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EFFECT OF DECARBOXYLASE INHIBITORS ON BRAIN *p*-TYROSINE LEVELS

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Abstract—A number of inhibitors of L-aromatic amino acid decarboxylase (AAD) and monoamine oxidase (MAO) were tested to determine whether they also inhibited tyrosine aminotransferase (TAT). The AAD inhibitors carbidopa, NSD-1015, NSD-1034 and Ro4-5127 inhibited liver TAT. Carbidopa inhibited brain AAD and liver TAT equally well. In contrast, other AAD inhibitors (Ro4-4602 and α-monofluoromethyldopa) did not inhibit TAT. Phenelzine, an MAO inhibitor, inhibited liver TAT, but other MAO inhibitors (tranylcypromine and isocarboxazid) did not. Systemic administration of those drugs that were found to be inhibitors of TAT *in vitro* caused significant increases in rat brain *p*-tyrosine levels.

Phenelzine (PLZ*, β-phenylethylhydrazine), an antidepressant, is usually thought to be an inhibitor only of monoamine oxidase (MAO) A and B [1]. Though this appears to be its most pronounced action, PLZ also inhibits aromatic L-amino acid decarboxylase (AAD, DOPA/5-HTP carboxy-lyase, EC 4.1.1.28) and tyrosine aminotransferase (TAT, L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) [2]. Intraperitoneal administration of PLZ to rats raises the brain levels of *p*-tyrosine [2]. This action was attributed to the inhibition of tyrosine transamination in the peripheral organs by PLZ. A number of other MAO inhibitors and AAD inhibitors are chemically similar to PLZ (see Fig. 1); thus, it is feasible that they too might be inhibitors of TAT.

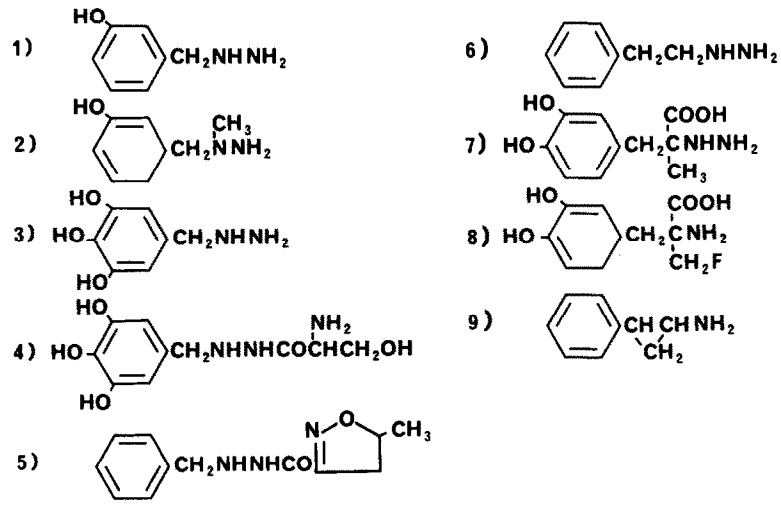
The purpose of the following study was 2-fold: (i) to determine whether other hydrazines, that are known to be inhibitors of either MAO or AAD, also inhibit TAT, and (ii) if so, to determine whether systemic administration of these drugs increases brain *p*-tyrosine levels.

MATERIALS AND METHODS

TAT assay. Liver obtained from Male Wistar rats (200–250 g) (Charles River Canada, Montreal, Quebec) killed between 11:30 a.m. and 1:30 p.m. was homogenized in 4 vol. of ice-cold potassium phosphate buffer (0.2 M, pH 7.6) containing 2.5 μM pyridoxal phosphate. The homogenate was centrifuged for 30 min at 2° at 30,000 *g*. The resulting supernatant fraction was stored at −70° and used for up to four weeks. TAT activity was assessed using a radiochemical assay [3, 4]. The reaction mixture for the assay contained 25 μl liver supernatant fraction, potassium phosphate buffer (0.2 M, pH 7.6), pyridoxal-5′-phosphate (2.5 μM), L-*p*-tyrosine (5.6 mM), L-*p*-tyrosine-[ring-3,5-^{3}H] (54.2 Ci/mmole, 1 μCi) and α-ketoglutaric acid (1.1 mM) in a total volume of 750 μl. The samples were preincubated for 20 min at 37° before the addition of α-ketoglutaric acid. After this, the samples were incubated for 20 min. TAT activity under these conditions is linear for a least 30 min and up to 100 μl enzyme. The enzyme reaction was stopped by the addition of 100 μl of 15 mM 2,4-dinitrophenylhydrazine in 1 M H_2SO_4. After 20–30 min, the hydrazone derivative of *p*-hydroxyphenylpyruvic acid was extracted into 750 μl toluene–ethyl acetate (10:1, v/v). The organic layer was aspirated and discarded. The organic layer was washed with 500 μl of 0.3 M HCl, removed and added to 10 ml counting solution (4 g Omnifluor/l toluene), and radioactivity was assessed by liquid scintillation spectrometry. Blanks using enzyme that had been placed in a boiling water bath for 5 min were run.

