benzylamine by human liver mitochondria were measured, over the indicated range of concentrations. The results are presented as a double reciprocal Lineweaver-Burk plot. Each point represents the mean value from three separate experiments.

Figure 6. Determination of the Kinetic Parameters of Pure Ox Liver Mitochondrial MAO-B towards FCE 25692

a) Time Courses of Oxidation of FCE 25692 by Pure Ox Liver Mitochondrial MAO-B at Different Concentrations of Each: The reactions of pure ox liver mitochondrial MAO-B and FCE 25692, (□) 2, (○) 2.5, (●) 3, (▲) 3.5, (▲) 4, and (■) 4.5 mM, were followed spectrophotometrically. The ratio between the enzyme concentration and FCE 25692 concentration was fixed. The points shown are the results from six representative experiments.

b) Half-time Plot for the Mechanism Based Inhibition of Pure Ox liver Mitochondrial Monoamine oxidase-B by FCE 25692: The plot of \([I_o \cdot t]^{1/2}\) against \([I_o]\) for a series of experiments as shown above (a) in which \(e_o / [I_o]\) was kept fixed where \(e_o\) is the initial concentration of pure ox MAO-B and \(I_o\) is the initial concentration of FCE 25692. The points shown are the mean values ± range from two separate experiments.
Scheme 1: The mechanism of FCE 25692 oxidation by MAO-B as would be expected since FCE 25692 acts as both a 'suicide' substrate and specific MAO-B inhibitor. E-I represents the irreversibly inhibited species.