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INHIBITION OF RAT LIVER TRYPTOPHAN PYRROLASE ACTIVITY AND ELEVATION OF BRAIN TRYPTOPHAN CONCENTRATION BY ADMINISTRATION OF DL-α-AMINO-β-PYRIDINEPROPANOIC ACID (PYRIDYLALANINE) ANALOGS

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Single doses of DL-α-amino-β-(2-pyridine)propanoic acid (2-PA, 100 mg/kg) significantly decreased the holoenzyme and apoenzyme activities of rat liver tryptophan pyrrolase (TP) and increased brain tryptophan, serotonin (5-HT) and 5-hydroxyindole-3-ylacetic acid concentrations. 2-PA had no inhibitory effect on either of the enzyme activities *in vitro*, but its expected metabolites were effective. Single doses of DL-α-amino-β-(3-pyridine)propanoic acid (3-PA, 100 mg/kg) decreased only the holoenzyme activity and elevated brain tryptophan and its metabolites levels in rats. 3-PA and its metabolite, 3-pyridylpyruvate, inhibited only the holoenzyme activity *in vitro*. DL-α-Amino-β-(4-pyridine)propanoic acid (4-PA) caused significant changes in liver TP (holo- and apoenzyme forms) activity and brain tryptophan concentration only after repeated administration (100 mg/kg/day). 4-PA was a weak inhibitor of the holoenzyme, but its metabolites apparently inhibited the holo- and apoenzyme activities *in vitro*. These findings suggest that PA analogs (and/or their metabolites) increased brain tryptophan (and hence 5-HT synthesis) by directly inhibiting liver TP activity.

KEY WORDS: DL-α-Amino-β-pyridinepropanoic acid analogs, liver tryptophan pyrrolase inhibition, increases in brain tryptophan and serotonin.

INTRODUCTION

Tryptophan is mainly metabolized *via* the kynurenine-anthranilate pathway, which is initiated by liver tryptophan pyrrolase (EC 1.13.11.11, TP). This enzyme is quantitatively the most important tryptophan-degrading enzyme in man and rat.[1,2] Tryptophan is the precusor of the neurotransmitter, serotonin (5-HT), and has been used for several years in the medical treatment of depression based on evidence that brain 5-HT or its turnover is reduced in this disorder.[3,4] Furthermore, serotonergic neurons are likely to be involved in blood pressure regulation since histochemical evidence has revealed that neuronal cell bodies and nerve terminals containing 5-HT are located in brain areas known to be cardiovascular regulatory centers.[5] However, the efficacy of tryptophan in depression[6–8] and blood pressure regulation[9,10] is still controversial. A problem of tryptophan treatment is that large amounts of tryptophan are metabolized

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by TP to kynurenine in the liver, so that relatively little is available for the synthesis of brain 5-HT.[11] Therefore, inhibition of the activity of liver TP, which is induced by its substrate, tryptophan, may increase 5-HT synthesis in the brain.

We have previously reported that a synthetic amino acid, DL-α-amino-β-(3-pyridine)propanoic acid (DL-3-pyridylalanine, 3-PA) decreased liver TP total enzyme activity and significantly increased brain 5-HT concentration in rats,[12] and that the combined administration of tryptophan with 3-PA caused a significant increase in rat brain 5-HT compared to a single administration of tryptophan.[13] Furthermore, we have shown that allopurinol and nicotinamide, which have been suggested to have potential use as *in vivo* TP inhibitors and have been used with tryptophan to treat depression,[14,15] did not increase brain 5-HT levels after administration with tryptophan.[13] However, the effects of the other PA analogs, DL-α-amino-β-(2-pyridine)-propanoic acid (2-PA) and DL-α-amino-β-(4-pyridine)propanoic acid (4-PA), on liver TP activity and brain 5-HT concentration have not been investigated. We therefore performed a detailed investigation of the effects of PA analogs on the various activities of liver TP (holoenzyme, apoenzyme and total enzyme) *in vitro* and *in vivo* and on brain tryptophan concentration and metabolism, as a part of the development of a potent TP inhibitor. The present paper also describes the effects of expected metabolites of PA analogs on TP activity *in vitro* and discusses them with relation to the mechanisms of action of PA analogs.

