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Effects In-vitro of Procarbazine Metabolites on Some Amine Oxidase Activities in the Rat

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Abstract—The effects were examined of four metabolites of the anticancer agent, procarbazine (*N*-isopropyl-α-(2-methyl hydrazino)-*p*-toluamide hydrochloride) on semicarbazide-sensitive amine oxidase (SSAO) and monoamine oxidase-A and -B (MAO-A and -B) activities in rat brown adipose tissue and liver homogenates, respectively. Azoprocarbazine (AZO) and monomethylhydrazine (MMH) inhibited selectively the deamination of benzylamine by SSAO, when compared with their effects on MAO activities. The IC50 values against SSAO, of 32·7 nM (AZO) and 7·0 nM (MMH), were more than three orders of magnitude lower than those exhibited against MAO. Neither isomer of azoxyprocarbazine was an effective inhibitor of rat amine oxidase activities. The inhibition of SSAO by AZO was reversed very slowly by dialysis, in contrast to results seen for MMH. The non-competitive kinetics of MMH and the ability of B24, a rapidly reversible SSAO inhibitor, to protect SSAO against inhibition by MMH are consistent with the view that this compound binds to the enzyme cofactor at, or near, the active site.

Metabolism in-vivo of the carcinostatic agent, procarbazine (*N*-isopropyl-α-(2-methyl hydrazino)-*p*-toluamide hydrochloride), is reported largely to be due to hepatic cytochrome P450 and monoamine oxidase (MAO; EC 1.4.3.4) activities (Dewald et al 1969; Coomes & Prough 1983; Prough et al 1984). The complex pathways of metabolism appear to be similar in both rat and man, with almost 20 distal metabolites or intermediates thought to be formed through the common proximal metabolite, azoprocarbazine (*N*-isopropyl-α-(2-methyl azo)-*p*-toluamide; AZO; see Tweedie et al (1987) for review). The anticancer and mutagenic properties of procarbazine are not exhibited if its metabolism is prevented, either by using inhibitors of the metabolic systems (Zijlstra & Vogel 1988) or in cultured cells, essentially devoid of the necessary enzymes (Erikson et al 1989). Furthermore, induction of cytochrome P450 activity with isozyme-specific agents can increase selectively the rate of formation of certain metabolites (Prough et al 1984; see Tweedie et al 1987). An understanding of the metabolic pathways followed by procarbazine is therefore essential if its pharmacological effects are to be understood.

Recently, we have found procarbazine to be a potent and selective inhibitor of semicarbazide-sensitive amine oxidase (SSAO; EC 1.4.3.6) in rat brown adipose tissue, when compared with its effects on rat hepatic MAO activities (Holt et al 1992). The physiological functions of most SSAO enzymes are presently unknown and this can be attributed, in part, to a lack of selective inhibitors which could be used in both in-vitro and in-vivo studies. The half-life of procarbazine in plasma after intravenous administration has been measured as 7 min in man and 24 min in rats (Raaflaub & Schwartz 1965). This is due predominantly to its rapid oxidation to AZO in the liver. It is thus possible that in-vivo levels of inhibition of SSAO enzymes by procarbazine might be lower than those predicted from in-vitro studies, where the drug is pre-incubated with the enzyme in, for example brown adipose tissue, for at least 2 h before addition of substrate (Holt et al 1992). We have therefore examined the effects of some metabolites of procarbazine on rat amine oxidase activities, both in order to ascertain whether any active metabolite might augment the inhibition of SSAO by procarbazine and to determine whether any of the compounds studied might be suitable for use as inhibitors in investigations of SSAO function. The metabolites examined, namely AZO, monomethylhydrazine (MMH), benzylazoxyprocarbazine (AZOXY I)* and methylazoxyprocarbazine (AZOXY II)*, have all been identified in blood plasma or urine following the in-vivo administration of procarbazine to rats (Schwartz 1966; Weinkam & Shiba 1978).

