In-vivo Effects of (E)-2-(3',4'-dimethoxyphenyl)-3-fluoroallylamine (MDL 72145) on Amine Oxidase Activities in the Rat. Selective Inhibition of Semicarbazide-sensitive Amine Oxidase in Vascular and Brown Adipose Tissues

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Abstract—One hour after MDL 72145 ((E)-2-(3',4'-dimethoxyphenyl)-3-fluoroallylamine) (2·5 mg kg^{-1}) was given by intraperitoneal injection, the semicarbazide-sensitive amine oxidase (SSAO) activity of rat aorta and brown adipose tissue measured in-vitro was reduced by more than 95% of its control value, whereas the monoamine oxidase (MAO-A) activity remained virtually unaffected. The action of this drug on amine oxidases in the liver at this dose was less selective. The in-vitro effect of MDL 72145 on the soluble enzyme diamine oxidase from rat intestine was 100 fold less potent than that of semicarbazide but about equipotent with semicarbazide on sheep plasma amine oxidase. Overall MDL 72145 was selectively more active against membrane bound SSAO enzymes that deaminate primary monoamines. Although MDL 72145 does inhibit MAO-B activity these results suggest that this compound may be used to study the effect of selective inhibition of SSAO activity on the pharmacological responses of appropriate preparations in-vitro.

Of the enzymes which oxidatively deaminate biogenic amines the monoamine oxidases (MAO; EC 1.4.3.4) have been intensively studied, while the semicarbazide-sensitive amine oxidases (SSAO; EC 1.4.3.6), forming a heterogeneous group of enzymes (see Kapeller-Adler 1970; Callingham & Barrand 1987 for reviews) are quite often neglected. Unlike MAO, the SSAO enzymes found in the vascular and brown adipose tissue (BAT) of the rat (Coquil et al 1973; Barrand & Callingham 1982) reside in the plasma membrane of smooth muscle cells and adipocytes, respectively (Wibo et al 1980; Barrand & Callingham 1982). The biochemical properties of these enzymes have been studied but little is known about their possible physiological significance.

A potent and irreversible inhibitor of SSAO activity would provide a useful experimental tool with which to examine the physiology and pharmacology of these enzymes. However, many currently available potent inhibitors of SSAO also strongly inhibit MAO as well as having other pharmacological properties (see Lyles 1984).

Allylamine, an unsaturated aliphatic amine is metabolized to acrolein by vascular SSAO (Nelson & Boor 1982) and this may be the mechanism of its toxicity. A derivative of this amine, MDL 72145 ((E)-2-(3',4'-dimethoxyphenyl)-3-fluoroallylamine), is a selective and irreversible inhibitor of MAO-B (McDonald et al 1984), and has also been shown to be a potent irreversible inhibitor of rat vascular SSAO in-vitro (Lyles & Fitzpatrick 1985). In-vitro, the inhibition of MAO-B by MDL 72145 has been shown to occur both at

lower concentrations of the drug and at a faster rate than inhibition of MAO-A (Zreika et al 1984). In-vivo administration of this drug has also been shown to inhibit SSAO at lower doses than those required to inhibit MAO-A (Flucker et al 1986).

Since rat blood vessels and BAT have very little MAO-B activity (Barrand & Callingham 1982; Callingham et al 1983), it would seem that MDL 72145 might prove a useful drug for the selective inhibition of SSAO activity in these tissues in pharmacological studies of such enzymes. In addition, the fact that the interaction between MDL 72145 and SSAO appears to be irreversible (Lyles & Fitzpatrick 1985) should allow determinations of the half life of the enzyme and thus its turnover.

We have examined the effects of in-vivo administration of this drug on the SSAO and MAO-A activities in the rat blood vessels and BAT to see if a suitable selective dose regime could be found and half lives determined. Studies have also been undertaken on the in-vitro effects of MDL 72145 on two other members of the SSAO group of enzymes, diamine oxidase (DAO; EC 1.4.3.6) and sheep plasma amine oxidase (SPAO; EC 1.4.3.6) to determine the selectivity of this drug within this heterogeneous group of enzymes. Enzymes described as EC 1.4.3.6 are believed to contain copper and are sensitive to inhibition by carbonyl reagents. This classification is therefore very broad and will include a somewhat heterogeneous group of tissue enzymes both bound on or within the tissues or free in the circulating blood plasma. Although there are indications that more than one membrane bound SSAO activity might occur in the rat (Clarke et al 1982) there is no clear evidence that would justify any subclassification to be made at present. In this paper SSAO is used in the singular.