MATERIALS AND METHODS

Chemicals

2-PA, 3-PA and 4-PA were synthesized by the method of Nieman *et al.*[16] 2-Pyridylacetic acid hydrochloride, 2-(2-aminoethyl)pyridine and 3-pyridylacetic acid hydrochloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). 3-Pyridylpyruvic acid and 3-(2-aminoethyl)pyridine hydochloride were gifts from Dr Y. Tomioka, Fukuoka University, Japan. 4-(2-Aminoethyl)pyridine, 4-pyridylacetic acid sodium salt, 5-hydroxyindole-3-ylacetic acid (5-HIAA) and L-tryptophan were from Jansen Chimica (Beerse, Belgium), Tokyo Kasei (Tokyo, Japan), Sigma (St. Louis, MO) and Kyowa Hakko Kogyo (Tokyo, Japan), respectively. Bovine blood hematin and 5-HT creatinine sulfate were purchased from E. Merck (Darmstadt, West Germany). All other chemicals were of the purest grade available from Wako Pure Chemicals (Osaka, Japan).

Animals and Drug Treatments

Male Wistar rats (140–160 g), obtained from the Animal Center of Fukuoka University, were housed in a room maintained at 23 ± 1°C with a strictly controlled 12 h diurnal lighting cycle (7:00 a.m.–7:00 p.m.). When tested *in vitro*, PA analogs and their expected metabolites were dissolved in 0.2 M potassium phosphate buffer, pH 7.4. When examined *in vivo*, PA analogs were dissolved in 0.9% NaCl and were neutralized (pH 7.4) before subcutaneous injection. When the drug was given repeatedly, the injection was carried out once daily. Control animals were given an equal volume (5 ml/kg) of saline alone. The animals were killed by decapitation at the same time of day (2:00–2:30 p.m.) to minimize the influence of possible diurnal variation. Brain and

other tissues were dissected out rapidly, washed in ice-cold saline, blotted dry and immediately used for assay.

Enzymic and Other Determinations

TP activity was determined in fresh liver homogenate either in the absence (holoenzyme activity) or in the presence (total enzyme activity) of 2 μM hematin.[17] The apoenzyme activity was calculated from the difference between total and holoenzyme activities. The holoenzyme and apoenzyme are the heme-containing and heme-free forms of TP in rats, respectively, and the activity of the total enzyme is the sum of the activities of the other two forms. For the determination of the inhibition constant (K_i) of PA analogs, the substrate (tryptophan) was varied in the range 0.2–1.2 mM and PA analogs were kept at a constant concentration (12 mM). L-Tryptophan 5-hydroxylase (EC 1.14.16.4), 5-hydroxy-L-tryptophan (5-HTP) decarboxylase (EC 4.1.1.28) and monoamine oxidase (EC 1.4.3.4, MAO) were determined as described preivously.[12] Brain tryptophan and 5-hydroxyindole acetic acid (5-HIAA) concentrations were determined fluorometrically by the method of Bloxam and Warren[18] and the method of Curzon and Green,[19] respectively. Brain and the other tissue 5-HT concentrations were measured as described previously.[12]

Statistical Analysis

Statistical analysis of results was performed by the use of Student's t test with $p < 0.05$ considered significant.

RESULTS

Effects of Administration of PA analogs on Rat Liver TP Activity

The effects of PA analogs on the activity of TP in rat liver at 24 h after administration are shown in Figure 1. Liver TP holoenzyme and total enzyme activities decreased at 50 mg/kg or more of 3-PA, with statistically significant decreases at the 100 and 150 mg/kg doses. However, the apoenzyme activity was not altered by any of the doses of 3-PA. On the other hand, 2-PA caused a significant decrease in liver TP apoenzyme activity at the 100 or 150 mg/kg dose. Significant decreases of the holoenzyme and total enzyme activities were also observed with 50, 100 and 150 mg/kg of 2-PA. All forms of liver TP activity slightly decreased after the administration of 4-PA, but not significantly.