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Materials and Methods

Materials

Substrates for the radiochemical amine oxidase assays were [7-^{14}C]benzylamine hydrochloride, from ICN Flow (High Wycombe, Bucks, UK) and 5-hydroxy[G-^{3}H]tryptamine creatinine sulphate, from Amersham International (Amersham, Bucks, UK). Unlabelled benzylamine was obtained as the free base from Sigma Chemical Co. (Poole, Dorset, UK) and crystallized as the hydrochloride in the laboratory. Unlabelled 5-hydroxytryptamine creatinine sulphate was obtained from Sigma. Opti-fluor O (liquid scintillation fluid) was obtained from Canberra Packard (Pangbourne, Berks, UK).

Azoprocarbazine, dissolved before use with an equivalent amount of HCl in distilled water and the methyl and benzyl isomers of azoxyprocarbazine, dissolved in an aqueous solution (1% v/v) of dimethylsulphoxide (Sigma), were

* The nomenclature used for the azoxy isomers is based on the IUPAC Tentative Rules, whereby the position of the oxygen atom is specified by the infixes -NNO- or -ONN-. The formulae, *N*-isopropyl-α-(2-methyl-NNO-azoxy)-*p*-toluamide (AZOXY I) and *N*-isopropyl-α-(2-methyl-ONN-azoxy)-*p*-toluamide (AZOXY II) therefore correspond to the benzyl and methylazoxy isomers of procarbazine, respectively. The nitrogen numbering system is based on that used for the parent compound, 2-methyl-1-benzylhydrazine.

donated by Dr Garold Yost, University of Utah. Monomethylhydrazine, purchased from Aldrich Chemical Co. (Gillingham, Dorset, UK), was obtained as the free base and dissolved with an equivalent amount of HCl in aqueous solution. B24 (3,5-ethoxy-4-aminomethylpyridine) was a gift from Professor Franca Buffoni, University of Florence. All other reagents were of analytical grade, where possible. Male Wistar rats, 200–400 g, were supplied by A. J. Tuck and Son (Rayleigh, Essex, UK).

Preparation of homogenates

Rats were killed by stunning followed by decapitation. Livers were excised, washed and stored at −20°C until required. Pads of interscapular brown adipose tissue (brown fat) were removed and placed on ice. A pooled homogenate of brown fat, obtained from a minimum of 3 animals, was made (1 in 40 w/v in 0·2 M potassium phosphate buffer, pH 7·8) using a mechanical homogenizer (Polytron Mark 5, Kinematica). The homogenate was stored in 1 mL portions at −70°C until required. Liver homogenates were made, immediately before use, (1 in 40 w/v in 1 mM potassium phosphate buffer, pH 7·8) using a Polytron homogenizer.

Radiochemical amine oxidase assay

Amine oxidase assays were based on the method described by Lyles & Callingham (1982) and summarized by Holt et al (1992). Substrates used were, for SSAO, [^{14}C]benzylamine (10 μM, sp. act. 10 μCi μmol^{-1}), for MAO-A, [^{3}H]5-hydroxytryptamine (250 μM, sp. act. 2 μCi μmol^{-1}) and for MAO-B, [^{14}C]benzylamine (250 μM, sp. act. 2 μCi μmol^{-1}).

Inhibitor selectivity and potency

Experiments were performed to determine the selectivities of AZO, MMH, AZOXY I and AZOXY II for SSAO over MAO activities. Following preliminary studies to establish optimum pre-incubation times for inhibitors with tissue homogenates, samples were pre-incubated at 37°C for 15 min, with aqueous inhibitor solutions or distilled water (controls) before remaining enzyme activity was assayed by addition of labelled substrate.

Dialysis experiments

For dialysis studies, brown fat homogenate (1:40, 500 μL) was pre-incubated with 500 μL samples of distilled water (controls), 20 μM AZO or 20 nM MMH, at 37°C for 15 min and then 0·75 mL samples were dialysed under appropriate conditions (see Results) for 24 h (AZO) or 3, 6 or 9 h (MMH). SSAO activity in the remaining 250 μL (undialysed) portion and in each dialysed sample was then assayed.

Kinetic experiments

The kinetics of the inhibition by MMH of benzylamine metabolism were studied by examining the deamination of [^{14}C]benzylamine (2–20 μM) in the presence of MMH (0, 3, 6 or 10 nM) following pre-incubation of homogenate samples at 37°C for 15 min with inhibitor solutions.

