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A NEW TIME-DEPENDENT REVERSIBLE INHIBITOR OF MONOAMINE OXIDASE

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INTRODUCTION

Monoamine oxidase [MAO; monoamine oxidoreductase (deaminating) (flavin-containing); EC 1.4.3.4] occurs in two forms, A and B, the products of separate genes.[1] The enzymes metabolise a range of endogenous and synthetic aralkylamines. The products of oxidation of amines such as RCH_2NH_2 by MAO are an aldehyde, RCHO, and NH_3 and it is assumed that the reaction proceeds via an intermediate imine (see e.g. Silverman *et al.*[2]). Numerous inhibitors of the enzyme, reversible and irreversible, have been examined as potential therapeutic agents for treatment of conditions such as depressive illness and Parkinson's disease.[3–5] *N*-(2-aminoethyl)-*p*-chlorobenzamide (**2**) (Figure 1) is one of a number of related aminoalkyl benzamides developed by Hoffman-La Roche that are potent, time-dependent inhibitors of MAO B. We have previously shown that this compound and some analogues display slow-binding kinetics with MAO B. In addition, we have discovered that compounds in which the NH of the amide bond is replaced by O, giving aminoethyl benzoate esters, e.g. 2-aminoethyl-*p*-chlorobenzoate (**3**) (Figure 1) are much more potent inhibitors of the enzyme.[6] It may be noted that the esters are not subject to the conformational constraint imposed on the amides by virtue of the electron delocalisation in the amide bond and this may account for the greater potency of the esters. We now find that an analogous urethane, *N*-phenyl-(2-aminoethyl)carbamate (**1**) (Figure 1) also inhibits MAO B in a time-dependent, reversible manner and conforms to a kinetic mechanism of the slow-binding type.

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(1) **(2)** **(3)**

FIGURE 1 Structure of the urethane *N*-phenyl-2-aminoethyl carbamate (**1**) and the analogous *N*-(2-aminoethyl)-*p*-chlorobenzamide (**2**) and 2-aminoethyl-*p*-chlorobenzoate (**3**).

METHODS

Synthesis of Inhibitors

N-*t*-boc ethanolamine was prepared by stirring together ethanolamine (2 g), di-*t*-butyl dicarbonate (7 g) and $NaHCO_3$ (5.5 g) in 40 ml of 20% aqueous tetrahydrofuran (THF) at room temperature for 16 h. The THF was removed by evaporation and the product was extracted from the aqueous residue into ethyl acetate. This was washed with 5% $NaHSO_4$ solution, then with water. The ethyl acetate was dried ($MgSO_4$) and evaporated to yield 4.2 g of a non-crystallisable viscous liquid which was used without further purification. It had a strong IR absorbance at 1680 cm^{-1}, indicative of the urethane carbonyl group.

This product was heated with phenyl isocyanate (3.3 g) for 10 min at 70°C and then cooled to about 50°C before adding 60°–80° petroleum ether (ca. 50 ml) with stirring. The resulting white solid (6.8 g) was collected and crystallised from ethyl acetate/petrol ether to yield 5 g of N-*t*-boc-$NHCH_2CH_2OCONHPh$. This was deprotected by stirring for 1 h in a mixture of dichloromethane (25 ml) and TFA (10 g). The residue remaining after evaporation of solvent and TFA was partitioned between ethyl acetate and sodium carbonate solution. The organic phase was washed with water, dried and evaporated. The residue was converted to an HCl salt and crystallised from isopropanol/methanol. Yield of (**1**), 1.35 g, m.p. 197–199°C. Found: C, 49.79; H, 6.13; N, 12.71. $C_9H_{13}N_2O_2Cl$ requires C, 49.88; H, 6.01; N, 12.93%.

Its structure was confirmed by ^{1}H-nmr. δ (ppm) [^{2}H DMSO] (TMS = 0) 3.1 [2H, t, CH_2-N]; 4.3 [2H, t, O-CH_2]; 7.0 [1H, *p-aryl*]; 7.3 [2H, *m-aryl*]; 7.5 [2H, *o-aryl*]; 8.2 [3H, s, NH_3^+]; 9.8 [1H, s, N*H*].

The *N*-methyl derivative was prepared in an analogous manner, starting from *N*-methyl-ethanolamine. The HCl salt had m.p. 163–165°C. Found: C, 52.15; H, 6.53; N, 11.98. $C_{10}H_{15}N_2O_2Cl$ requires C, 52.06; H, 6.5; N, 12.04%. ^{1}H nmr: δ (ppm) [^{2}H DMSO] TMS = 0. 2.6 [3H, s, CH_3]; 3.2 [2H, t, CH_2N]; 3.35 [2H, t, OCH_2]; 7.0 [1H, *p-aryl*]; 7.3 [2H, *m-aryl*]; 7.5 [2H, *o-aryl*]; 9.0 [2H, s, NH_2]; 9.8 [1H, s, N*H*CO].