The time courses of the effects of PA analogs (each 100 mg/kg) on liver TP activity are shown in Figure 2. Two hours after administration of 2-PA or 3-PA, significant decreases in liver TP holoenzyme and total enzyme activities were observed. These decreases in the enzyme activities were maintained throughout the duration of the experiment. The apoenzyme activity significantly decreased at 2 h after the administration of 2-PA, but the enzyme activity returned to the control level at 48 h. In contrast, 3-PA had little effect on the apoenzyme at any of the times examined. No significant change in liver TP activity was found in rats given 4-PA at any of the times examined.

Figure 3 shows the effects of repeated administration of PA analogs (100 mg/kg/

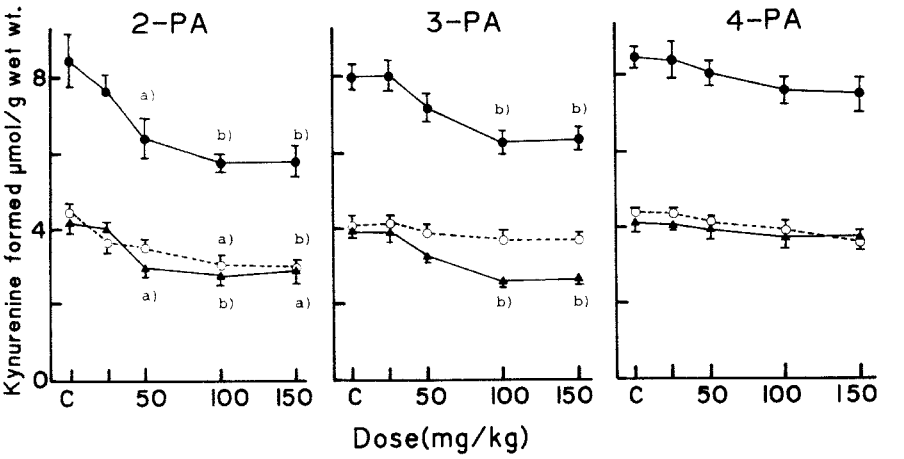

FIGURE 1 Effects of various doses of PA analogs on rat liver tryptophan pyrrolase (TP) activity. Rats were sacrificed 24 h after receiving a subcutaneous injection of saline (control) or various doses of PA analogs (25–150 mg/kg). Liver TP activity was determined either in the absence (holoenzyme activity, ▲——▲) or in the presence (total enzyme activity, ●——●) of added hematin. The apoenzyme activity (O- - - - -O) was calculated by difference (total activity − holoenzyme activity). Points represent the means ± S.E.M. for 6 animals per group. Statistical significance of differences compared to controls; (a) $p < 0.05$, (b) $p < 0.01$.

day) on liver TP activity. The enzyme activity was determined at 24 h after the last injection. Liver TP activity in chronically 2-PA- or 3-PA-treated rats was not statistically different from that in the animals that had received a single dose of 2-PA or 3-PA. The repeated administration of 4-PA (for 5 and 7 days) caused significant decreases in liver TP holo-, apo- and total enzyme activities as compared with control levels. The effect of 4-PA increased on increasing the number of administrations.