Protection of the active site

Initial experiments had shown that SSAO activity in brown fat could be inhibited almost completely by 100 μM B24, an active site-directed inhibitor (Banchelli et al 1990), and that dialysis for 3 h could restore activity almost to control levels. Samples of brown fat (1:40, 500 μL) were pre-incubated, at 37°C for 15 min, with 500 μL water (controls), MMH (200 nM, 500 μL) or B24 (200 μM, 500 μL). A further 500 μL sample of brown fat (1:40) was pre-incubated with B24 (400 μM, 250 μL) for 15 min, followed by the addition of MMH (400 nM, 250 μL). All samples were then further pre-incubated at 37°C for 10 min. Portions of 600 μL were dialysed for 3 h under appropriate conditions (see Results) and the remaining 400 μL portions were stored at dialysis temperature for 3 h. SSAO activity in each sample was then measured by the addition of labelled substrate.

Protein assays

Protein contents of homogenates were measured by the method of Lowry et al (1951) and enzyme activities are expressed as nmol of product formed h^{-1} (mg protein)$^{-1}$.

Statistical analysis

Sigmoid curves were fitted to the data, when appropriate, using the nonlinear regression facility of GraphPAD InPlot, Version 3·0 (GraphPAD Software, San Diego, CA, USA).

Results

Potency and selectivity of procarbazine metabolites against some amine oxidase activities

Homogenates of brown fat or liver were pre-incubated for 15 min with inhibitor concentrations from 100 pM to 1 mM (AZO), 10 pM to 1 mM (MMH) or 1 μM to 1 mM (AZOXY) and remaining amine oxidase activities were determined. Figs 1–3 illustrate the concentration-dependent inhibition of enzyme activities compared with activity in control samples. IC50 values, obtained following fitting of sigmoid curves to the data points by nonlinear regression (see Materials and

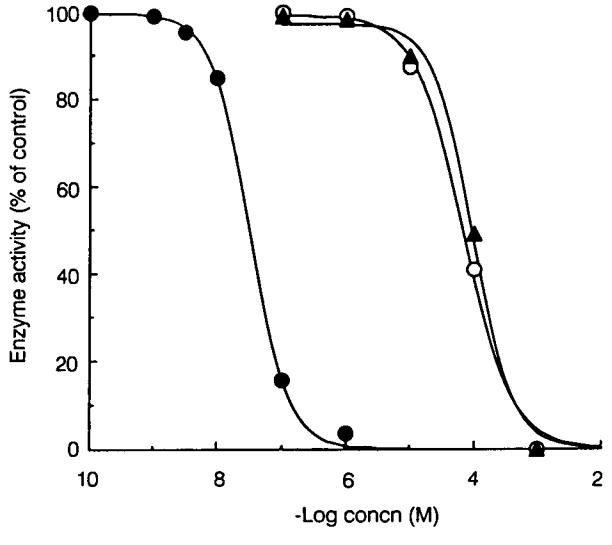

FIG. 1. Effect of AZO on rat brown adipose tissue SSAO (●), liver MAO-A (○) and MAO-B (▲) activities of the rat. Samples of homogenates were pre-incubated with AZO for 15 min at 37°C before addition of labelled substrate. Deaminating activities are expressed as a percentage of those in control samples pre-incubated without inhibitor. Each point is the mean of 2 determinations, each in triplicate, on homogenates of pooled tissue.

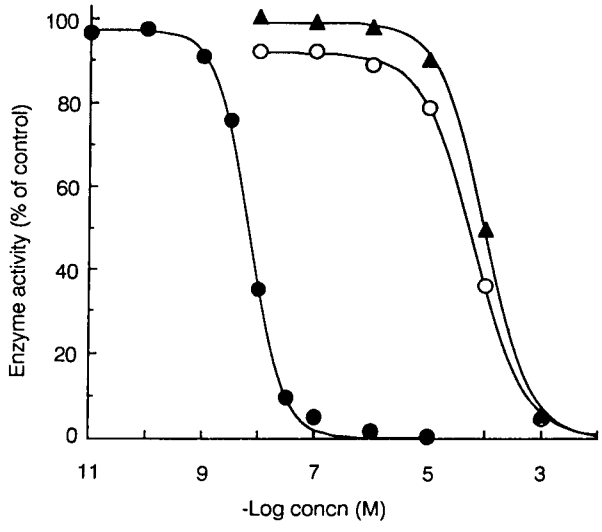

FIG. 2. Effect of MMH on rat brown adipose tissue SSAO (●), liver MAO-A (○) and MAO-B (▲) activities of the rat. Experimental conditions were as described in the legend to Fig. 1. Each point is the mean of 3 or 4 determinations, each in triplicate, on homogenates of pooled tissue.