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Materials and Methods

Materials

Substrates for the amine oxidase assays were 5-hydroxy-[G-³H]tryptamine creatinine sulphate and [1,4-¹⁴C]putrescine dihydrochloride (Amersham International plc, Amersham, UK), [7-¹⁴C]benzylamine hydrochloride (Dupont UK Ltd, Stevenage, Herts), and unlabelled substrates, 5-hydroxytryptamine creatinine sulphate (5-HT), putrescine dihydrochloride and spermidine trihydrochloride (Sigma Chemical Company Ltd, Poole, Dorset, UK). Unlabelled benzylamine hydrochloride was prepared by crystallization of the hydrochloride from solutions of free base (Sigma) following the addition of hydrochloric acid. Semicarbazide hydrochloride, phenobarbitone sodium, 2,4-dichlorophenol, 4-aminoantipyrine and horse radish peroxidase (Sigma type II) were all obtained from Sigma. MDL 72145 was a gift from Merrell Dow Research Institute, Strasbourg, France. Other reagents were of analytical grade where possible. Male Wistar rats (200–400 g) were purchased from A. J. Tuck & Son, Rayleigh, Essex.

Methods

MDL 72145, dissolved in distilled water (1 mg mL⁻¹) was administered to groups of 5 rats by intraperitoneal injection (i.p., 2·5 and 5 mg kg⁻¹). Age and weight-matched controls were injected with the same volume of distilled water. The animals were killed 1 and 24 h later and the BAT, aorta and liver of each rat removed for assay. The time course for recovery of the enzymes was obtained following i.p. injection of MDL 72145 (5 mg kg⁻¹), by killing groups of 5 animals 2 h, 1, 2, 4, 7, 11 days later. The BAT, aorta and ileum were removed and stored at −20°C until used for assay, with the exception of rat ileal samples that were always assayed immediately due to the risk of degradation by proteolytic enzymes (see Barwell & Canham 1988). The tissue homogenates were prepared in 1 mM potassium phosphate buffer, pH 7.8. Each was centrifuged at 600 g for 10 min to remove unbroken cells, nuclei and debris.

SSAO and MAO activities were assayed radiochemically by a method modified from that of Callingham & Laverty (1973) by extraction of radioactive deamination products with toluene. The substrates were 5-HT (500 μM for MAO-A) benzylamine (1 mM) for MAO-B and benzylamine (10 μM) for SSAO in the aorta and BAT. In the intestine, benzyla-

mine (5 μM) was used as the substrate for SSAO. At this concentration the effect of deamination of benzylamine by MAO-B is small (Callingham et al 1985). The protein content of each tissue homogenate was assayed by the method of Lowry et al (1951). The enzyme activities were expressed as nmoles of product produced h⁻¹ mg⁻¹ of protein in the homogenate. The treated groups were compared with their controls using Student's *t*-test.

To determine if active drug was still present during the assay, homogenates from control animals were preincubated for 30 min at 37°C with equal volumes of homogenates from treated animals before assay. No inhibitory effects were evident (see Table 1).

The in-vitro effects of MDL 72145 on DAO from the rat ileum, a tissue with substantial activity of this enzyme (Shakir et al 1977), were studied using a method based on that of Thomasset et al (1982). Putrescine (50 μM) in 0·2 M potassium phosphate buffer (pH 7·8) containing phenobarbitone sodium (10 μM) was used as the substrate. In these experiments, the MDL 72145 (0·1 μM to 1 mM) was preincubated with homogenates of ileum before the addition of putrescine to allow time for any irreversible complex to be formed. The effect of semicarbazide (1 μM to 1 mM) on the enzyme activity was also examined by preincubation with homogenate before the addition of substrate.