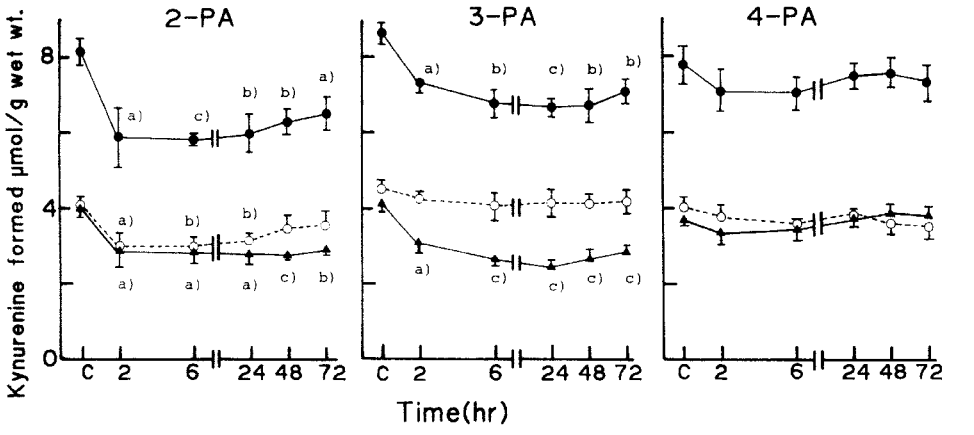

FIGURE 2 Time courses of effects of PA analogs on rat liver TP activity. Rats were sacrificed at various times following a subcutaneous injection of 100 mg/kg dose of PA analogs. TP holoenzyme (▲——▲), apoenzyme (O- - - - -O) and total enzyme (●——●) activities were assayed as described in the legend to Figure 1. Points represent the means ± S.E.M. for 6 animals per group. Statistical significance of differences compared to controls; (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$.

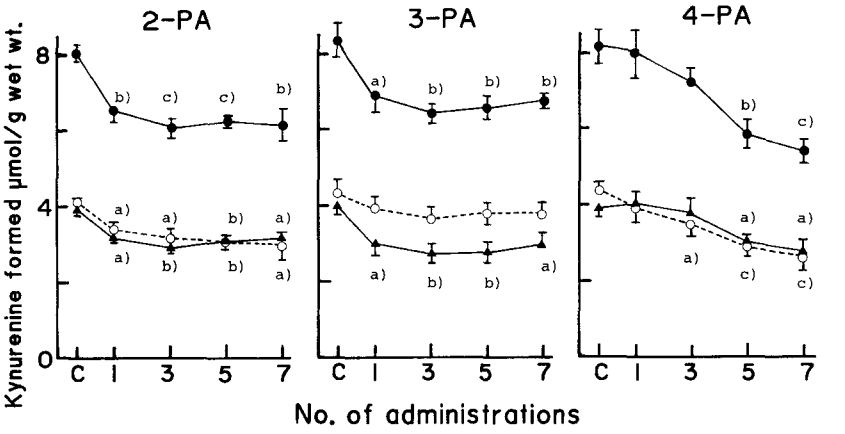

FIGURE 3 Effects of repeated daily administration of PA analogs on rat liver TP activity. Control rats were injected with 0.9 % NaCl. When PA analogs (100 mg/kg/d, each) were given repeatedly, subcutaneous injection was carried out once daily. TP activity was assayed at 24 h after the last injection of PA analogs. The symbols are the same as described in the legend to Figure 1. Points represent the means ± S.E.M. for 6 animals per group. Statistical significance of differences compared to controls; (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$.

Effects of Administration of PA Analogs on Rat Brain Tryptophan, 5-HT and 5-HIAA Concentrations

Figure 4 shows the concentrations of brain tryptophan, 5-HT and 5-HIAA in rats given 2-PA, 3-PA or 4-PA at four dose levels. Brain tryptophan, 5-HT and 5-HIAA significantly increased at the doses of 100 and 150 mg/kg of 2-PA or 3-PA. In contrast, 4-PA had little effect on the concentrations of these compounds in the brain even at a dose of 150 mg/kg.