AAD assay. Whole rat brains were homogenzed in 10 vol. of ice-cold sodium phosphate buffer (0.05 M, pH 8.0) containing 7.5 μM pargyline hydrochloride. The homogenate was centrifuged at 2° for 30 min at 30,000 *g*, and the resulting supernatant fraction was decanted and stored at −70° for up to 4 weeks. AAD activity was assayed radiochemically [5]. The incubation mixture consisted of 100 μl brain supernatant fractions, sodium phosphate buffer (0.05 M, pH 8.0), pyridoxal-5′-phosphate (7.5 μM), DL-5-[3-^{14}C]hydroxytryptophan (59 mCi/mole, 3.6 μM) and 5-hydroxytryptamine-[G-^{3}H]creatinine sulfate (500 mCi/mmole, 3.2 nCi added to determine recovery) in a total volume of 500 μl. The samples were preincubated at 37° for 15 min prior to the addition of the substrate. Under these conditions, AAD activity is linear with up to 300 μl enzyme and up to 30 min of incubation [2]. After this addition, samples were incubated for 10 min. The reaction was terminated by the addition of 500 μl of ice-cold, pH 6.0, sodium phosphate buffer; 500 μl of 0.1 M diethylhexylphosphoric acid in $CHCl_3$ was added, and the mixture was shaken. The aqueous layer (containing the substrate) was discarded, and the chloroform layer (containing the extracted serotonin) was

* Abbreviations: AAD, L-aromatic amino acid decarboxylase; Car, carbidopa; Isocar, isocarboxazid; MAO, monoamine oxidase; MFMD, α-monofluoromethyldopa, PLZ, phenelzine; and TAT, tyrosine aminotransferase.



1) NSD-1015
2) NSD-1034
3) Ro4-5127
4) Ro4-4602
5) Isocarboxazid
6) Phenelzine
7) Carbidopa
8) α-Monofluoromethyldopa
9) Tranylcypromine

Fig. 1. Chemical structures of the drugs used.

washed with 500 μl sodium phosphate buffer, pH 6.0; then the radioactivity in the chloroform layer was assessed by liquid scintillation spectrometry. Enzyme blanks were run in each experiment using samples that had been placed in a boiling water bath for 5–6 min before the AAD assay.

p-Tyrosine and tryptophan determinations. p-Tyrosine and tryptophan concentrations in the rat brains were measured using reverse phase high pressure liquid chromatography with electrochemical detection (HPLC–ECD) [6]. Tissues were homogenized in 0.1 N perchloric acid containing 0.1 mM $Na_2S_2O_5$, 0.25 mM EDTA and a known amount of isoproterenol as internal standard. The homogenate was centrifuged for 15 min at room temperature in a high speed microcentrifuge (Fisher, model 235B). *p*-Tyrosine levels in the supernatant fractions were detected on a carbon paste electrode (model TL-3, Bioanalytical Systems, West Lafayette, IN) set at 0.9 V versus an Ag/AgCl reference electrode. The mobile phase used in this system was composed of 75 mM NaH_2PO_4, 1 mM sodium octyl sulfate, 0.5 mM disodium EDTA, 12% acetonitrile adjusted to pH 2.8 with phosphoric acid. Samples of the drugs were also run to check for possible interference.

