Biochemical Pharmacology, Vol. 24, pp. 1889–1891. Pergamon Press, 1975. Printed in Great Britain.

MONOAMINE OXIDASE—III

FURTHER STUDIES OF INHIBITION BY PROPARGYLAMINES

CARVELL H. WILLIAMS and JILL LAWSON

Department of Mental Health, Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland

(*Received* 16 *January* 1975; *accepted* 10 *March* 1975)

Abstract—The pK_a values and partition coefficients have been determined for a number of propargylamines. It is shown that in a number of cases there is a close correlation between the partition coefficient and the effectiveness of inhibition of mitochondrial monoamine oxidase as measured by I_{50} values. Using ^{14}C-labelled pargyline and clorgyline it is shown that, *in vitro*, these substances fail to bind to proteins other than MAO, and that a number of other irreversible inhibitors prevent pargyline from binding to this enzyme.

The inactivation of monoamine oxidase, MAO [monoamine O_2 oxido-reductase (deaminating), EC 1.4.3.4] by *N*-substituted propargylamines proceeds with markedly varying efficiencies with different compounds of this class [1, 2] and there does not at first sight appear to be any simple relationship between structure and activity. We have observed, however, that a compound such as *N*-(2,4-dichlorobenzyl)-*N*-methyl propargylamine is a much better inhibitor than its non-halogenated analogue, pargyline, and that the latter is more effective than its desmethyl derivative, *N*-benzyl propargylamine [1]. Differences between pargyline and its dichloroanalogue must be attributable to the electronic, steric or solubility differences conferred by the chlorine atoms. Similar arguments apply pargyline and its desmethyl derivative.

Recent work on MAO has produced indications that multiple forms of the enzyme may exist [3]. The two principal criteria used to support this idea are the separation of MAO into several bands by electrophoresis and the selectivity of certain MAO inhibitors. It has been suggested that such multiple forms of MAO might be due to different amounts or types of lipid material adhering to the enzyme [4, 5]. We have therefore investigated the possibility that the effectiveness of propargylamines in inhibiting MAO may be a function of their lipophilic character as measured by their partition coefficients.

Interaction of pargyline with MAO is now thought to occur at the flavin moiety of the enzyme, partly by mimicking the action of substrates [6]. It is also reported that pargyline reacts only with MAO [7], which suggests that the catalytic action of the enzyme may be a prerequisite for inhibition by this compound. We have used ^{14}C-labelled pargyline and clorgyline to study the interaction of propargylamines with proteins other than MAO, and to investigate their possible binding to the enzyme after inhibition with other types of irreversible inhibitor.

MATERIALS AND METHODS

The propargylamines were prepared as previously described [1]. MAO for gel filtration studies was prepared from rat liver as described by Houslay and Tipton [5]. Gel filtration media were from Pharmacia Ltd. Tranylcypromine and modaline were gifts from Smith, Kline and French and Warner–Lambert Laboratories respectively. Iproniazid phosphate was from Ralph N. Emanuel Ltd. All other chemicals were of Analar quality, or the highest quality available.

pK_a values of propargylamines at 30° were measured by potentiometric titration as described by Albert and Sarjeant [8].

Partition coefficients were measured by distributing the propargylamines between water and hexane, the concentration in each phase being calculated from the differences in optical densities before and after partition. Optical density measurements were made at 260 nm.

Binding studies with ^{14}C-labelled inhibitors. Radioactive pargyline and clorgyline were prepared by introduction of a ^{14}C-labelled methyl group into the appropriate secondary propargylamine. It is hoped to publish full details of these syntheses elsewhere.

[^{14}C]pargyline (0·1 μCi, sp. act. 32 μCi/mole) was incubated at 30° for 45 min with glucose oxidase (1 mg) in a total vol of 1 ml of 0·05 M phosphate buffer, pH 7·4. The sample was then applied to a column of Sephadex G25 (30 × 1·5 cm) pre-equilibrated with the same buffer. Material was eluted from the column at a flow rate of 20 ml/hr and collected in 2-ml fractions. A 1-ml sample of each fraction was assayed for radioactivity using liquid scintillation counting. Each fraction was also assayed for protein by the method of Lowry *et al.* [9].

This procedure was repeated with D-amino acid oxidase, bovine serum albumen, succinate dehydrogenase and a sample of MAO from rat liver. The series of experiments was repeated using [^{14}C]clorgyline instead of pargyline.