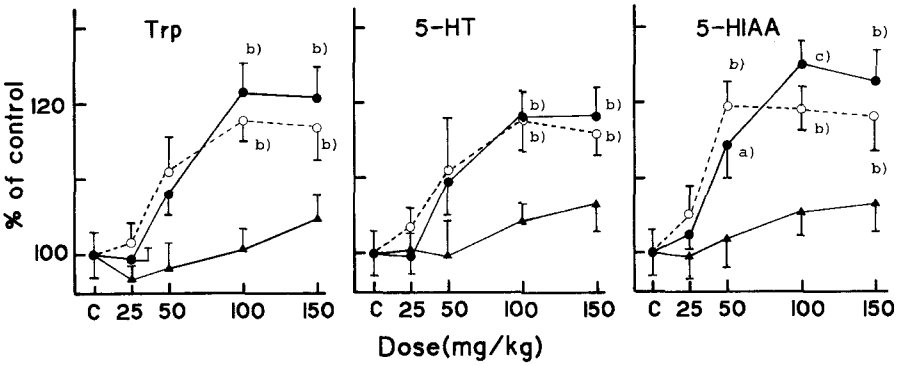

FIGURE 4 Effects of various doses of PA analogs on rat brain tryptophan (Trp), serotonin (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) concentrations. Rats were injected with 2-PA (O- - - - -O), 3-PA (●——●) or 4-PA (▲——▲) as described in the legend to Figure 1. Brain Trp, 5-HT and 5-HIAA concentrations were determined at 24 h after the administration of PA analogs. Points represent the means ± S.E.M. (6 animals per group). Statistical significance of differences compared to controls; (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$. Control values (μg/g wet wt.) are as follows; Trp 4.04 ± 0.12, 5-HT 0.406 ± 0.012, 5-HIAA 0.446 ± 0.014.

As shown in Figure 5, 2-PA or 3-PA (100 mg/kg) caused a significant increase in brain tryptophan levels at 2 h after administration, and the effect was still apparent at 72 h. A similar pattern was seen in the concentrtions of 5-HT and 5-HIAA in the brain. 4-PA (100 mg/kg) did not elevate the concentrations of brain tryptophan and its metabolites at any of the time intervals examined.

The repeated administration of 2-PA or 3-PA (100 mg/kg) did not produce any further increases in brain tryptophan, 5-HT and 5-HIAA concentrations compared with those in animals given a single dose of 2-PA or 3-PA (Figure 6). On the other hand, the concentrations of brain tryptophan, 5-HT and 5-HIAA in rats administered 4-PA (100 mg/kg) for 5 or 7 days were significantly higher than those in animals injected with saline or a single dose of 4-PA.

Although the data are not shown here, L-tryptophan 5-hydroxylase, 5-HTP decarboxylase and MAO activities in the brain were not significantly changed by the administration of PA analogs as compared with those in control animals.

Effects in Vitro of PA Analogs and Their Expected Metabolites on Rat Liver TP Activity

PA analogs and their expected metabolites were examined *in vitro* for a possible direct inhibitory effect on liver TP activity. As shown in Table I, 3-PA decreased the total enzyme activity without altering the apoenzyme activity which demonstrated that this compound specifically inactivated only the holoenzyme. As shown in Figure 7, 3-PA competitively inhibited the holoenzyme activity and its K_i value was 6.5 mM calculated from a Lineweaver-Burk plot. 3-Pyridylpyruvic acid, which may be produced by the deamination of 3-PA, also inhibited only the holoenzyme activity. The extent of this inactivation by 3-pyridylpyruvic acid was almost the same as that shown by 3-PA. On the other hand, 3-(2-aminoethyl)pyridine, the decarboxylated compound of 3-PA, decreased only the apoenzyme activity, but its effect was very weak. Surprisingly, 2-PA had no inhibitory effect on the holoenzyme and apoenzyme activities *in vitro*. In contrast, 2-(2-aminoethyl)pyridine apparently decreased both enzyme activities.