Inhibition Studies

Protein was measured by the method of Lowry *et al.*[7] All experiments were carried out at 30°C and pH 7.5 (50 mM Na/K phosphate buffer) in a total volume of 1 ml, unless otherwise indicated. Spectrophotometric assays were conducted in a Cary

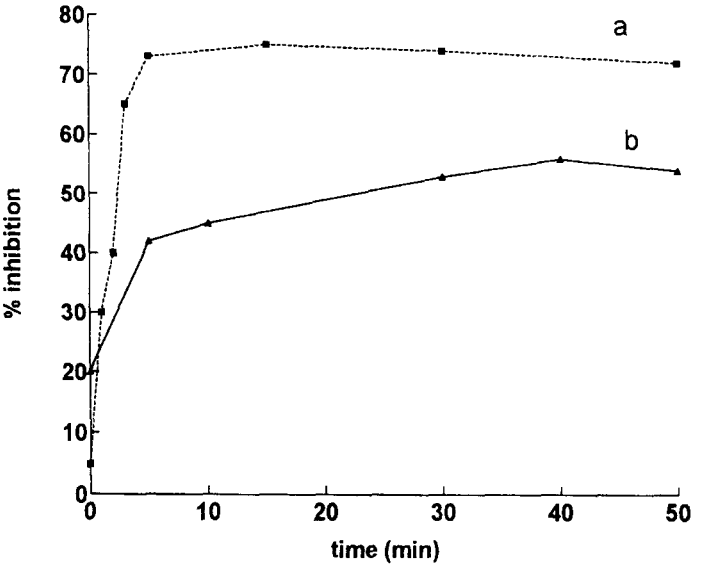

FIGURE 2 Effect of pre-incubation time on the inhibition of mitochondrial MAO B in rat liver by the urethanes (a) *N*-phenyl-(2-aminoethyl)carbamate (5 μM) and (b) *N*-phenyl-(2-methylaminoethyl)carbamate (500 μM). Inhibitor was added to the enzyme at zero time and the MAO B activity was measured at various times thereafter by addition of (E)-cinnamylamine (0.05 mM).

spectrophotometer. Substrate was either (E)-cinnamylamine HCl or benzylamine HCl. The former is predominantly a substrate for MAO B[8] but is metabolised slowly by MAO A, so in all experiments with either substrate, samples were preincubated for 20 min with the irreversible MAO A inhibitor clorgyline (10 nM), so that measurements of enzymic activity were confined to MAO B. Initial studies to test for time-dependence of the inhibitory process for the primary and secondary amines were carried out with mitochondria from rat liver, isolated as previously described,[9] as a source of MAO, using (E)-cinnamylamine HCl (0.5 mM) as substrate. Enzyme (0.5 mg of mitochondrial protein) and inhibitor in buffer (0.95 ml) were pre-incubated for different times with a fixed inhibitor concentration (Figure 2) before adding substrate solution (0.05 ml) to start the enzymic reaction, which was followed by observing the increase in absorbance at 290 nm (ε, 24500) due to formation of (E)-cinnamaldehyde. As can be seen from Figure 2, the secondary amine was a very much less potent inhibitor than its primary amine analogue and its properties were not examined further.

The time-dependent inhibition of MAO B by the primary amine was tested for reversibility as follows. Triplicate 3 ml samples of the enzyme preparation (0.2 mg of protein/ml) were incubated for 30 min at 30°C with and without the inhibitor (10^{-5} M). Samples of 0.95 ml were removed and assayed for MAO activity (initial velocity) using (E)-cinnamylamine (0.1 mM) as substrate in a total volume of 1 ml.[8] Samples were then centrifuged at 15000 $\times$ g and 20°C for 15 min. The pelleted enzyme preparations were washed three times by

