genase are similar. Despite the evidence that calmodulin is involved in insulin–receptor autophosphorylation [18], and in the ATP-dependent Ca^{2+} transport of adipocyte plasma membranes [6, 8, 9], there does not appear to be an obligatory step in insulin signalling that is obliterated when adipocytes are incubated with trifluoperazine.

Trifluoperazine does inhibit the effect of oxytocin to stimulate phospholipid methyltransferase, suggesting that calmodulin may be involved in mediating the effect of oxytocin on this enzyme.

The data from this study suggest a reevaluation of the role of calmodulin in insulin action as determined by studies with trifluoperazine [10, 11], the most potent inhibitor of calmodulin [12].

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Substituted phenylpiperidines and phenylpyridines as reversible selective inhibitors of monoamine oxidase type A in rodent brain and liver

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Clinically effective antidepressant monoamine oxidase (MAO) inhibitors share the property of inhibiting MAO-A, which is characteristic of monoaminergic nerve terminals [1]. These agents as well as the newer, selective propargylamine inhibitors of MAO-A (clorgyline) and MAO-B ([-]deprenyl) are irreversible agents with prolonged actions [1]. The older, clinically effective non-selective MAO inhibitors and the selective anti-MAO-A agents also potentiate the pressor effects of indirect sympathomimetics such as tyramine, sometimes dangerously, by complex and incompletely defined mechanisms [1, 2]. Several new reversible short-acting inhibitors of MAO-A (in addition to the older harmala alkaloids) are currently under investigation (cimoxatone, moclobemide, and others) [2]. At least one of these, the benzofuranyl piperidine derivative brofaromine (CGP-11-305-A) [3], may be a clinically effective antidepressant and also have less risk of inducing hypertension than the older drugs [2]. Further leads to additional novel, selective and reversible MAO inhibitors may arise from recent studies of the pharmacologic effects of certain neurotoxic phenylpyridines [4].

Inhibitors of MAO, especially of type B, reportedly

protect animals against the dopamine-depleting neurotoxic effects of 1-methyl-4-phenyl-tetrahydropyridine (MPTP) [4, 5] and prevent conversion of MPTP to the toxic metabolite, 1-methyl-4-phenyl-pyridinium ion (MPP^+) [5–7], the dopamine-depleting effects of which are not prevented by MAO inhibitors [8]. In addition, binding of [^{3}H]MPTP to tissues correlates highly with the distribution of MAO, and MAO inhibitors compete for this binding [9]. Moreover, recent observations suggest that MPTP can inhibit MAO activity *in vitro* with some selectivity for the enzyme in monoaminergic nerve terminals (presumably MAO-A) [10] and, in selective assays, MAO-A over MAO-B [11–14]; *in vivo* inhibitory effects of this agent have been reported on rodent brain MAO-A [12, 15] but also on MAO-B [15, 16]. Several structural analogs of MPTP and MPP^+ also have selective effects on MAO types A and B [15]. These findings indicate that pharmacologically important interactions occur between MPTP-like compounds and specific forms of MAO, and encourage additional explorations of the potential of analogous compounds as selective MAO inhibitors.

Materials and methods

Whole-brain and liver tissue was obtained from young adult (200–300 g) male S-D rats and liver was obtained from adult (30–40 g) male C-D mice (Charles River, Wilmington, MA). Tissue was removed rapidly to ice, rinsed with ice-cold 0.32 M sucrose, homogenized in 5 vol. of the isotonic sucrose, and centrifuged at 1,000 *g* (4°, 15 min) and then at 17,000 *g* (30 min). The resulting mitochondria-rich P_2 pellet was taken up in 5 vol. (vs original tissue wt) of the sucrose (containing 0.1 M KH_2PO_4, pH 7.4) and frozen (−20°) until assay of MAO [17], when the thawed P_2 preparation was diluted 4-fold with isotonic KCl (1.15% [w/v]) just before use.