Samples of sheep blood plasma obtained from four male mixed breed sheep were assayed for the oxidative deamination of spermidine by a peroxidase linked colorimetric assay based on the method of Yamada et al (1979). 700 μM spermidine in 0·2 M potassium phosphate buffer (pH 7·8) containing 2,4-dichlorophenol, 4-aminoantipyrine and horse radish peroxidase (all at 0·2 mg mL⁻¹) was used as the substrate. The blood plasma was preincubated with MDL 72145 (0·1 μM to 30 μM) for 30 min at 37°C and the colorimetric assay was then used to estimate the amount of enzyme activity remaining. The effect of semicarbazide was again examined for comparison.

Results

At 5 mg kg⁻¹, MDL 72145 inhibited SSAO, MAO-A and MAO-B in a relatively non-selective manner so that 1 and 24 h after this dose the enzyme activities were all significantly reduced to less than 50% of their control values (see Fig. 1). At 2·5 mg kg⁻¹ a more selective inhibition of SSAO in the

Table 1. The effect of preincubating tissue homogenates from control rats with those of treated animals for 30 min at 37°C on their amine oxidase activities. The result obtained is compared with that predicted by combining the activities measured in the two homogenates alone. Each value given is the mean of triplicate estimations carried out on tissue homogenates from a pair of animals.

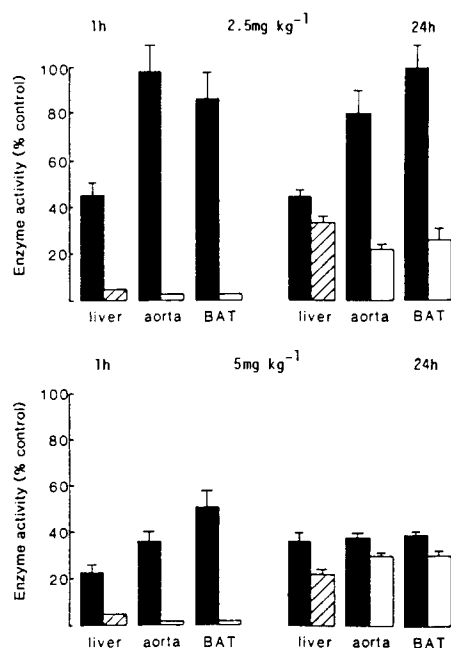| Tissue and Enzyme	Time after MDL 72145 (h)	Specific activity of control tissue (nmol h⁻¹ (mg prot.)⁻¹)	Specific activity of treated tissue (nmol h⁻¹ (mg prot.)⁻¹)	Combined activity (nmol h⁻¹ (mg prot.)⁻¹) Measured	Combined activity (nmol h⁻¹ (mg prot.)⁻¹) Predicted
Ileum; MAO-A	2	65·6	15·6	40·5	40·6
	24	44·0	16·7	34·6	30·4
Ileum; MAO-B	2	23·5	2·1	12·6	12·8
	24	31·1	8·0	21·1	19·6
Ileum; SSAO	2	1·82	0	1·3	0·9
	24	2·05	0·3	2·4	1·7
Aorta; SSAO	2	79·8	4·7	41·3	42·3
	24	87·8	30·8	60·3	59·3

FIG. 1. Amine oxidase activities in homogenates of liver, aorta and brown adipose tissue (BAT) of rats, 1 and 24 h after i.p. injection of 2·5 or 5 mg kg⁻¹ MDL 72145 (MAO-A solid columns; MAO-B hatched columns; SSAO clear columns). Results are expressed as the percentage (±s.e.r.) of enzyme activity remaining in the treated group compared with its control (n = 5 in each group). Only MAO-A in aorta and BAT failed to show any significant reduction after 2·5 mg kg⁻¹ MDL 72145. (Significance was determined on the absolute values of enzyme activities.)

aorta and BAT was seen, when 1 h after this dose SSAO activity was reduced to below 5% of its control value whereas MAO-A was not significantly different from the control. After 24 h the SSAO activity had recovered to between 20 and 30% of its control level whilst MAO-A values remained not significantly different from control. This dose of MDL 72145 in the liver, however, significantly inhibited both MAO-A and MAO-B by more than 50% at both 1 and 24 h.