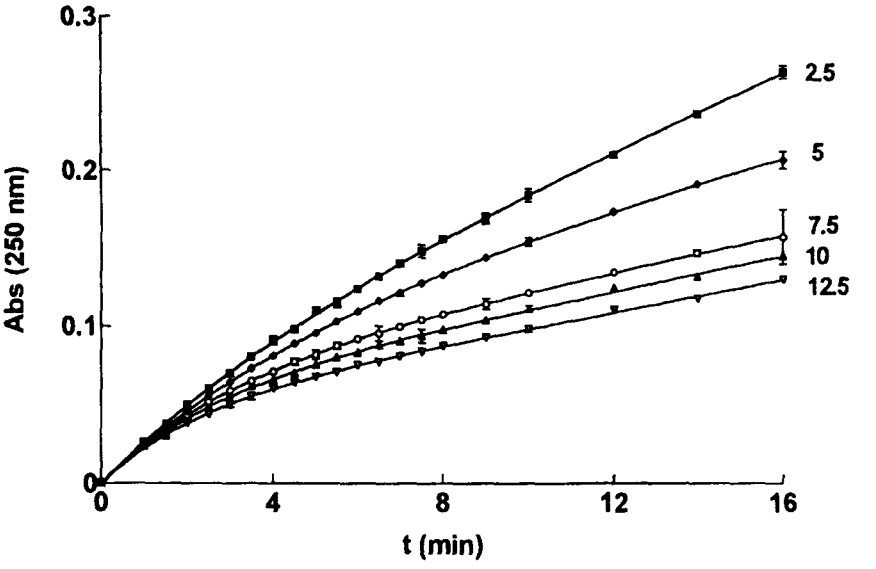

FIGURE 3 Progress curves for the oxidation of benzylamine (0.5 mM) by MAO B in rat liver mitochondria in presence of indicated concentrations (μM) of *N*-phenyl-(2-aminoethyl)carbamate (**1**, Figure 1). Substrate and inhibitor were added simultaneously to the enzyme preparation at zero time and the reaction was followed by observing the change in absorbance at 250 nm. Curves were generated by fitting data to equation (1) using mean values of data points taken from the experimentally determined curves. These are the points appearing in this figure.

resuspension and centrifugation in buffer (3 ml) at 20°C. Final pellets were resuspended in buffer and enzyme activities were re-measured.

The inhibitory properties of the primary amine were examined in more detail, as follows. Different concentrations of inhibitor in the range 2.5 to 12.5 μM were added simultaneously with substrate (benzylamine HCl, 0.5 mM) to 0.5 mg of protein and the progress of the reaction was followed by the change in absorbance at 250 nm, due to enzymic formation of benzaldehyde. These assays were carried out in duplicate. Progress curves were initially non-linear, eventually reaching a final steady-state rate (Figure 3). The K_m value for benzylamine (158 μM) under the conditions used were also measured, the latter by Lineweaver-Burk analysis.

Analysis of Data

The progress curves in Figure 3 are typical of those displayed by slow-binding inhibitors, with an initial nonlinear component and a final steady-state component. Results were analysed essentially as described by Williams and Morrison[10] by fitting data by a non-linear curve-fitting procedure to the rate equation

$$[P]_t = V_s t + (V_o - V_s)(1 - e^{-kt})/k + [P]_o \qquad (1)$$

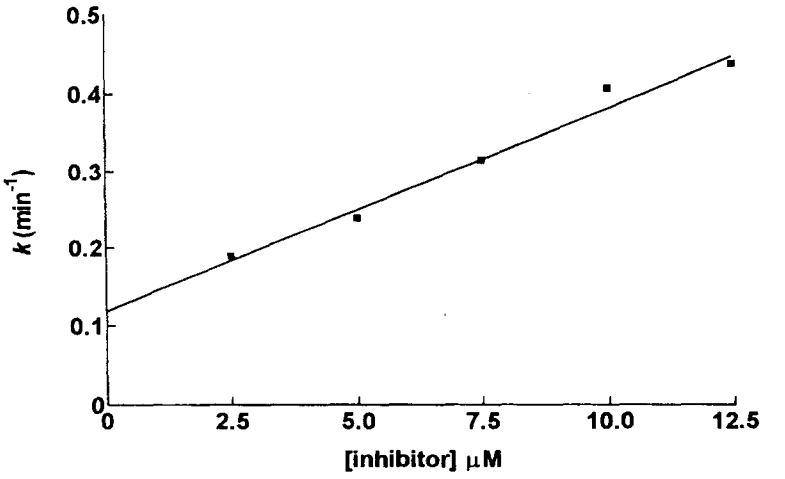

FIGURE 4 Plot of inhibitor concentration against the apparent first order rate constant for the inhibition of MAO B by *N*-phenyl-(2-aminoethyl)carbamate. A value for K_i was obtained from this curve, using equation (2), as described in the text.

where V_o and V_s are initial and final velocities, $[P]_t$ and $[P]_o$ are concentrations of product at times t and zero respectively and k is the apparent first order rate constant for the inhibitory process. Mean values for $[P]$ at times t were read directly from duplicate progress curves, as were initial estimates of the parameters V_o, V_s and k (the reciprocal of the value of t at the point of intersection of the estimated slopes of V_o and V_s), for use in the non-linear regression analysis. In conditions where the concentrations of inhibitor used are such that the initial velocities of the enzymic reaction remain essentially invariant, the values for the rate constants for association (k_3) and dissociation (k_4) of the slow-binding E-I complex are related to k as follows.[11]

$$k = k_4 + k_3[I]/(1 + [S]/K_m) \tag{2}$$

Hence plots of k against $[I]$, seen in Figure 4, give k_4 (intercept) directly and a value for k_3 is obtained from the slope, from which K_i (i.e. k_4/k_3) is calculated.