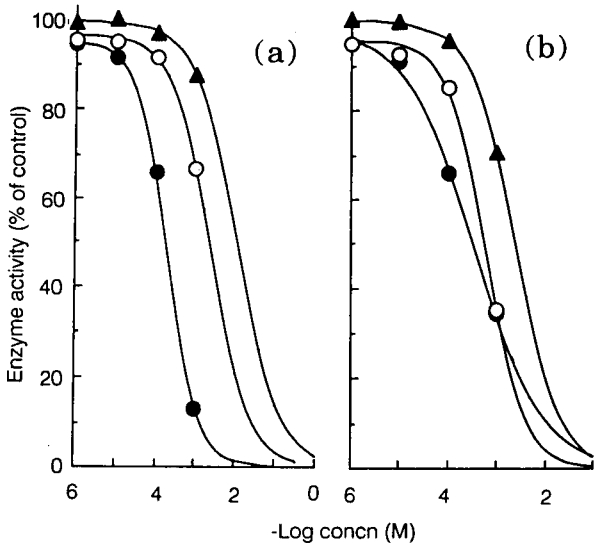

FIG. 3. Effects of AZOXY I (a) and AZOXY II (b) on rat brown adipose tissue SSAO (●), liver MAO-A (○) and MAO-B (▲) activities of the rat. Experimental conditions were as described in the legend to Fig. 1. Each point is the mean of 2 determinations, each in triplicate, on homogenates of pooled tissue.

Methods), are shown in Table 1. Both AZO and MMH are highly potent inhibitors of SSAO activity in this tissue and exhibit a considerable degree of selectivity when compared with their effects on either MAO-A or -B. However, these characteristics are not evident in either of the azoxy isomers. The small amounts of these azoxy compounds available to us precluded examination of their effects on amine oxidase activities at drug concentrations greater than 1 mM.

Table 1. IC50 values for the inhibition of rat amine oxidase activities by metabolites of procarbazine. Values were obtained by nonlinear regression of data presented in Figs 1–3.

Metabolite	SSAO	MAO-A	MAO-B
AZO	32·7 nM	73·5 μM	97·7 μM
MMH	7·0 nM	62·9 μM	97·7 μM
AZOXY I	0·20 mM	n/d	n/d
AZOXY II	0·34 mM	0·62 mM	n/d

Data are means of triplicate values obtained from 2 experiments (AZO and AZOXY) or 3 or 4 experiments (MMH). n/d = could not be determined.

Comparison of rates of reversal of inhibition by AZO and MMH under different dialysis conditions

Following substantial inhibition of SSAO by the use of AZO or MMH, samples were dialysed to determine the rate of recovery of deaminating activity. We considered that the chemical stability of these compounds might influence this rate and dialysis conditions were chosen accordingly. After pre-incubation of homogenates with 10 μM AZO, samples were dialysed against 1 L of 1 mM potassium phosphate buffer, pH 7·8, for 24 h at 37°C, with buffer changes at 3 and 9 h. A further sample was stored at 37°C for the duration of dialysis. SSAO activities were then measured and compared against control samples, preincubated with water in place of inhibitor. Stored samples contained 1·7% of the activity of controls, while dialysis increased this activity to 23·3%. These results, the means from 2 experiments, indicate that inhibition of SSAO by AZO is very slowly reversible by dialysis. After pre-incubation of homogenates with 10 nM MMH, samples were dialysed against 1 L of 1 mM potassium phosphate buffer, pH 7·8, for 3, 6 or 9 h at 25°C, with buffer changes at 1, 3 and 6 h. A further sample was stored for 9 h at 25°C. SSAO activities, expressed as a percentage of control values, were 21·0% (undialysed), 37·1% (3 h), 47·2% (6 h) and 53·5% (9 h). These results, the means from 3 experiments, indicate that recovery of enzyme activity by dialysis following inhibition by MMH is initially quite rapid, although complete reversal at 25°C might require dialysis for a prolonged period.