Assay mixtures contained 0.2 ml of 0.67 M KH_2PO_4 buffer (final pH 7.4); 50 μl of the P_2 fraction (equivalent to 2.5 mg of original tissue, with linear dependency on tissue found between 10 and 100 μl of the P_2 preparation); and 50 μl each of test agent and radiolabeled substrate ([^{14}C]serotonin for MAO-A and [^{14}C]phenethylamine for MAO-B assays)—both in isotonic KCl, which was also used to make up the final volume to 0.5 ml in Kimax screw-cap glass centrifuge tubes (1.6 × 10 cm). Tissue routinely was preincubated for 30 min at 37° with either (-)deprenyl (25 nM) or clorgyline (25 nM) to minimize substrate-nonselectivity in assays of MAO-A and MAO-B respectively. (The latter conditions were determined in pilot experiments in which concentration–effect curves for both inhibitors were tested with both substrates to permit selection of a concentration of deprenyl that provided >95% inhibition of the deamination of [^{14}C]phenethylamine but <5% inhibition with [^{14}C]serotonin, and the opposite with clorgyline; the optimal values of 25 nM selected for each specific inhibitor agree well with other similar experiments [18].) Reactions were started by addition of substrate (typically, in test agent concentration–effect experiments, to provide a 100 μM concentration of [^{14}C]serotonin for MAO-A and a 5.5 μM concentration of [^{14}C]phenethylamine for MAO-B assays [18]), continued for 30 min for MAO-A and 5 min for MAO-B (providing linearity vs time), and terminated by rapid transfer to an ice bath and adding 0.1 ml of 4 N HCl. "Blank" reactions had the HCl added at the start of the incubation (with results indistinguishable from those obtained by omitting tissue, incubating at 4°, or including a 10 μM concentration of the MAO inhibitor pargyline which is not specific at this concentration); blanks typically were less than 10% of total counts of product.

The deaminated products, 5-hydroxyindoleacetic acid and phenylacetic acid, were extracted into 5 ml of a mixture of toluene and ethyl acetate (5:2, v/v) and, after vigorous mixing and centrifugation at 1000 *g*, the aqueous phase was frozen on dry ice and the organic phase decanted into counting vials containing 8 ml of Liquiscint (National Diagnostics, Somerville, NJ), and counted in a Packard liquid scintillation spectrometer at 89% efficiency for ^{14}C. The ^{14}C-labeled deaminated products extracted and counted were >90% isochromatographic with authentic unlabeled compounds by paper and silica TL chromatography in butanol–acetic acid–water (25:4:10, by vol.).

Preliminary concentration isotherm experiments yielded values of apparent K_m for serotonin (MAO-A) of 188 and 142 μM for rat brain and liver and, for phenethylamine (MAO-B), of 5.0 and 10 μM for rat brain and liver respectively. Similar values were found with mouse liver (115 and 6.8 μM with MAO-A and MAO-B assays respectively). Inhibition functions were determined with at least six concentrations of each test agent, assayed in triplicate, and repeated at least twice. Data were fit to curves to permit determination of values for $IC_{50} \pm$ SEM by programs of DeLean *et al.* [19] adapted to a microcomputer [20], and converted to values of K_i based on preliminary determinations of apparent K_m in each tissue, evaluated (as illustrated in the control function in Fig. 1) using four to eight concentrations of each substrate in triplicate and pooling values of substrate K_d from at least two independent replications. The effects of inhibitors on substrate concentration isotherms were evaluated by determination of slope and y-intercept functions by linear, least-squares regression of [reaction velocity]$^{-1}$ vs [substrate concentration]$^{-1}$.

Materials used (see Table 1) included MPP iodide (Compound I), MPTP base (II), clorgyline-HCl, and L(-)deprenyl-HCl, all from Research Biochemicals (RBI, Natick, MA). Compounds III, V, and VI were prepared at Northeastern University as described elsewhere [21]. The *N*-ethylindolyl derivative AHR-1709 (IV) was donated by A.H. Robins (Richmond, VA). Pargyline-HCl was donated by Abbott Laboratories (North Chicago, IL). 2-Phenethylamine HCl was from ICN-K & K Biochemicals (Plainview, NY) and 5-hydroxytryptamine-creatinine sulfate (serotonin), 5-hydroxyindoleacetic acid, and phenylacetic acid were from Sigma Biochemicals (St. Louis, MO). Radiochemicals were [2-^{14}C]-5-hydroxytryptamine binoxalate (56.3 mCi/mmol) and [*ethyl*-1-^{14}C]-2-phenethylamine-HCl (50.2 mCi/mmol) from DuPont-New England Nuclear Corp. (Boston, MA).

Results and discussion

All six test compounds inhibited MAO activity in the rat brain mitochondrial preparation and all were selective versus MAO-A (Table 1); the most active versus rat brain MAO-A were MPP^+ and MPTP. MPP^+ was about 34 times more potent versus rat brain MAO-A than MAO-B; it was also 38 times more active versus rat liver MAO-A ($K_i \pm$ SEM = 0.56 ± 0.05 μM) than MAO-B (K_i = 21.4 ± 2.3 μM). MPTP and AHR-1709 also showed similar preference for MAO-A in rat liver (K_i of MPTP = 2.0 ± 0.2 μM vs MAO-A and 66.9 ± 5.1 μM vs MAO-B; K_i of AHR-1709 = 7.5 ± 0.5 μM vs MAO-A and 20.0 ± 1.7 μM vs MAO-B) as in rat brain (Table 1). Results obtained with mouse liver tissue showed a similar rank-order of drug potency as in rat tissues. For example, in mouse liver, the potencies (K_i) of MPP^+ and MPTP versus MAO-A were 1.0 and 5.2 μM.