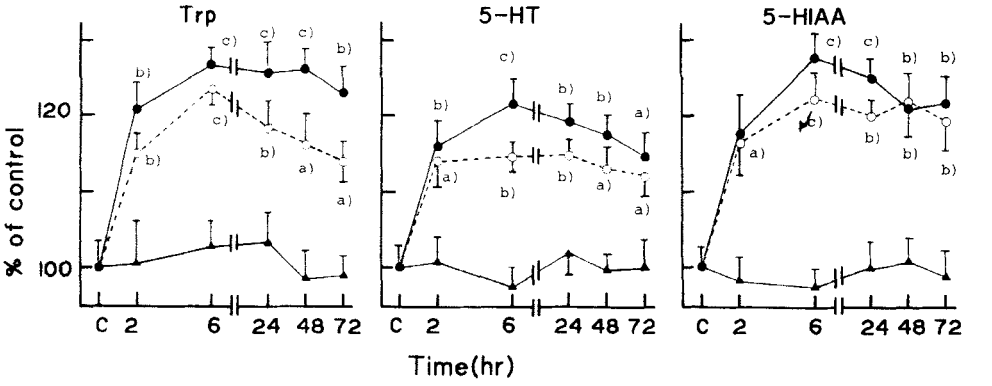

FIGURE 5 Time courses of effects of PA analogs on the concentrations of brain Trp and its metabolites. Rats were injected with 2-PA (○- - - -○), 3-PA (●——●) or 4-PA (▲——▲) as described in the legend to Figure 2. Points represent the means ± S.E.M. (6 animals per group). Statistical significance of differences compared to controls: (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$. Control values (μg/g) are as follows: Trp 3.85 ± 0.14, 5-HT 0.410 ± 0.013, 5-HIAA 0.396 ± 0.013.

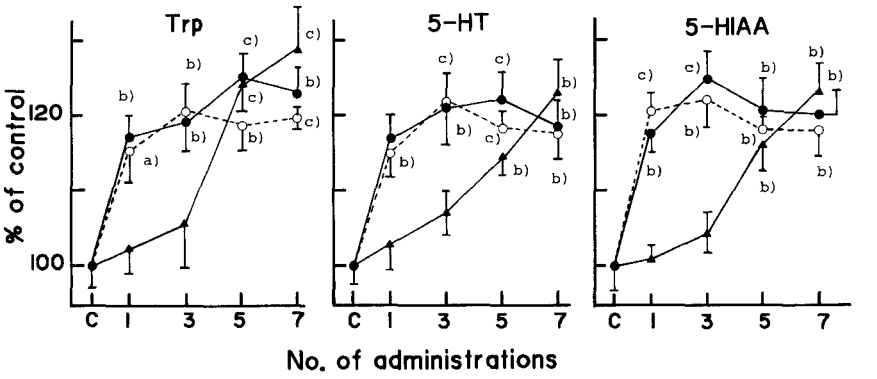

FIGURE 6 Effects of repeated administration of PA analogs on the concentrations of Trp and its metabolites. Rats were treated with 2-PA (O------O), 3-PA (●——●) or 4-PA (▲——▲) as described in the legend to Figure 3. Points represent the means ± S.E.M. (6 animals per group). Statistical significance of differences compared to controls; (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$. Control values (μg/g) are as follows; Trp 3.97 ± 0.11, 5-HT 0.383 ± 0.010, 5-HIAA 0.423 ± 0.014.

2-Pyridylacetic acid, which may be produced by the decarboxylation of 2-pyridylpyruvic acid and by the oxidation of 2-(2-aminoethyl)pyridine, was a weak inhibitor of the holoenzyme and apoenzyme. 4-PA competitively inhibited only the holoenzyme activity (data not shown), but its inhibitory effect (K_i = 26.5 mM) was considerably weaker than that of 3-PA. Liver TP apoenzyme activity was not inhibited by 4-PA itself. However, the expected metabolites of 4-PA, 4-(2-aminoethyl)pyridine and 4-pyridylacetic acid apparently inhibited the apoenzyme and holoenzyme activities.