Effect of MMH on kinetic constants for [^{14}C]benzylamine deamination

Assays of [^{14}C]benzylamine metabolism (2–20 μM) were carried out in the presence of MMH (3, 6 or 10 nM) following pre-incubation with brown fat for 15 min. A parallel experiment used water in place of inhibitor. Fig. 4 shows a Hanes-Woolf plot of the results. The common intercept on the abscissa is indicative of a non-competitive interaction between MMH and SSAO.

Determination of the site of action of MMH by protecting the active site with a reversible inhibitor

The active site of SSAO was protected by pre-incubating brown fat homogenate with B24, an active site-directed inhibitor which can be removed rapidly by dialysis. Following further pre-incubation with 100 nM MMH, a concentration sufficient to reduce enzyme activity by more than 90%, a portion of the sample was dialysed against 1 L of potassium phosphate buffer, pH 7·8, at 20°C for 3 h, with buffer changes

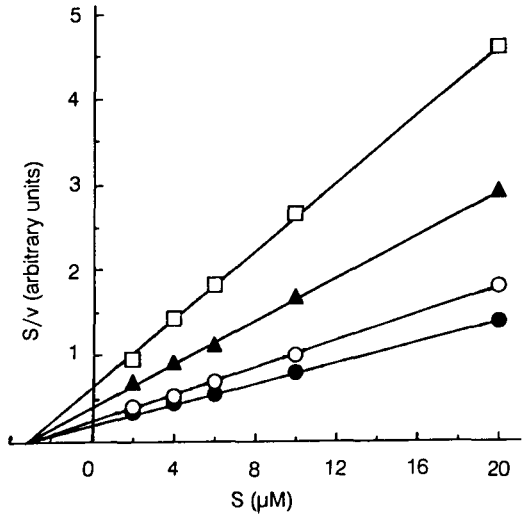

FIG. 4. Hanes-Woolf plot showing inhibition of [^{14}C]benzylamine metabolism by MMH in rat brown adipose tissue homogenates. Substrate concentration is represented as S and reaction velocity as v. Final MMH concentrations, pre-incubated with homogenates for 15 min at 37°C before addition of labelled substrate, were 0 (●), 3 (○), 6 (▲) and 10 nM (□). Each point is the mean of 2 or 3 determinations, each in triplicate, on homogenates of pooled tissue.

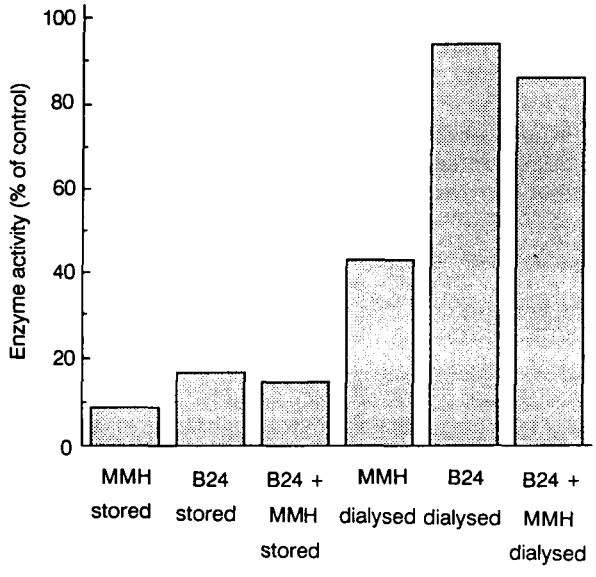

FIG. 5. Protective effect of B24 on the inhibition of SSAO in rat brown adipose tissue by MMH. Homogenate samples were pre-incubated, for 15 min at 37°C, with MMH, B24 or B24 followed by MMH. Samples were then divided and portions were either stored, or dialysed, for 3 h at 20°C. SSAO activity in each sample was then measured by the addition of labelled substrate and expressed as a percentage of those in control samples, pre-incubated without inhibitors. Results are the means of 3 determinations, each in triplicate, on homogenates of pooled tissue.

at 30 and 90 min. The remainder of the sample was stored at 20°C for the duration of the dialysis. Remaining SSAO activity was determined in both portions by the addition of labelled substrate and results were expressed as a percentage of activity in control samples, pre-incubated with water in place of inhibitors. These activities were then compared with activities measured in parallel experiments, where either B24 or MMH had been replaced by water. The results (Fig. 5) indicate that B24 and MMH share a common site of action and therefore that MMH probably interacts with SSAO at, or close to, the active site of the enzyme.

