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STEREOSELECTIVITY AND ISOENZYME SELECTIVITY OF MONOAMINE OXIDASE INHIBITORS

ENANTIOMERS OF AMPHETAMINE, *N*-METHYLAMPHETAMINE AND DEPRENYL

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Abstract—The enantiomers of amphetamine, *N*-methylamphetamine and deprenyl were studied, using a solubilised rat liver mitochondrial monoamine oxidase (MAO) preparation, as competitive inhibitors of MAO-A and MAO-B (5-hydroxytryptamine and β-phenylethylamine as substrate respectively). Only in the case of deprenyl enantiomers inhibiting MAO-B was a preference shown towards the [*R*]-configuration enantiomer justifying the use of [*R*]-(−)-deprenyl (as compared to the racemate) for the specific inhibition of MAO-B. Recalculation of the observed K_i values in terms of the base form of the inhibitor indicated that the activity of all enantiomers fell within a narrow, approximately 25-fold range when inhibiting MAO-B. The selectivity of inhibition of MAO-B by [*R*]-(−)-deprenyl cannot therefore be attributed to any specific structural features of the MAO-B isoenzyme form but rather to a lack of affinity of this enantiomer towards MAO-A.

The presence of isoenzyme forms of the enzyme monoamine oxidase (MAO) [monoamine: O_2 oxidoreductase (deaminating) (flavin containing) EC 1.4.3.4] within a tissue or enzyme preparation is demonstrated by the use of isoenzyme selective or specific substrates and inhibitors (see Refs. 1 and 2 for review). Thus, MAO-A in the presence of 5-hydroxytryptamine (5-HT) (MAO-A specific substrate) is selectively inhibited by clorgyline, and MAO-B in the presence of β-phenylethylamine (PEA) or benzylamine (MAO-B selective substrates) is selectively inhibited by L[*R*]-(−)-deprenyl. Although the above methods are established as a means of identifying the presence of the different isoenzyme forms within a tissue or enzyme preparation, the stereoselectivity (or stereospecificity) of the isoenzyme forms towards enantiomeric inhibitors of defined absolute configuration is less clear (for recent comment on this problem see Ref. 3). In this context, detailed information on the activity or isoenzyme selectivity of D[*S*]-(+)-deprenyl is not available.

The compounds (±)-amphetamine, (±)-*N*-methylamphetamine and (±)-deprenyl [(±)-*N*-methyl-*N*-propargyl-amphetamine] may all be considered analogues of the MAO-B selective substrate PEA. Whereas [*R*]-(−)-deprenyl selectivity inhibits MAO-B, the enantiomers of (±)-amphetamine are more potent inhibitors of MAO-A than MAO-B with [*S*]-(+)-amphetamine being the more active enantiomer [4]. Of the enantiomers of (±)-*N*-methylamphetamine only the [*S*]-(+) isomer has been studied and this compound also is a more potent inhibitor of MAO-A than MAO-B [5].

The mechanism of inhibition and, as a consequence, the kinetics of the inhibition differ between deprenyl and the simple amphetamines. Amphetamine and *N*-methylamphetamine are competitive reversible inhibitors of MAO and thus modify the apparent rate of abstraction of a proton from the α-carbon atom of the substrate (PEA or 5-HT) in the normal, enzyme-catalysed, reaction. Inhibition by deprenyl (and other propargylamine derivatives), on the other hand, although initially competitive, shows time-dependent, non-competitive irreversible kinetics mediated by proton abstraction from the α-carbon atom of the propargylamine side chain followed by covalent bond formation with the reduced flavin moiety of the enzyme [3]. It follows, therefore, that comparison of the stereospecificity/stereoselectivity or isoenzyme selectivity of enantiomeric inhibitors of MAO can only reasonably be compared if the kinetics of the inhibition are identical for all compounds studied. Accordingly, the present paper reports the inhibition, under conditions of competitive kinetics, of enantiomers of amphetamine, *N*-methylamphetamine and deprenyl.

MATERIALS AND METHODS

Clorgyline and L[*R*]-(−)-deprenyl were the gifts of May & Baker (Canada) Ltd. and Professor J. Knoll, Semmelweis University of Medicine (Budapest, Hungary) respectively. Synthesis and resolution of amphetamine and conversion to enantiomers of *N*-methylamphetamine and deprenyl were performed under the authority of Federal license C/83/Z/123 issued to the author. All syntheses were performed using standard laboratory procedures,