DISCUSSION

The results shown in Figure 1–3 demonstrate the ability of PA analogs to decrease liver TP activity in rats. 3-PA competitively inhibited TP holoenzyme activity *in vitro* (Figure 7). Therefore, the inhibitory effect of 3-PA on liver TP *in vivo* is probably due

TABLE I

Effects of PA analogs and their expected metabolites on liver TP activity *in vitro*. The data are based on single experiments in which 10 mM of each compound was tested and compared to TP activity in the absence of the compound. The concentration of tryptophan was 1.0 mM

Compounds	% inhibition		
	Holoenzyme	Apoenzyme	Total enzyme
DL-2-PA	0	0	0
2-Pyridylacetic acid	5	8	7
2-(2-Aminoethyl)pyridine	40	18	28
DL-3-PA	46	3	23
3-Pyridylacetic acid	4	2	3
3-Pyridylpyruvic acid	42	3	21
3-(2-Aminoethyl)pyridine	0	9	5
DL-4-PA	18	4	11
4-Pyridylacetic acid	28	44	39
4-(2-aminoethyl)pyridine	26	21	23

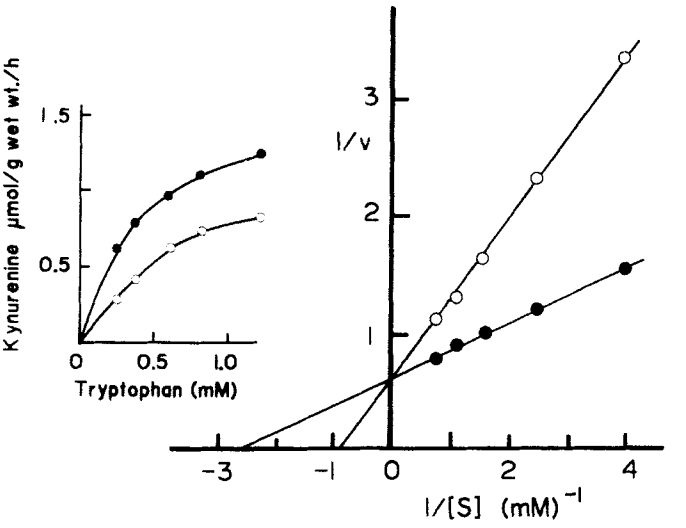

FIGURE 7 Lineweaver-Burk plot for inhibition of liver TP holoenzyme activity *in vitro* by 3-PA. Various amounts of Trp were incubated either in the absence (●——●) or in the presence (○——○) of 12 mM 3-PA. Data shown are from a typical experiment. Each point represents the average of duplicate determinations.

to a direct inhibition of the holoenzyme. Furthermore, that 3-pyridylpyruvic acid, one of the expected metabolites of 3-PA, may contribute to the decrease in the holoenzyme activity is suggested by its inhibitory property *in vitro* (Table I). Indeed, 3-pyridylpyruvic acid significantly decreased the activities of the total enzyme (84 % of control) and the holoenzyme (70 % of control) without affecting the apoenzyme activity at 24 h after administration (100 mg/kg, Shimeno *et al.*, unpublished data). Although 2-PA decreased the holoenzyme and apoenzyme activities *in vivo*, this drug itself had no inhibitory effect *in vitro* on these enzyme forms. This suggests that the decrease in TP activity in rats given 2-PA is caused by the metabolites of 2-PA, (2-(2-aminoethyl)pyridine and 2-pyridylacetic acid), which directly inhibited the holoenzyme and interfered with the conjugation of the apoenzyme with its cofactor heme *in vitro* (Table I). 4-PA was a weak inhibitor of the holoenzyme *in vitro*, whereas its metabolites, 4-(2-aminoethyl)pyridine and 4-pyridylacetic acid considerably inhibited both the holoenzyme and apoenzyme activities *in vitro* (Table I). Therefore, the decrease in TP activity (both enzyme forms) upon the administration of 4-PA seems to be mainly caused by the metabolites of 4-PA. A decrease in enzyme activity was caused by only the repeated administration of 4-PA (Figure 3), suggesting that 4-PA may be slowly metabolized and its metabolites accumulated in the body. These results suggest that the ability of PA analogs to decrease TP activity is due to the direct inhibitory effects of these analogs and/or their metabolites. We have not examined the effects of PA analogs, synthetic amino acids, on the synthesis of the enzyme protein or cofactor heme. Therefore, we cannot ignore the possibility that these effects may contribute to the decrease in liver TP activity *in vivo*.