Drugs. PLZ sulfate and NSD-1015 were purchased from the Sigma Chemical Co., St. Louis, MO, and tranylcypromine hydrochloride (TCP) from ICN Biochemicals, Plainview, NY. The following were donated by the indicated companies: α-monofluoromethyldopa (MFMD, Merrell International, Strasbourg, France); isocarboxazid (Isocar), Ro4-4602 and Ro4-5127, Hoffmann-LaRoche Ltd., Etobicoke, Ontario; Carbidopa (Car), Merck Frosst Canada, Dorval, Quebec; and NSD-1034 (Sandev Ltd., Essex, UK). When preparing drug solutions for the enzyme assays, it was necessary to dissolve isocarboxazid in a small volume of 1 N NaOH and, similarly, MFMD and carbidopa were dissolved in a minimum volume of 1 N HCl. The appropriate buffer was then added and the pH of the solutions adjusted, if necessary, before the solutions were brought up to the desired volumes.

RESULTS

Inhibition of liver TAT. A number of drugs that are known to be inhibitors of either MAO or AAD were tested to determine whether they also inhibited TAT (see Fig. 1 for chemical structures). Carbidopa was found to be the most potent inhibitor of TAT. The concentration required to inhibit TAT to 50% of control activity (IC_{50}) was 3.2 μM (Fig. 2). Several other AAD inhibitors also inhibited TAT, though they were less potent than carbidopa. Their IC_{50} values were: 30 μM for NSD-1015, 80 μM for NSD-1034 and 50 μM for Ro4-5127. The MAO inhibitor, PLZ, had a similar potency (IC_{50} = 60 μM). The other drugs tested were either very weak or inactive TAT inhibitors. The IC_{50} values were: MFMD, 1 mM; isocarboxazid, 1 mM; Ro4-4602, 0.9 mM; and TCP, >30 mM.

Inhibition of brain AAD. Most of the drugs tested are presumed to be primarily inhibitors of AAD. To determine their relative potencies in inhibiting AAD and TAT, their IC_{50} values for inhibiting AAD were determined. NSD-1015 was the most potent inhibitor of AAD. Its IC_{50} was 0.015 μM (Fig. 3). Ro4-5127, MFMD, Ro4-4602, NSD-1034 and carbidopa were also potent AAD inhibitors. Their respective IC_{50} values were: 0.08, 0.15, 0.24, 0.32 and 1.0 μM.

PLZ inhibited AAD with an IC_{50} of 18 μM. Very high concentrations of the MAO inhibitors isocarboxazid and TCP were required to inhibit AAD.

Comparison of the IC_{50} values. The most striking finding was that carbidopa inhibited TAT and AAD

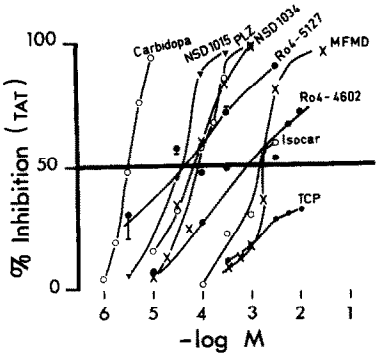


Fig. 2. Percent inhibition of liver TAT by the various Isocar drugs. Values are mean ± SEM (N = 6). Abbreviations: Isocar, isocarboxazid; TCP, tranylcypromine; PLZ, phenelzine; and MFMD, α-monofluoromethyldopa.

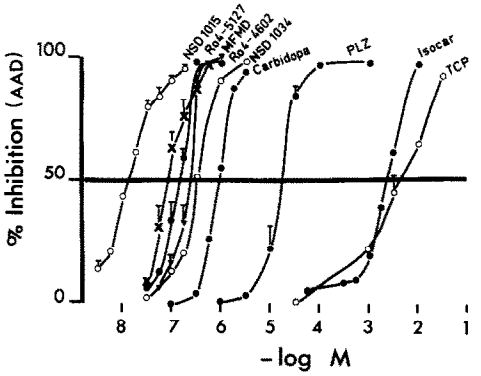


Fig. 3. Percent inhibition of brain AAD by the various drugs. Values are mean ± SEM (N = 6). Abbreviations: Isocar, isocarboxazid; TCP, tranylcypromine; PLZ, phenelzine; and MFMD, α-monofluoromethyldopa.

in vitro equally well. The other AAD inhibitors that were reasonably potent TAT inhibitors were also monosubstituted hydrazines, whereas the doubly substituted hydrazines (Ro4-4602 and isocarboxazid) were poor inhibitors of TAT *in vitro*. Similarly, TCP and MFMD, which are not hydrazines, were not innibitors of TAT.