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and the products all displayed i.r. and NMR spectra consistent with the proposed structures. [*S*]-(+)-Amphetamine hydrochloride [m.p. 153–4°; $[\alpha]_D^{20}$ = +25.5° (c, 4.522, H_2O)] (for assignment of absolute configuration see Ref. 6) was obtained following resolution of (±)-amphetamine using (+)-tartaric acid [7] and was employed to synthesise [*S*]-(+)-*N*-methylamphetamine hydrochloride [m.p. 171.5–172.5°; $[\alpha]_D^{20}$ = +15.95° (c, 4.116, H_2O)] and [*S*]-(+)-*N*-methyl-*N*-propargyl-amphetamine hydrochloride ([*S*]-(+)-deprenyl) [m.p. 140–1°; $[\alpha]_D^{20}$ = +9.53° (c, 2.433, H_2O)] (mixed m.p. with authentic L[*R*]-(−)-deprenyl = 123–7°). [*R*]-(−)-Amphetamine hydrochloride [m.p. 151–2°; $[\alpha]_D^{20}$ = −26.5° (c, 5.007, H_2O)] was obtained by resolution of (±)-amphetamine using (−)-tartaric acid and used to prepare [*R*]-(−)-*N*-methylamphetamine hydrochloride [m.p. 171.5–172.5°; $[\alpha]_D^{20}$ = −16.33° (c, 4.782, H_2O)] and [*R*]-(−)-*N*-methyl-*N*-propargyl-amphetamine hydrochloride (L[*R*]-(−)-deprenyl) [m.p. 140.5–142°; $[\alpha]_D^{20}$ = −11.56° (c, 2.240, H_2O)] (mixed m.p. with authentic L[*R*]-(−)-deprenyl = 140–141.5°). ^{14}C-Labelled β-phenylethylamine hydrochloride (50 mCi/mmole) and ^{14}C-labelled 5-hydroxytryptamine binoxalate (50 mCi/mmole) were purchased from New England Nuclear, Boston, MA.

Livers (43 g) were obtained from two male Sprague–Dawley rats. All subsequent operations were performed at 0–4°. The livers were homogenised (4 min) in a Waring Blender in a solution of sucrose (0.25 M) in phosphate buffer (10 mM; pH 7.4) (10 ml/g tissue) and the suspension was centrifuged at 840 *g* for 10 min. The supernatant fraction was centrifuged (8200 *g*; 10 min), and the mitochondrial pellet was suspended in sucrose solution (0.25 M) and centrifuged at 8200 *g* for 10 min. The resulting pellet was gently stirred at 0° for 90 min in a solution of Triton X-100 (3%, w/v) in phosphate buffer (10 mM; pH 7.4) (40 ml) and then centrifuged at 20,000 *g* for 30 min. To the supernatant fraction was added ammonium sulfate to 40% saturation, and the mixture was stirred at 0° for 1 hr and then centrifuged at 14,500 *g* for 30 min. The sediment was suspended in phosphate buffer (10 mM; pH 7.4) (10 ml) and dialysed against two changes of phosphate buffer (10 mM; pH 7.4) containing sucrose (0.25 M) total volume 51; time 4 hr). The dialysed enzyme was centrifuged at 100,000 *g* for 1 hr, and the supernatant fraction was stored at −20° until used.

Protein content of the solubilised enzyme preparation was determined by the Biuret method [8] using bovine serum albumin as standard.

Enzyme activity was determined by radiochemical methods [9, 10]. Immediately prior to use, the solubilised enzyme was diluted with an appropriate volume of phosphate buffer (10 mM; pH 7.4) (1 ml diluted to 2 ml for MAO-A studies; 1 ml diluted to 50 ml for MAO-B studies). In studies employing [*R*]-(−)- or [*S*]-(+)-deprenyl, the inhibitor (50 μl) was preincubated at 37° for 3 min in the presence of substrate in phosphate buffer (10 mM; pH 7.4), and the enzyme-catalysed reaction was initiated by addition of enzyme solution (50 μl). (Final volume of incubation mixture was 300 μl.) The reaction was allowed to proceed for 3 min and was stopped by addition of 2 N HCl (200 μl). In studies employing the enantiomers of amphetamine or *N*-methylamphetamine, enzyme (50 μl) and inhibitor (50 μl) in phosphate buffer (10 mM; pH 7.4) were preincubated at 37° for 3 min, and the enzyme-catalysed reaction was initiated by addition of substrate (50 μl or 100 μl) (final volume of incubation mixture, 300 μl), allowed to proceed for 10 min and stopped by addition of 2 N HCl (200 μl). A 10-fold range of substrate concentration was employed, and enzyme activity was determined in the presence and absence of three fixed concentrations of inhibitor, the concentrations of which were determined from preliminary studies. All determinations were carried out in duplicate.