The non-selective dose of 5 mg kg⁻¹ was chosen to look at the time course for recovery of the tissue amine oxidase enzymes. The results obtained for the 3 enzymes in the intestine are shown in Fig. 2. The pattern was the same in the aorta and BAT. Maximum inhibition of SSAO and MAO-B (only present in the intestine) was seen at 2 h. However, MAO-A activity in all tissues continued to fall between 2 and 24 h after which recovery took place.

The data from the time course experiments were used to calculate rate constants of degradation of the amine oxidase enzymes in-vivo and therefore their half lives as described in Table 2. This assumes that synthesis of new enzyme is required to regenerate the enzyme activity and that this process is zero order so that the time to achieve recovery of enzyme activity is dependent solely upon the rate constant of 1st order degradation (Schimke & Doyle 1970; Della Corte & Callingham 1977).

The in-vitro effects of MDL 72145 and of semicarbazide on the DAO activity of rat intestinal homogenates are shown in Fig. 3a. Semicarbazide was a much more effective inhibitor of DAO than MDL 72145 with a dose of 1 μM giving greater than 50% inhibition whereas the IC50 value for MDL 72145 upon DAO was 100 μM. Similarly, the in-vitro effect of these
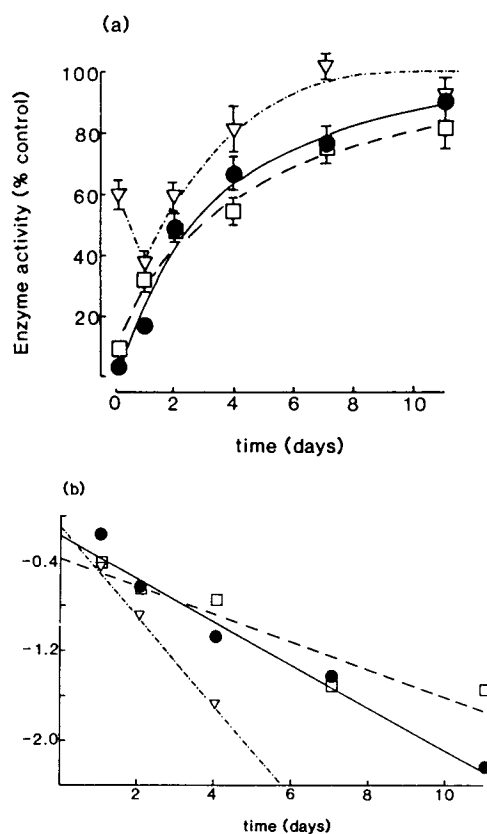

FIG. 2. (a) Time course of recovery of MAO-A (∇--∇), MAO-B (□- -□) and SSAO (●——●) in the rat ileum following i.p. injection of 5 mg kg⁻¹ MDL 72145. Control rats received distilled water. Animals were killed 2, 24 h, 2, 4, 7 and 11 days later (n = 5 per group) and homogenates of ileum were made and assayed immediately for the 3 enzyme activities. Each point represents the mean enzyme activity in the treated group as a percentage of its control group (±s.e.r). (b) Time course of recovery replotted in terms of ln(1-f) where f is the fractional activity remaining. The regressions are shown as the unweighted lines of best fit from which the rate constants and half lives were calculated and shown in Table 2.

agents on sheep plasma amine oxidase is shown in Fig. 3b. The IC50 values are 3 and 10 μM for MDL 72145 and semicarbazide, respectively. Dilution of these inhibitors by 10 fold following preincubation with the plasma did not alter the degree of inhibition seen in the final assay indicating that their effects were not easily reversed.

Table 2. The rate of recovery of the amine oxidase enzymes of the aorta, BAT and ileum following in-vivo inhibition by MDL 72145 (5 mg kg⁻¹ i.p.). The rate constant for degradation and half life for each enzyme were calculated by plotting the unweighted line of best fit for ln(1-f) against time (see Fig 2b) where f is the fraction of the enzyme activity remaining (see Della Corte & Callingham 1977).