RESULTS AND DISCUSSION

(E)-cinnamylamine is an excellent substrate for measurements of initial velocities of MAO activity,[8] such as those depicted in Figure 2, and it is used routinely in this laboratory for that purpose. However, it cannot be used for continuous monitoring of time-dependent inhibitory processes because it slowly inactivates MAO B.[12] Hence benzylamine was used for such experiments. Both the primary and secondary amine inhibited MAO B in a time-dependent manner (Figure 2), but it can be seen that the latter is much less efficient, since even at a concentration 100 times greater than the primary amine it is still much the poorer inhibitor.

Its properties were not examined further. Although the action of the primary amine was time-dependent, it was found that the inhibition was relieved by the washing procedure, as indicated by the following enzymic reaction rates. Control sample (no inhibitor): before washing: 0.72±0.11 nmol/min; after washing, 0.81±0.3 nmol/min. Inhibited enzyme: before washing, 0.28±0.07 nmol/min; after washing, 0.8±0.22 nmol/min. This reversibility is consistent with the shape of the curves shown in Figure 3 which are characteristic of slow-binding inhibitors. Kinetic analysis of these data showed that the curves conform to equation (1). The validity of this rate equation is dependent on the substrate concentration remaining essentially constant during the experimental periods.[11,13] In the present case, less than 2% of substrate was consumed in any of the experiments shown in Figure 3, and this was ignored in the calculations. The analysis gave values for k_3 and k_4 of 110956 $min^{-1}M^{-1}$ and 0.1178 min^{-1} respectively from which a K_i value of 1.06 μM was calculated for the primary amine (**1**, Figure 1).

The compound (**1**) in Figure 1 is another example of an apparently slow-binding inhibitor of MAO, as judged by the kinetic data, to add to the aminoethyl esters and aminoethyl amides previously shown to conform to this kinetic mechanism.[6] Using a radiolabelled form of (**2**) Cesura *et al.*[14] found that this amide acts as a very poor substrate for MAO. They showed that end products (e.g. aldehyde) were not responsible for the inhibitory effects and postulated that an intermediate imine may become stabilised by H-bonding of an imino H to the amide carbonyl group, thus significantly retarding its conversion to products. However it was later shown by the same workers[15] that treatment of the inhibited enzyme with sodium cyanoborohydride resulted in formation of a stable covalent adduct between enzyme and inhibitor. Since the substrate itself would be unlikely to form a covalent bond with the enzyme in such circumstances, this observation suggests an interaction between an intermediate product (possibly an imine?) and some functional group on the enzyme. Thus the inhibition of MAO could be explained if such an interaction produced a reversible, slowly dissociating, covalent complex, obviating the need to invoke an intramolecularly H-bonded imine. Such inhibition is best seen as mechanism-based. The ester and the urethane (Figure 1) presently under study could obviously behave similarly.

The much reduced potency of the secondary amine (see Figure 2) is not readily explicable on the basis of this hypothesis, since MAO normally tolerates *N*-methyl secondary amines well; they often have K_m and V_{max} values similar to those of their primary amine counterparts. It is possible that the urethane group, -NHCOO- (and the amide and ester group of the analogous structures in Figure 1) places some steric constraint on the ability of MAO B to efficiently metabolise the primary amine and that this is exacerbated by the additional steric influence of the *N*-methyl group in the secondary amine.

It is usually assumed that the kinetic behaviour of slow-binding enzyme inhibitor is due to a two stage process; rapid formation of a reversible E-I complex, followed by slow formation of a second, more stable complex. The latter may or may not be covalent. In the present case, if the urethane is being very slowly turned over, as appears to happen with the related aminoethyl benzamides,[14] it seems likely that the observed kinetics of inhibition are due either to slow release from the enzyme, or slow catalytic turnover, of an intermediate species. Examples are known of enzyme inhibitors which show the kinetic characteristics of slow-binding inhibition for these reasons (see, for example, cases cited by Morrison and Walsh[16]).

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