The effect of MAO inhibitors on the binding of pargyline and clorgyline to MAO. 1-ml samples of MAO from rat liver, containing 5 mg of protein/ml of 0·05 M phosphate buffer, pH 7·4 were treated with 1 ml samples of the following MAO inhibitors in the same buffer to give final concentrations as indicated;

Table 1. pK_a values and partition coefficients (K) of propargylamines

Propargylamine	pK_a	K
(1) *N*-β-phenylethyl	7·47	9·2
(2) *N*-benzyl	7·12	12·3
(3) *N*-γ-phenyl-*n*-propyl	7·85	33·1
(4) *N*-δ-phenyl-*n*-butyl	8·12	58·3
(5) *N*-benzyl-*N*-methyl	6·61	146·3
(6) *N*-(2,4-dichlorobenzyl)	6·26	553
(7) *N*-(2,4-dichlorobenzyl)-*N*-methyl	5·80	5010
(8) *N*-propargyl-4-phenylpiperidine	6·99	88·9

iproniazid phosphate (10^{-4} M), tranylcypromine (10^{-4} M), unlabelled pargyline (10^{-4} M). In the case of modaline, which requires 'activation' by mixed function oxidases before it will inhibit MAO, a slightly different procedure was used. In a final volume of 2 ml of buffer was included modaline (10^{-4} M), a microsomal preparation from rat liver (10 mg of microsomal protein), NADPH (10^{-4} M) and the MAO preparation (1 ml). Control samples, in which buffer replaced the inhibitor solution, were also prepared. All samples, in duplicate, were incubated at 30° for 45 min in air, followed by dialysis at 4° against three changes, each of 2 litres, of 0·05 M phosphate buffer, pH 7·4. Aliquots (0·5 ml) of each sample were then assayed for MAO activity using [^{14}C]tyramine as previously described [1]. One ml of the remainder of each sample was then incubated at 30° for 45 min in the presence of [^{14}C]pargyline as described above, followed by gel filtration as already outlined, in order to separate protein-bound from unbound [^{14}C]pargyline. Fractions (2 ml) were collected and aliquots assayed for radioactivity and protein.

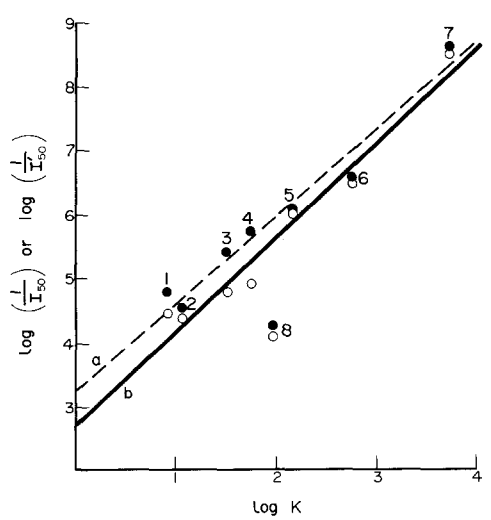

Fig. 1. Linear regression curves showing relationship between hydrophobicity (K) and inhibitory potency of propargylamines. I_{50} refers to total concentration of inhibitor required to produce 50% inhibition (○), and I'_{50} to the corresponding concentration of unionised inhibitor (●). Numbering of points corresponds to numbering of compounds in Table 1. Point no. 8 was not used in computing the curves and is included only for comparison.

RESULTS

Ionisation constants and partition coefficients (K) appear in Table 1. Hansch [10] has described a relationship between biological response, C, and hydrophobic character, K, of the form log 1/C = a. log K + b. Figure 1 is a graphical presentation of the data, using this relationship, where the I_{50} values of the inhibitors are used for the parameter C. Values of C were calculated from our previously published data on these inhibitors [1]. Linear regression curves were fitted to points calculated by a computerised unweighted least squares method. Correlation coefficients calculated for curves (a) and (b) were 0·984 and 0·981 respectively.

Figure 2 shows the result of attempts to bind [^{14}C]pargyline to MAO after treatment with MAO inhibitors. The residual MAO activity in these pre-inhibited samples was, for pargyline pre-treatment, 5%, iproniazid, 11%, tranylcypromine, 0%, modaline, 13%.

Gel filtration of MAO on Sephadex G-25, following its incubation with either [^{14}C]pargyline or [^{14}C]clorgyline, gave two peaks of radioactivity in the eluate, as shown for pargyline Fig. 2. The smaller