Brain p-tyrosine and tryptophan levels. The amounts of *p*-tyrosine present in the rat brain 2 hr after intraperitoneal injections of 0.1 mmole/kg of carbidopa, Ro4-4602, Ro4-5127 or MFMD were compared to control (Table 1). It can be seen that brain *p*-tyrosine levels were increased significantly (2- to 3-fold) by carbidopa, Ro4-4602 and Ro4-5127. In contrast, MFMD did not alter *p*-tyrosine levels. Tryptophan levels were not affected by administration of any of the AAD inhibitors.

Samples of Ro4-4602, Ro4-5127, carbidopa and MFMD were also analyzed by the HPLC–ECD method to assess possible interference. MFMD was detected very early in the run (retention time <4 min compared to tyrosine, 5.26 min and tryptophan, 30.6 min); thus, it could not be mistakenly identified as *p*-tyrosine or tryptophan. Similarly, carbidopa was not problematic (retention time, 6.11 min). In the brain samples, either a very small or no carbidopa peak was observed. Ro4-5127 was detected close to, but was resolvable from, *p*-tyrosine (4.92 vs 5.26 min). No Ro4-5127 peak was detected in the brain samples. On the other hand, Ro4-4602 was detected at 5.34 min and was not separated from *p*-tyrosine (5.26 min). Hence, this *p*-tyrosine peak could potentially be a mixture of these two compounds; however, since virtually none of the other AAD inhibitors (injected in equimolar doses) was detected in the brain samples, such a possibility seems unlikely. Nevertheless, to be certain that Ro4-4602 was not contributing to the *p*-tyrosine peak from brain samples the procedure was modified by subjecting the samples to strongly alkaline conditions under which di- and trihydroxybenzyl compounds decompose. Samples (of brain and of the drugs) were homogenized in 6 ml of 0.1 N perchloric acid; then, 2 ml of 1 N NaOH was added. Fifteen minutes later, 2 ml of 1 N HCl was added followed by 10 ml of 0.1 N perchloric acid containing $Na_2S_2O_5$, EDTA and isoproterenol, as before. The internal standard, isoproterenol, was added after the HCl so that it would not be oxidized by the alkaline treatment. When the samples were analyzed, the retention time of isoproterenol was not changed. The profile of Ro4-4602 was different from before, illustrating that the alkaline treatment had oxidized it. Similarly, dopamine in the brain samples was destroyed. Samples of Ro4-4602 no longer contained a peak that co-chromatographed with *p*-tyrosine. The *p*-tyrosine levels in brain samples were not different when they were treated with alkali; therefore, it is clear that the drugs did not interfere with the quantification of brain *p*-tyrosine.

Table 1. Effects of inhibitors of AAD on rat brain *p*-tyrosine levels

Treatment	*p*-Tyrosine (μg/g)	Tryptophan (μg/g)
Saline	9.7 ± 0.5	3.63 ± 0.32
Carbidopa	19.7 ± 3.1*	4.03 ± 0.26
Ro4-4602	31.5 ± 2.8*	3.63 ± 0.08
Ro4-5127	21.3 ± 2.7*	4.44 ± 0.54
MFMD	11.6 ± 0.7	3.94 ± 0.27

Values are mean ± SEM (N = 6). Rats were injected with AAD inhibitors (0.1 mmole/kg) 2 hr before being killed. The data were analyzed by one-way analysis of variance followed by Dunnett's *t*-test

* $P < 0.05$ compared to saline treatment.

DISCUSSION

The foregoing results document the efficacy of various presumed MAO and AAD inhibitors as inhibitors of TAT *in vitro*. The few previous studies [7, 8] which were interested in similar problems adopted an indirect approach and injected the presumed AAD inhibitor into an animal and then measured the effect on enzymatic activity *in vitro*. Burkard *et al.* [7] reported that 3 mmoles/kg Ro4-4602 decreased liver TAT activity (though the data were not shown). Similarly, Moran and Sourkes [8] surmised from their data that carbidopa (0.4 mmole/kg) inhibits TAT *in vivo*. Another study [9] noted (data not shown) that 0.17 mM Ro4-4602 inhibits rat liver TAT *in vitro* to about 50% of control