Discussion

The rapid oxidation in-vivo of procarbazine to its azo derivative is the initial transformation in a complex series of metabolic steps resulting in the formation of many intermediates and metabolites. All known therapeutic actions of the drug have been attributed to one or more of these metabolites. For instance, hydrogen peroxide, released during formation of AZO, was thought originally to be responsible for damage to DNA (Berneis et al 1963). However, the amount formed is insufficient to account for the extent of damage caused by procarbazine (Gale et al 1967). More recently, it has been demonstrated that the cytotoxic agent responsible for most of the anticancer activity against L1210 leukaemia cells is methylazoxyprocarbazine (AZOXY II) (Swaffar et al 1989). Furthermore, methyl radicals, capable of DNA alkylation (Augusto et al 1990) and extraction of hydrogen atoms from lipids, are produced from procarbazine by rat hepatic microsomal enzymes in-vitro (Sinha 1984). Therefore, apart from reducing biogenic amine metabolism by competition for MAO (De Vita et al 1965), procarbazine has rarely been considered as a compound possessing significant pharmacological activity in its own right.

Recent studies in our laboratory have provided evidence that procarbazine is a suicide inhibitor of SSAO in rat brown adipose tissue in-vitro (Holt et al 1992). However, the results presented here suggest that inhibition of SSAO following administration of procarbazine in-vivo might be due, not only to the parent drug, but also to the action of metabolites. Although many metabolites have been isolated from the blood and urine of animals and man after administration of procarbazine, we have chosen to examine the effects on rat amine oxidase activities of four compounds in particular. The proximal metabolite, AZO and the two azoxy compounds thought to be largely responsible for the anticancer activity of procarbazine (Erikson et al 1989) were studied, along with the putative metabolite, MMH. The hydrazine structure of MMH suggested that this compound might prove to be a powerful inhibitor of carbonyl-containing enzymes such as SSAO.

Experiments to determine the potency and selectivity of these compounds for SSAO showed that AZO and MMH were both more potent than procarbazine in this respect, but showed a similar degree of selectivity over MAO to that seen with procarbazine (Holt et al 1992). Neither azoxy isomer had a marked effect against any of the amine oxidase activities examined and further investigations did not therefore involve these compounds. MMH proved to be the most potent of the procarbazine metabolites in inhibiting SSAO, possibly because its small molecular size allows easy access to the cofactor or the active site, which, in SSAO, are thought to lie close together (Janes et al 1990). Furthermore, the rapid onset of inhibition seen with both AZO and MMH suggest that inhibition of SSAO in-vivo would be almost immediate, bearing in mind the short plasma half-life of procarbazine.

Dialysis experiments were used to give some possible indication of the duration of inhibition of SSAO following administration of procarbazine in-vivo. It was apparent that, while MMH was extremely potent, its action could be reversed by more than 50%, by dialysing at 25°C for 9 h. However, AZO was only very slowly removed from the enzyme by dialysis at 37°C, indicating that a single dose of procarbazine might result in long-lasting inhibition of SSAO activities in-vivo. AZO is known to be lipid soluble (Baggiolini et al 1969) and this might prolong the time to clear the drug from the body and consequently the duration of inhibition of SSAO. Following administration of a single intraperitoneal dose of [^{14}C]procarbazine to rats, more than 10% of the radioactivity remained and could be detected in the animal after 4 days (Schwartz 1966); however, the tissue distribution of radioactivity was not examined. Any concentration of this remaining radioactivity in the smooth muscle surrounding blood vessels, or in brown adipocytes, could be indicative of an association of procarbazine or its metabolites with SSAO enzymes, since these cell types are a prominent source of SSAO when compared with those in most other tissues (Barrand et al 1984; Lyles & Singh 1985).