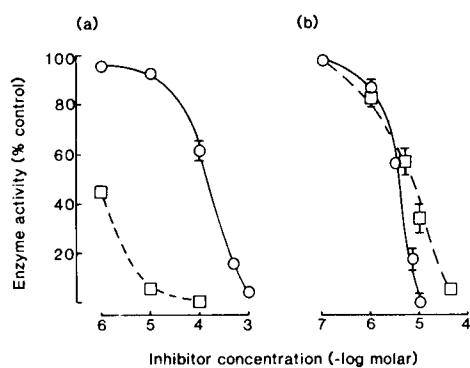| Tissue	Enzyme	Elimination rate constant (d⁻¹; mean ± confidence limits at P = 0·05)	Half life (d)	Correl. coeff. of plot
Aorta	SSAO	0·284 ± 0·064	2·44	0·997
	MAO-A	0·226 ± 0·164	3·06	0·973
BAT	SSAO	0·113 ± 0·079	6·15	0·948
	MAO-A	0·198 ± 0·135	3·50	0·975
Ileum	SSAO	0·192 ± 0·063	3·62	0·984
	MAO-A	0·402 ± 0·049	1·73	0·999
	MAO-B	0·123 ± 0·084	5·64	0·993

FIG. 3. The effect of MDL 72145 (O——O) and semicarbazide (□- - -□) in-vitro on rat ileum diamine oxidase (DAO) and sheep plasma polyamine oxidase (SPAO) activities. (a) Homogenates of rat ileum were preincubated with MDL 72145 or semicarbazide for 30 min at 37°C before the addition of 50 μM putrescine as the substrate for the measurement of DAO activity. Each point represents the mean enzyme activity (±s.e.r. when exceeding size of symbol) as a percentage of control samples preincubated without inhibitor (n=4). (b) Sheep blood plasma was preincubated with MDL 72145 or semicarbazide for 30 min at 37°C before the addition of 700 μM spermidine as the substrate for the measurement of SPAO activity. Each point represents the mean enzyme activity (±s.e.r. when exceeding size of symbol) as a percentage of control samples preincubated without inhibitor (n=4).

Discussion

MDL 72145, at a dose of 2·5 mg kg^{-1}, caused selective inhibition of SSAO in the aorta and BAT. However, in the liver less selectivity was obtained with such a dose, a finding which is consistent with those of Fozard et al (1985). These authors found that MDL 72145 administered orally at 2·5 mg kg^{-1} daily for 5 days was selective for MAO-B versus MAO-A in the brain and duodenum but not in the heart and liver.

The studies reported here, may also suggest that the rates of inhibition of MAO-B and of SSAO are greater than that of MAO-A. This finding is supported by the in-vitro data of Zreika et al (1984) and Lyles & Fitzpatrick (1985). Both these groups of authors showed that the rate of formation of the enzyme inhibitor complexes was much faster for MAO-B and SSAO than for MAO-A.

By the use of MDL 72145 (2·5 mg kg^{-1}) followed by killing the animal 1 h later, it was possible to achieve almost complete inhibition of SSAO with minimal inhibition of MAO-A in vascular tissue and BAT. It should therefore, be possible to use MDL 72145 to study the effect of selective inhibition of SSAO activity on the pharmacological responses of preparations in-vitro. However, this drug would not be useful for pharmacological studies in the whole animal. The dose required to inhibit SSAO under these conditions would also inhibit MAO-B which is present in many tissues. Reducing the dose further may help but another related compound may prove more promising in this respect. In-vitro data shows that (E)-2-phenyl-3-chloroallylamine (MDL 72274) may have a greater selectivity for inhibition of SSAO versus both MAO-A and MAO-B (Lyles et al 1987).