The data in Table 1 suggest some tentative structure–activity relationships. The aromatic, quaternary, phenylpyridinium ion (MPP^+, Compound I) was the most potent anti-MAO-A agent (K_i = 0.75 μM), and was 4 times more potent than its partially hydrogenated congener MPTP (II) with rat brain tissue. The presence of a quaternary pyridine nitrogen atom, itself, did not provide high potency against MAO-A since the *N,N*-dimethyl analog (VI) of MPTP was 35 times *less* potent than MPP^+, and virtually inactive versus MAO-B. Adding a methyl substituent on the pyridine ring had little effect on MAO-A potency but markedly reduced

Table 1. Effect of substituted phenylpiperidines and phenylpyridines on rat brain mitochondrial MAO activity

	Compound	MAO-A K_i (μM)	MAO-B K_i (μM)
I	*N*-Methyl-4-phenylpyridinium iodide (MPP^+)	0.75 ± 0.06	25.7 ± 3.2
II	*N*-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	3.0 ± 0.3	16.9 ± 2.4
III	*N*-Methyl-4-phenylpiperidine	8.8 ± 0.7	56.9 ± 1.8
IV	1-[2-(3-Indolyl)ethyl]-4-phenyl-1,2,3,6-tetrahydropyridine (AHR-1709)	9.9 ± 0.7	51.7 ± 4.7
V	1,5-Dimethyl-4-phenyl-1,2,3,6-tetrahydropyridine	10.0 ± 0.7	127.0 ± 16.5
VI	*N*,*N*-Dimethyl-4-phenyl-1,2,3,6-tetrahydropyridine	26.0 ± 1.7	>500

Each experiment included four to six concentrations of test agent, each assayed in triplicate. Substrates were 100 μM serotonin and 5.5 μM phenethylamine for MAO-A and MAO-B assays respectively. Data are means ± SEM.

potency versus MAO-B (compare V and II in Table 1). The *N*-indolylethyl analog AHR-1709 (IV) also retained moderate activity versus MAO-A.

When inhibition of rat brain MAO-A by MPP^+ and MPTP was evaluated further in concentration isotherm experiments (Fig. 1), there were large increases in slopes with little or no change in y-intercept, indicating competitive inhibition versus brain MAO-A by MPTP and mainly competitive inhibition by MPP^+. The y-intercept (apparent [maximum velocity]$^{-1}$) with these agents increased very little with 6 μM MPTP (0.011 vs 0.013) and slightly more with 1.5 μM MPP^+ (0.016), but slope increased by 53% and 55% respectively (Fig. 1). Similar results were obtained with rat brain MAO-B (y-intercept = 0.035, 0.045, and 0.055 for control, 34 μM MPTP, and 51 μM MPP^+, respectively, while slope increased by 111 and 132% with these treatments). In further experiments, preincubation of the rat brain mitochondrial fraction for 30 min at 37° with 1 μM MPP^+ or 6 μM MPTP, separated by centrifugation followed by washing with a 100-fold excess fresh, drug-free buffer and recentrifugation, led to removal of ≥95% of the inhibition produced with unwashed tissue similarly treated (N = 4). These observations, taken together, support the impression that MPTP and MPP^+ are mainly competitive, reversible inhibitors selective for MAO-A.

The results of this study indicate that MPP^+ is a particularly potent inhibitor of MAO-A in rodent brain and liver tissue, as has been suggested previously [11, 12]. MPP^+ and MPTP appeared to inhibit MAO-A competitively and reversibly. No other compound tested was as potent as MPP^+ against MAO-A, and no test agent was found to be highly selective for MAO-B. The rank-order of anti-MAO potency of the agents tested in this study bore no significant correspondence to their other pharmacological effects. Thus, potencies of Compounds I–IV against brain MAO-A and MAO-B activities (Table 1) did not correlate with their potencies against uptake of [^{3}H]dopamine by striatal synaptosomes [21] ($r = -0.14$ and -0.10 respectively).