As described above, the inhibitory mode of PA analogs on liver TP activity was slightly different to each other. However, when the enzyme activity was significantly decreased by the administration of PA analogs, apparent increases in brain trypto-

phan, 5-HT and 5-HIAA concentrations were observed (Figures 4–6). *In vitro* determination of the K_m of tryptophan 5-hydroxylase suggested that the enzyme in the brain is not normally saturated with tryptophan.[20] This implies that an increase in the availability of tryptophan to the brain would result in an increase of brain 5-HTP synthesis. There is no evidence that the decarboxylation of 5-HTP to 5-HT is rate-limiting, and there is a considerably greater activity of 5-HTP decarboxylase than of tryptophan 5-hydroxylase in the brain.[21] Therefore, brain tryptophan may play an important role in 5-HT synthesis. There is evidence in favor of this concept, as well as evidence of a direct relationship between the concentration of free tryptophan in the serum (or plasma) and that of brain tryptophan.[22–24] Furthermore, PA analogs had little effect on the activities of three enzymes involved in the synthesis and catabolism of 5-HT (tryptophan 5-hydroxylase, 5-HTP decarboxylase and MAO) in the brain. The changes in brain 5-HIAA concentration after the administration of PA analogs (Figures 4–6) also indicate that brain MAO activity was not inhibited by PA analogs and/or their metabolites. These findings strongly suggest that PA analogs increase brain tryptophan concentration by increasing tryptophan availability to the brain secondarily to the inhibition of liver TP activity, and that the elevation of brain tryptophan levels by the administration of PA analogs causes the increases in brain 5-HT and 5-HIAA. A similar inverse relationship between liver TP activity and brain 5-HT synthesis has been reported under condition involving TP inhibition by acute administration of antidepressants[25] or by chronic administration of ethanol.[26] Among PA analogs, 3-PA caused significant increases in brain tryptophan, 5-HT and 5-HIAA concentrations, although this drug decreased only the holoenzyme activity. On the other hand, when 4-PA was administered to rats for 3 days, only the apoenzyme activity significantly decreased (Figure 3). However, significant increases in brain tryptophan, 5-HT and 5-HIAA were not observed (Figure 6). Therefore, a decrease in liver TP holoenzyme activity seems to be more important than that in the apoenzyme activity for increasing brain 5-HT synthesis. In addition to the mechanism described above, it is also known that brain tryptophan concentration can be increased when the availability of the circulating amino acid to the brain is enhanced by any of the following peripheral mechanisms: (1) increased release of protein-bound serum (or plasma) tryptophan,[27] (2) a decrease in concentration of plasma neutral amino acids that compete with tryptophan for the same cerebral uptake mechanism.[28] We have not examined the effects of PA analogs and/or their metabolites on tryptophan displacement from serum protein and the ratio of tryptophan to plasma neutral amino acids. Therefore, although we are not certain of all of the effects of PA analogs, we can infer that the decrease in liver TP activity plays an important role in the increase in brain tryptophan (and hence 5-HT synthesis) after administrations of PA analogs.

The present work was performed as a part of the development of a potent TP inhibitor, and we found out that PA analogs (and/or their metabolites) increase rat brain 5-HT concentration by decreasing liver TP activity. Human liver TP appears to resemble the rat enzyme in that both the holoenzyme and apoenzyme forms are present in roughly equal proportions.[29] PA analogs, therefore, may also inhibit the human enzyme. However, PA analogs were not very strong inhibitors of TP *in vitro* and only caused significant inhibition of the enzyme after the administration of relatively large doses (50 mg/kg and above). Therefore, the development of more potent inhibitors than PA analogs is desirable. The ability of TP inhibitor to elevate brain 5-HT synthesis by a tryptophan-mediated mechanism may be useful for inves-

tigating the effect of brain 5-HT on some neuropsychiatric disorders and on blood pressure regulation. On the basis of the present results, several efforts towards the synthesis of a potent TP inhibitor are in progress in our laboratory.

Acknowledgements

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