activity. The present report clearly confirms these earlier reports and extends the list of TAT inhibitors to include NSD-1015, NSD-1034, PLZ and Ro4-5127 (the active metabolite of Ro4-4602 [7]). Furthermore, it shows that MFMD can be used experimentally to inhibit AAD without any complicating effects on TAT. While the earlier studies indicated that some AAD inhibitors could also inhibit TAT, the evidence was not as substantial as the present report. Furthermore, the present report compares the abilities of the various inhibitors to inhibit AAD and TAT to obtain some idea of relative selectivity. This study quite clearly demonstrates that carbidopa was as good an inhibitor of TAT as it was of AAD. The other drugs which inhibited both TAT and AAD did so with much less efficacy for TAT than AAD compared to carbidopa.

The findings presented here have important implications with respect to published and prospective studies. It is clearly necessary to take into account the significant TAT inhibitory properties of several presumed AAD inhibitors, especially carbidopa, when planning experiments designed to inhibit AAD specifically. Similarly, the interpretation of published material can be made clearer with this new information. It has already been outlined concisely and elegantly that the failure to take into account the inhibition of TAT by carbidopa and Ro4-4602 can be misleading [8, 9]. Such an oversight has led to the erroneous conclusion that decarboxylation of tyrosine is a major pathway [10]. Decarboxylation of tyrosine by AAD is a very slow process and represents only a minor portion of its overall metabolism [8, 9, 11–13].

Further studies are necessary to elucidate the mechanism of inhibition of TAT by these hydrazine drugs. It seems likely that they interact with pyridoxal-5′-phosphate, the coenzyme for TAT.

The second major finding presented here is that the levels of *p*-tyrosine in the brain can be increased by the systemic administration of carbidopa, Ro4-4602 and Ro4-5127. Since transamination of *p*-tyrosine is a major metabolic pathway in peripheral tissues, it appears likely that inhibition of TAT increased the circulating level of *p*-tyrosine which, in turn, increased the transport of this amino acid into the brain. Clearly, inhibition of AAD alone by injection of MFMD had no such effect. It may, at first sight, seem surprising that Ro4-4602 increased brain *p*-tyrosine levels, since it was a poor TAT inhibitor *in vitro*. However, Ro4-4602 is the seryl derivative of Ro4-5127 (see Fig. 1) and is hydrolyzed *in vivo* to produce Ro4-5127, the active metabolite [7], which inhibited TAT *in vitro*. In fact, Ro4-4602 increased brain *p*-tyrosine levels to a significantly (Newman–Keuls test) greater extent than Ro4-5127. While one might have expected that Ro4-5127 would increase *p*-tyrosine levels to the same or even to a greater extent than Ro4-4602, since Ro4-5127 is the active metabolite, this expectation is probably too simplistic. It does not take into account the rate of formation of Ro4-5127 from Ro4-4602, the rate of degradation of Ro4-5127, and the possibility that Ro4-5127 is formed *in vivo* in a place devoid of, or deficient in, drug-metabolizing enzyme systems.

The finding that hydrazine AAD inhibitors can increase brain *p*-tyrosine levels has some implications experimentally and clinically. For example, in experiments designed to study whether the rate of catecholamine synthesis in the brain varies with the amount of *p*-tyrosine available, it would undoubtedly be best to use a dopa decarboxylase inhibitor devoid of TAT inhibitory properties. If peripheral TAT were inhibited, brain *p*-tyrosine levels would increase, and this increase might saturate tyrosine hydroxylase before the experimental insult (more *p*-tyrosine) is delivered. Thus, one could falsely conclude that catecholamine synthesis could not be increased by administering additional *p*-tyrosine. It has been suggested, however, that the availability of *p*-tyrosine does control catecholamine synthesis and that this precursor dependency is more pronounced in those nigrostriatal neurons that survive after partial lesions of the nigrostriatal pathway and in Parkinson's disease [14–16]. Perhaps the use of Ro4-4602 and carbidopa as adjuvants in the treatment of Parkinson's disease involves not only the protection of L-DOPA from decarboxylation in the periphery but also involves the provision of more *p*-tyrosine to the brain.

In summary, the present report shows that (1) many inhibitors of AAD are also inhibitors of TAT and (2) intraperitoneal administration of these drugs increased the brain levels of *p*-tyrosine. These findings are relevant to past and proposed studies where the abilities of these presumed AAD inhibitors to inhibit TAT must also be taken into account.

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