For studies of MAO-B activity (PEA as substrate), deaminated products were extracted by addition of toluene (6 ml); the mixture was extracted by shaking in a Vortex test tube mixer and then centrifuged. An aliquot (4 ml) of the toluene layer was added to scintillation fluid (5 ml) [Liquifluor, New England Nuclear, containing 0.4% (w/v) PPO* and 0.005% (w/v) POPOP in toluene] and counted in a Beckman LS 7500 liquid scintillation counter. For studies of MAO-A activity (5-HT as substrate), deaminated products were extracted into benzene/ethyl acetate (1:1) (6 ml) and, after vortex mixing and centrifuging, the mixture was refrigerated at −10° for 4 hr. An aliquot (4 ml) of the organic layer was added to scintillation fluid (5 ml) (Liquifluor, New England Nuclear) and counted as described above.

The data obtained were plotted as double-reciprocal plots, the best straight line fitting the experimental data points was obtained by a least squares regression analysis, and the data were used to calculate K_m and K_i values.

To test for the presence of MAO-A and MAO-B within the enzyme preparation, enzyme (50 μl) was incubated with a range of concentrations of the inhibitors clorgyline and deprenyl dissolved in phosphate buffer (10 mM; pH 7.4) for 30 min at 37° prior to addition of a fixed concentration of substrate (50 μl) (5-HT final concentration approx. 2.5×10^{-4} M; PEA final concentration approx. 1.0×10^{-5} M) (total volume of incubation mixture, 300 μl). The enzyme-catalysed reaction was allowed to proceed for 10 min, stopped by the addition of 2 N HCl (200 μl), and the deaminated products extracted and counted as described above.

RESULTS AND DISCUSSION

Preparation of a solubilised rat liver MAO preparation by the method reported above yielded an enzyme preparation displaying both MAO-A and MAO-B activity as shown by the pI_{50} values (irreversible inhibition) of clorgyline and L[*R*]-(−)-deprenyl when using 5-HT or PEA as substrate [pI_{50} values of clorgyline were 7.60 (5-HT) and 5.50 (PEA); pI_{50} of L[*R*]-(−)-deprenyl were 4.35 (5-HT) and 7.95 (PEA)].

* PPO = 2,5-diphenyloxazole, and POPOP = 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene.

Table 1. K_i values (competitive inhibition) of enantiomeric amphetamine analogues towards MAO-A and MAO-B in a solubilised rat liver mitochondrial MAO preparation*

	MAO-A K_m (5-HT) = 225 (±30) μM	R/S	MAO-B K_m (PEA) = 5.5 (±1.25) μM	R/S	$\frac{\text{MAO-A}}{\text{MAO-B}}$
	K_i (μM)		K_i (μM)		
Amphetamine					
[*R*]-(−)-	203 ± 7	6.0	180 ± 3	1.12	1.13
[*S*]-(+)-	33.8 ± 4	$S > R$	161 ± 32	$S \simeq R$	0.21
N-Methylamphetamine					
[*R*]-(−)-	99 ± 1	0.9	343 ± 81	1.26	0.29
[*S*]-(+)-	110 ± 4	$S \simeq R$	272 ± 83	$S > R$	0.404
Deprenyl					
[*R*]-(−)	76 ± 5	5.42	0.325 ± 0.03	0.040	234
[*S*]-(+)	14 ± 0.7	$S > R$	8.1 ± 0.6	$R > S$	1.73

* Results are the mean value (±S.D.) of duplicate determinations at three inhibitor concentrations. Inhibition studies were performed at 37° and pH 7.4.

Because of the possibility of time-dependent, irreversible inhibition by the enantiomers of deprenyl, the enzyme-catalysed reaction was initiated by addition of enzyme, and the reaction was allowed to proceed for only 3 min. Using such conditions, the lines of best fit of the experimental data showed a common intercept on the y-axis of double-reciprocal plots and signified competitive kinetics for the inhibition (see also Ref. 11). For all other inhibitors, enzyme and inhibitor were preincubated prior to addition of substrate, and the enzyme-catalysed reaction was allowed to proceed for 10 min. Again, competitive kinetics were shown on double-reciprocal plots.

The calculated values of K_i, the enzyme–inhibitor equilibrium constant under conditions of competitive inhibition, for each of the inhibitors in the presence of either 5-HT or PEA are shown in Table 1, and typical results obtained with one of the inhibitors are shown in Fig. 1.

A previous study [4] of the stereoselectivity and isoenzyme selectivity of enantiomers of amphetamine has shown that solubilised rat liver MAO-A (30°; pH 7.2) is more readily inhibited than MAO-B, that MAO-A shows greater stereoselectivity than MAO-B, and that the [*S*]-(+)-enantiomer is a more potent inhibitor than the [*R*]-(−)-enantiomer. Although the conditions of enzyme assay used in the present work differ slightly from those employed in the above-mentioned study, similar conclusions can be made from the present results.

Of the enantiomers of *N*-methylamphetamine only the [*S*](+)-enantiomer has been studied as an inhibitor of MAO and found to be a more potent inhibitor of MAO-A than MAO-B [11]. Although this finding has been reproduced in the present work, it would appear that MAO-A is not stereoselective towards enantiomers of *N*-methylamphetamine while MAO-B shows only marginal stereoselectivity towards the [*S*]-(+)-enantiomer.