In view of the high potency shown by MMH against SSAO as well as its ready availability, the interaction between inhibitor and enzyme was examined in greater detail, to ascertain whether MMH might be suitable for studies designed to determine the effects of inhibiting SSAO enzymes in-vitro and in-vivo. Kinetic experiments showed that the interaction was non-competitive in nature, consistent with the view that the cofactor is the most likely site of attack by hydrazine reagents (Suva & Abeles 1978). Similar kinetic characteristics have previously been observed for procarbazine (Holt et al 1992). The active site of SSAO was protected by pre-incubating brown fat homogenate with the selective inhibitor, B24. Enzyme activity can subsequently be restored, almost to control levels, by dialysing samples at 25°C for 3 h. Although we found B24 to be rather less potent against SSAO here than in previous studies (Banchelli et al 1990), the concentration used was able to afford almost complete protection against the action of MMH. Previous experiments, using benzylamine to protect SSAO from procarbazine, suggested that the binding site of procarbazine is at, or near, the active site of the enzyme (Holt et al 1992) and the results presented here are consistent with the view that MMH and procarbazine share a common binding site. Although much confusion has surrounded the identity of the cofactor in SSAO enzymes, current evidence favours 6-hydroxydopa, incorporated into the active site (Janes et al 1990). This cofactor, which, when oxidized, presents carbonyl groups, should be inactivated readily by hydrazine derivatives such as procarbazine, AZO and MMH (see Blaschko 1974; Lyles 1984). The physiological consequences of SSAO inhibition in this manner remain unclear, since the roles of most SSAO enzymes are not known. However, the aliphatic amine, methylamine is a substrate for SSAO, but not MAO (Dar et al 1985), in-vitro (Precious et al 1988; Lyles et al 1990) and almost certainly in-vivo (Lyles & McDougall 1989). An endogenous metabolite of compounds such as sarcosine, adrenaline, choline and glycine (Kohn 1931), methylamine is normally metabolized in-vivo to CO_2 via formaldehyde (Boeniger 1987). Possible deleterious effects of raised plasma methylamine levels due to inhibition of SSAO by procarbazine (see Precious et al 1988) might be exacerbated by the more persistent inhibitory effects of metabolites such as AZO.

It has been proposed that methylamine might itself be an intermediate in the metabolism of procarbazine (Schwartz 1966). This possibility has been discounted by workers who were unable to detect methylamine in rat isolated livers perfused with procarbazine (Baggiolini & Bickel 1966). Furthermore, [^{14}C]methylamine metabolism in-vivo, measured by the production of [^{14}C]CO_2, was inhibited in rats co-administered procarbazine. However, [^{14}C]methylamine has been isolated from the urine of rats injected with [^{14}C]-procarbazine (Schwartz 1966), implicating some extra-hepatic metabolic system in the production of methylamine from procarbazine. The results presented here and elsewhere (Holt et al 1992) suggest that administration of procarbazine in-vivo might result in extensive inhibition of SSAO activities by procarbazine, AZO and perhaps other compounds such as MMH. This would prevent the metabolism of methylamine derived both from endogenous substrates and from procarbazine itself. In this respect, it is almost certainly correct to state that methylamine is not an intermediate in the production of CO_2 from procarbazine. However, it is possible that methylamine is the end product of one particular metabolic pathway. [^{14}C]Methylamine has also been isolated from the urine of rats injected with [^{14}C]MMH (Schwartz 1966), although the identity of the enzyme catalysing this reaction is unknown. Once this has been resolved, use of selective inhibitors of this enzyme might allow us to determine whether MMH is an intermediate in the breakdown of procarbazine to methylamine. [^{14}C]MMH has not been detected following administration of [^{14}C]procarbazine to rats, although one possible reason for this is that it is so rapidly degraded that none remains in the tissues.

Further experiments with procarbazine and its metabolites as potent and selective inhibitors of SSAO enzymes, both in-vitro and in-vivo, will provide some clues to the physiological roles of these enzymes. At the same time, a better understanding of both the metabolic pathways followed by procarbazine and the causes of some of the adverse effects seen in patients receiving this drug might be achieved (see Tweedie et al 1987; Pfefferbaum et al 1989).

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