In conclusion, MPP^+ and MPTP are potent, selective, and mainly competitive and reversible inhibitors of MAO-A in rodent brain and liver tissue; other analogs showed similar but less potent effects, while none of the six compounds tested was highly selective against MAO-B. Other analogs of MPTP (especially 4-phenylpyridine and 4[4-chlorobenzyl]-pyridine) have been reported recently to have some selectivity against MAO-B [15]. The non-quaternary ammonium compounds tested (II, III, IV, and VI) may be able to penetrate the blood–brain diffusion barrier and compounds III, IV, and V (unlike compound II, MPTP) have been found *not* to deplete dopamine from mouse brain tissue after large doses given *in vivo* [21]. Since there are few reversible inhibitors selective for specific types of MAO, a search for potent, non-polar and non-toxic analogs of the phenylpiperidines tested as lead compounds seems worthy of further consideration.

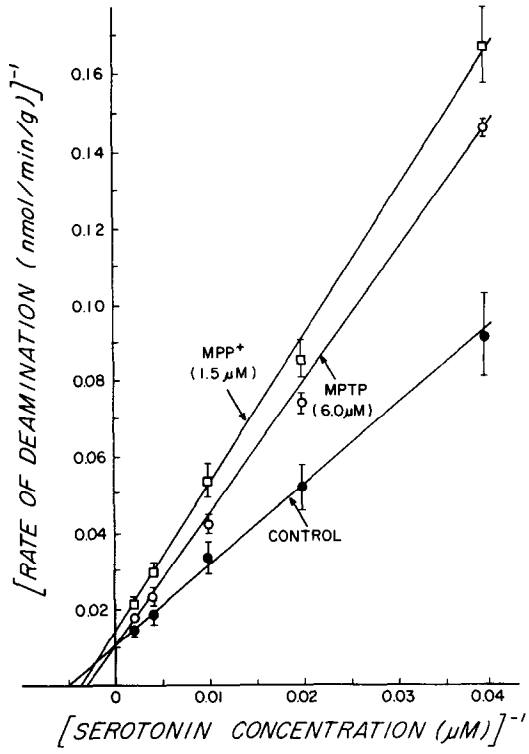

Fig. 1. Linearized analysis of inhibition of MAO-A by MPP^+ and MPTP at a range of substrate concentrations in a mitochondrial fraction of rat brain. [^{14}C]Serotonin (10–100 μM) was the substrate, and test agents were added at 1.5 or 6.0 μM. Data are means (±SEM) for three to six assays per point. The control condition (based on pooling six independent replications of the experiment) yielded values of apparent $K_m = 188$ μM and $V_{max} = 91.6 \pm 10.2$ nmol/min/g of original wet tissue (linear correlation, $r = +0.99$).

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Interaction of stanozolol with cytochrome P-450

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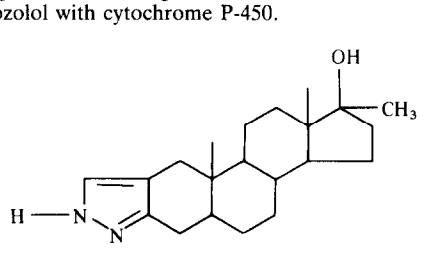
The biotransformation of drugs catalysed by cytochrome P-450 requires interaction of the drug with the enzyme as a substrate. This substrate-type interaction causes spectral changes of the oxidized form of cytochrome P-450 characterized by a trough at 420 nm and a peak at about 390 nm. The ligand type of interaction with cytochrome P-450, characterized by a trough at about 400 nm and a peak between 420 nm and 430 nm, has been found with lipophilic compounds possessing nitrogen or other hetero-atoms in the molecule which can coordinate the heme iron. Depending on binding affinity, compounds interacting as ligands to cytochrome P-450 can be strong inhibitors of the enzyme [1]. In our previous papers [2–5], we have shown that inhibition of the drug-metabolizing activity *in vivo* observed with H_2-receptor antagonist cimetidine can be attributed to its ligand binding properties caused by the structural features of the compound, i.e. by the presence of cyano and imidazole groups in the molecule [2, 4]. Studies of the interaction of drugs with cytochrome P-450 can provide information about the nature and mechanisms of the binding processes and give useful information when studying structural features of particular compounds.

Stanozolol, an anabolic steroid used for the treatment of aplastic anaemia [6], is also misused alone or in combinations by sportsmen, and the International Olympic Committee consider it a doping agent which is to be controlled in sport [7]. The very limited information on its *in vivo* and *in vitro* metabolism available as well as its interesting structural feature possessing, namely, the pyrazole ring as a potential ligand to cytochrome P-450 prompted us to investigate the nature of the interaction of Stanozolol with cytochrome P-450.



Stanozolol

Materials and methods

Preparation of liver microsomal fractions. The liver microsomal fractions from Sprague–Dawley rats (100–150 g) were prepared by differential centrifugation as described by Frommer *et al.* [8]. Phenobarbital was administered orally (1 mg/ml of drinking water) for one week.