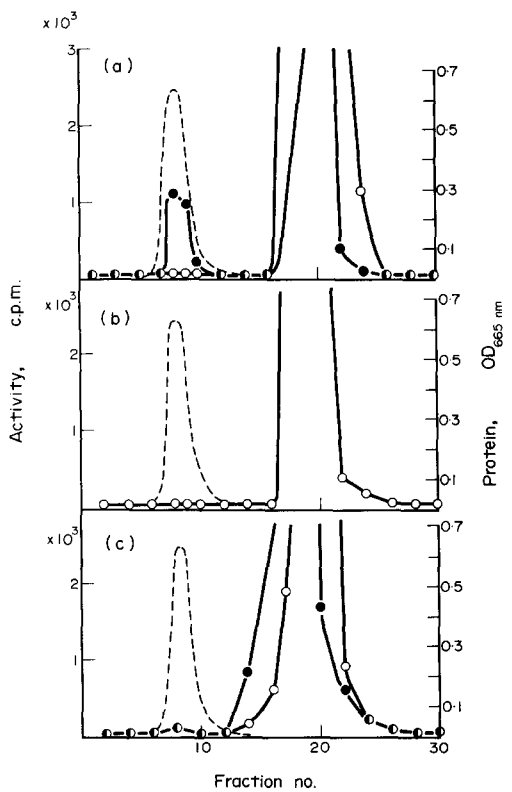

Fig. 2. Gel filtration of MAO on Sephadex G25, showing failure to bind [^{14}C]pargyline after inhibition with various inhibitors. Profiles show elution of protein (------) and radioactivity. (a) elution of [^{14}C]pargyline without pre-inhibition of MAO (●—●) and after pre-inhibition with unlabelled pargyline (○—○). (b) elution of [^{14}C]pargyline after pre-inhibition of MAO with tranylcypromine (○—○). (c) elution of [^{14}C]pargyline after pre-inhibition of MAO with modaline (○—○) or iproniazid (●—●). The small amount of radioactivity eluted with protein in (c) may reflect the incomplete inhibition of MAO by modaline and iproniazid.

peak corresponded with the elution of the protein, the larger peak being eluted later. In cases where other proteins were so treated, radioactivity was in all cases eluted as a single peak following the elution of protein, indicating lack of affinity for the MAO inhibitors.

DISCUSSION

The efficacy of propargylamines in inhibiting MAO is to some extent related to their lipophilic character, as indicated by Fig. 1. That other factors are involved is suggested by the failure of 4-phenyl-*N*-propargyl piperidine to fit the curves computed for the other inhibitors. This particular propargylamine is the only one not having an open chain and it is possible that steric requirements of the hetero-ring are responsible for its anomalous behaviour. There are at least two possible explanations for the results shown in Fig. 1. The rate of inactivation of MAO may be determined either by the ease of penetration of a particular inhibitor through a lipid barrier attached to the enzyme, or by its ability to bind to hydrophobic sites on the enzyme itself. Evidence is available which suggests that such sites exist at the substrate binding site of MAO [11–14]. It is currently suggested that the iso-enzymes of MAO may differ in their substrate and inhibitor specificities [3]. The nature of these iso-enzymes is still uncertain though it has been suggested that they may be artefacts produced by a single enzyme binding varying amounts of lipid. This view, first advanced by Veryovkina and Gorkin [4], has been strengthened by the finding that treatment of MAO to remove some lipid results in only a single band of enzyme activity on subsequent electrophoresis [5]. Prior to such treatment multiple bands of MAO were obtained. In addition, Olivecrona and Oreland [15] have shown that MAO freed from lipid material can complex with lipids of both mitochondrial and extra-mitochondrial origin.

The apparent relationship between lipophilicity and inhibitory potency of propargylamines now demonstrated suggests that penetration through lipid associated with MAO may be a factor in the selectivity of e.g., clorgyline towards the multiple forms of the enzyme. Such an explanation is reinforced by the finding by Houslay and Tipton [5] that the removal of lipid from MAO results in loss of the bimodal inhibition curve for clorgyline. The existence of such bimodal inhibitory characteristics has been advanced as evidence of multiplicity of MAO enzymes [16, 17]. Work now in progress in this laboratory suggests that there are marked differences between clorgyline and pargyline in their reversible binding to mitochondrial fractions from liver.

Erwin and Deitrich [7] have shown that pargyline binds exclusively to MAO *in vivo*. Our gel filtration studies with labelled pargyline and clorgyline confirm this high degree of specificity, which seems likely to be a feature of all such compounds. The fact that neither inhibitor bound to any of three other flavoenzymes, excluding MAO, suggests that the catalytic function of the MAO is involved in the inhibition by propargylamines. Such a conclusion can also be drawn from the attempts to bind pargyline and clorgyline to MAO previously inhibited by other types of irreversible inhibitors. As is evident in Fig. 2, after inhibition by tranylcypromine, iproniazid or modaline (or by pargyline itself) MAO fails to incorporate radioactivity from [^{14}C]pargyline, though under similar experimental conditions untreated enzyme incorporates ^{14}C as shown by the presence of radioactivity in the protein fraction after gel filtration. The mechanism of inactivation of MAO by the three inhibitors used here is unknown at present, though it can be concluded that if pargyline does indeed interact with the flavin portion of the enzyme then each of them prevents such interaction, either by similar action upon the flavin or by binding close enough to it to prevent access by pargyline.

Acknowledgements—We should like to thank Prof. D. T. Elmore for the use of facilities in his Department and to Mrs S. Turley for preparation of diagrams.

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