The fact that MAO-A inhibition continues in-vivo, yet no active free drug can be detected in the tissues at the same time, is surprising. It is possible that the drug has bound to

the enzyme but has still to be activated to complete its inhibitory effect. In this state it would appear that the enzyme is still capable of metabolizing its substrates. The in-vitro data of Zreika et al (1984) however, suggest that the initial complex formed between enzyme and inhibitor occurs at or near the active site since the presence of enzyme substrates will protect the enzyme from inhibition, making a pharmacokinetic explanation for the increasing inhibition of MAO-A with time rather more likely. It may be that the drug is sequestered in tissues and organs that have not been examined in this study and from which it can emerge when concentration gradients are favourable. It is possible that it is these pharmacokinetic factors that are responsible for the variations in the sensitivities of the enzymes between tissues to inhibition by MDL 72145 that have been reported by Flucker et al (1986) rather than any differences in the properties of the enzymes in the different tissues. This question may well be answered by examining the in-vitro action of MDL 72145 on a variety of tissue homogenates. However, it is possible that the effect is not due to simple sequestering of the drug but to concentration of the drug at a site where it may be metabolized into a product that is more, rather than less, potent as an enzyme inhibitor on one or more enzyme activities. For example, the MAO-A inhibitor moclobemide is more potent ex-vivo than in-vitro but still remains reversible, an effect almost certainly due to the formation of metabolites more active than moclobemide itself (Da Prada et al 1981; Keller et al 1987). Furthermore, the possibility that MAO itself may be involved in the conversion has been suggested since in-vitro production of an activated metabolite appears to be greatly reduced in tissues where MAO has been inhibited with pargyline (Burton et al 1984; Callingham & Ovens 1988). Unpublished work in our own laboratory suggests that in-vivo metabolism of some MAO inhibitors may have unforeseen consequences. Pargyline, which has no inhibitory action on SSAO in-vitro, causes substantial and apparently irreversible inhibition of this enzyme after in-vivo administration. Indeed it has been possible to use this agent to obtain values for the half life of SSAO in the intestine. A value of 2·97 d was found from a regression calculated from 5 groups of 4 rats. This finding suggests that care should be taken when using pargyline in-vivo, since inhibition of SSAO may contribute to the effects observed.

The results presented here for the half lives of MAO-A and MAO-B in the ileum determined from their recoveries following in-vivo MDL 72145 administration are in close agreement with results from earlier experiments following inhibition with pargyline, which also indicated that the MAO-A activity in the rat intestine was highest in the mucosal layers (Callingham et al 1985). MAO-A turnover was more rapid than for MAO-B, with no obvious deviation from a logarithmic recovery of MAO-A activity after inhibition with MDL 72145 in spite of the rapid turnover of the mucosal cells, in which it is largely contained. This may indicate that these short-lived cells also have a rapid turnover of their cellular proteins. As noted by Hasan et al (1988) the turnover rates of MAO-A and MAO-B vary between tissues. This also seems to be the case for SSAO where the half life in the aorta is considerably shorter than in the BAT.

The in-vitro studies performed showed that MDL 72145 is

not a potent inhibitor of DAO in the rat intestine. It should be remembered, however, that the properties of DAO enzymes vary between species and between tissues within a species (Kapeller-Adler 1970). Certainly intestinal DAO shows different inhibitor characteristics to SSAO. For example, in the rat aorta we have found MDL 72145 to be 100 times more potent than semicarbazide upon SSAO in-vitro (unpublished observations). It was felt important to compare relative potencies of MDL 72145 and semicarbazide when studying the inhibitors' effects in-vitro since the amount of non-specific binding may well vary between different tissue homogenates and assay conditions (Fowler & Callingham 1978). With the sheep plasma enzyme, MDL 72145 is almost equipotent with semicarbazide. The effects of this drug on the circulating plasma enzyme in man and on the tissue bound amine oxidase, lysyl oxidase (EC 1.4.3.13), should be studied so that the selectivity of this drug within the group of semicarbazide-sensitive amine oxidase enzymes can be determined.

In conclusion, these studies show that MDL 72145 can be used selectively to inhibit SSAO in blood vessels and BAT of the rat since these tissues contain little MAO-B. The study of SSAO in whole animal experiments will depend on the development of a drug, which in-vivo shows greater selectivity than MDL 72145 for SSAO. Extrapolation of drug selectivity from in-vitro studies to the in-vivo situation is not possible since pharmacokinetics and drug metabolism in-vivo may dramatically alter the drug's effects.

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