In considering the results obtained employing the enantiomers of deprenyl as inhibitors of MAO, significant stereoselectivity is shown by the isoenzyme forms of MAO. The selectivities of these forms, however, are towards different enantiomers (MAO-B selective towards the [*R*]-configuration; MAO-A selective towards the [*S*]-configuration). Such a finding adds significant justification to the use of [*R*]-(−)-deprenyl rather than the racemate as a selective irreversible inhibitor of MAO-B, since it is considered that the isoenzyme selectivity is determined predominantly by the reversible phase of the inhibition rather than by the time-dependent, irreversible phase [3, 12].

Consideration of the data presented of the inhibition of a particular isoenzyme form, e.g. MAO-B, suggests that the structural differences between amphetamine, *N*-methylamphetamine and deprenyl have resulted in an approximately 1000-fold change

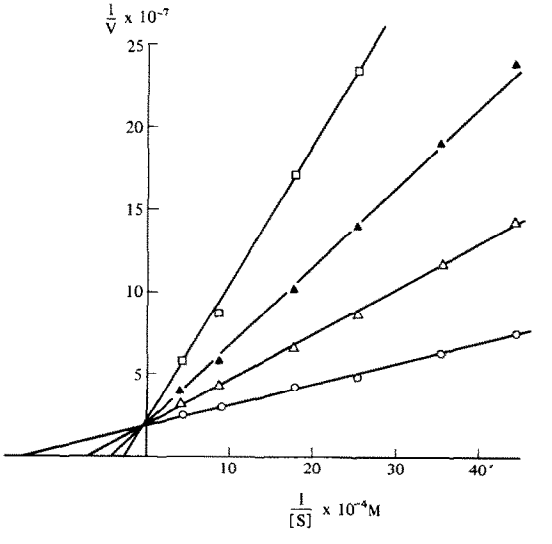

Fig. 1. Double-reciprocal plot of inhibition of oxidative deamination of PEA by [*R*]-(−)-deprenyl. Inhibitor concentrations are 0 μM (○), 0.446 μM (△), 0.891 μM (▲) and 1.782 μM (□). Experimental details are given in the text.

Table 2. $\bar{K}_i$ values (competitive inhibition) of enantiomeric amphetamine analogues corrected for degree of ionisation at pH 7.4 using equation $\bar{K}_i = K_i/1 + \text{antilog}(pK_a - pH)$ [14]

	Approx. pK_a	% Base at pH 7.4	$\bar{K}_i$ (μM) MAO-A		$\bar{K}_i$ (μM) MAO-B	
			[R]-(−)-	[S]-(+)-	[R]-(−)-	[S]-(+)-
Amphetamine	9.60	0.62	1.26	0.21	1.11	1.0
N-Methylamphetamine	9.80	0.39	0.385	0.43	1.33	1.05
Deprenyl	7.40	50.0	37.85	7.0	0.16	4.07

in inhibitory activity. However, the values of K_i reported are dependent upon both the pH of the incubation medium and the pK_a of the inhibitor and it is the unionised form which is considered to be the active inhibitory species [13–15]. Assuming a pK_a at 37° for amphetamine of 9.60 (pK_a at 25° = 9.92 [16]), the pK_a values of N-methylamphetamine and deprenyl would be 9.80 and 7.40, respectively, due to the known base-strengthening and base-weakening effects following successive alkylation of the amine with methyl and propargyl functions [17]. Accordingly, K_i values have been recalculated in terms of the concentration of unionised inhibitor at pH 7.4 (Table 2). Although such calculations have no influence upon the stereoselectivity displayed by enantiomeric forms of the inhibitors, the range of activity of the enantiomers when inhibiting MAO-B all fall within a much narrower 25-fold range. Thus, it would appear that structural variations, within the limited number of compounds tested in the present study, have little significant influence upon the inhibition of MAO-B. With one exception, i.e. [R]-(−)-deprenyl, a similar conclusion may be drawn from the inhibition of MAO-A, although the compounds are more potent inhibitors of this latter isoenzyme form. [R]-(−)-Deprenyl is considerably weaker as an inhibitor of MAO-A than the [S]-(+)-enantiomer or any of the enantiomers of amphetamine and N-methylamphetamine. Thus, the high inhibitory activity and isoenzyme selectivity in favour of MAO-B displayed by [R]-(−)-deprenyl cannot be attributed to structural features of MAO-B leading to a high affinity, since enantiomers of amphetamine and N-methylamphetamine are little different in activity when calculated in terms of the concentration of base form. Rather the N-propargyl function in [R]-(−)-deprenyl is presented to MAO-A in an unfavourable orientation, leading to a significant reduction of affinity.

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