Biochemical Pharmacology, Vol. 22, pp. 1379–1383. Pergamon Press, 1973. Printed in Great Britain.

SHORT COMMUNICATIONS

Effect of L-valine on renal excretion, blood concentrations and toxicity of cycloleucine in mice

(*Received* 17 *August* 1972; *accepted* 8 *December* 1972)

CYCLOLEUCINE, 1-aminocyclopentane-carboxylic acid, inhibits tumor growth in rodents[1] and exhibits immunosuppressive[2,3] and antimalarial[4] activities. The toxicity of this unnatural amino acid for a given species is related to the rate of excretion, since it is non-metabolizable. Species such as rodents and dogs, in which the excretory half-life of the drug is several weeks, are more adversely affected than monkeys and humans in which the half-life of the drug is only a few days.[5,6]

Our previous work in this area described the antagonism between L-valine and the antileukemic activity and toxicity of cycloleucine in mice.[7] Valine–cycloleucine interactions have also been observed by others in studies reporting on the incorporation of valine into proteins of the rat,[8] the supression of growth by cycloleucine in *Escherichia coli*[9] and chickens,[10] and the transport of cycloleucine in kidney slices[11] and in everted intestinal sacs.[12] More recently and subsequent to our previous reports on valine–cycloleucine antagonism,[7,13] it was demonstrated that the immunosuppressant activity of cycloleucine could also be reversed by L-valine.[14]

The present investigation was undertaken to explore the possible relationship between the enhanced renal excretion and reduced toxicity in mice given toxic doses of cycloleucine followed by high doses of L-valine. A preliminary account of some of our findings has been presented.[13]

Reagent grade cycloleucine, L-valine and the other natural amino acids were obtained from commercial sources. BDF_1 mice were dosed intraperitoneally with aqueous suspensions of cycloleucine and natural amino acids or saline (1 ml or less) as described below. Urine and feces were collected at indicated intervals from groups of 10–30 mice housed in stainless steel rat metabolism cages. Heparinized plasma was obtained from blood taken at sacrifice.

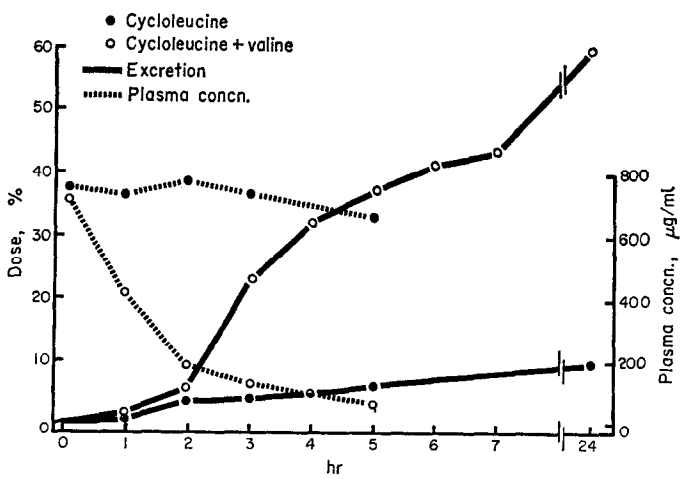

FIG. 1. Plasma concentration and renal excretion of cycloleucine in mice treated with L-valine at hourly intervals. Two groups of female mice were treated intraperitoneally with a single 2000 mg/kg dose of cycloleucine. One group received 2000 mg/kg of L-valine, i.p., each hr for 7 hr beginning immediately after the administration of cycloleucine; the other received an equivalent amount of saline at the same time intervals. Four mice in each group were sacrificed at hourly intervals to obtain blood for the estimation of cycloleucine concentrations in plasma.

All samples were stored frozen until assayed for cycloleucine by the gas chromatographic method described by Janssen *et al.*[15]

Less than 10 per cent of a single i.p. 2000 mg/kg dose of cycloleucine was excreted in the urine by mice in the 24-hr period after dosing. In contrast, nearly 60 per cent of the dose was excreted during this time interval when the cycloleucine administration was followed by seven doses of L-valine (2000 mg/kg each) given i.p. at hourly intervals (Fig. 1). The concentration of cycloleucine in plasma of mice receiving only this compound and seven doses of saline remained fairly constant over a 5-hr period, whereas in animals receiving massive doses of L-valine it declined to nearly zero during the same time interval (Fig. 1). Similarly, the concentrations of cycloleucine in several tissues were also greatly reduced by the hourly administration of high doses of L-valine (Table 1). At 24 hr after the cycloleucine dose, only small amounts remained within the tissues of the valine group. In the group given only cycloleucine and saline most of the drug was retained in the tissues. The difference is primarily attributable to the valine-enhanced renal excretion; fecal excretion accounted for relatively small amounts of the drug in either group.

TABLE 1. DISTRIBUTION OF CYCLOLEUCINE IN THE TISSUES OF MICE GIVEN A SINGLE DOSE OF CYCLOLEUCINE FOLLOWED BY HOURLY DOSES OF L-VALINE*

Sample	Control (cycloleucine)		Treated (cycloleucine + L-valine)	
	(mg/g)	(mg/mouse)	(mg/g)	(mg/mouse)
Liver	1·2	1·6	0·15	0·16
Kidney	0·86	0·25	0·12	0·04
Intestine and contents	1·3	3·3	0·18	0·42
Heart	0·98	0·23	0·14	0·03
Spleen	1·4	0·10	0·23	0·02
Lung	0·86	0·14	0·10	0·02
Carcass	1·1	15	0·13	1·7
Blood	0·67	1·0	0·05	0·07
Urine		3·8		24
Feces		0·70		1·2
Total		26·1		27·7

* Two groups of female BDF_1 mice described in Fig. 1 were sacrificed at 24 hr after the cycloleucine dose. The pooled tissues from 6–20 animals were weighed and homogenized in a blender or by glass tissue grinder. Cycloleucine in the homogenates was determined as described for serum.[15] The amount of cycloleucine per mouse for a given tissue was obtained by dividing the total quantity of cycloleucine by the number of animals making up the pooled sample. The concentration in blood was obtained at 5 hr. The amounts in urine and feces were the cumulative 24-hr excretion.

A different excretion pattern was observed when L-valine doses were given daily instead of hourly. Rather than the continuous response noted in the hourly dosed animals shown in Fig. 1, a stepwise excretion of cycloleucine was observed in the animals treated once each day with valine (Fig. 2). Thus, a high rate of cycloleucine excretion was observed in the first sampling period (7 hr) after each dose of valine. However, during the subsequent interval of 7–24 hr after each valine dose, the rate of cycloleucine excretion returned to that seen in animals receiving saline. The concentration of cycloleucine in the plasma of these animals, also shown in Fig. 2, declined in response to the valine treatment but the decrease was not as pronounced as that noted for the hourly dosed animals (Fig. 1). We have no explanation for the decline in both groups in Fig. 2 at 48 hr.

In addition to L-valine, two other branched amino acids, L-leucine and L-isoleucine, also increased the excretion of cycloleucine (Table 2). Glycine, L-alanine and the basic amino acids, L-ornithine, L-lysine and L-arginine, did not significantly enhance the excretion of cycloleucine.

Renal excretion and toxicity of cycloleucine in mice treated with branched amino acids after a single dose of cycloleucine are shown in Table 3. The enhanced excretion of cycloleucine after treatment with the branched amino acids is accompanied by reduced toxicity in these groups compared to controls. Mice treated with L-valine or L-isoleucine were protected from the toxic effects of the cyclic amino acid to a greater extent than the group treated with L-leucine.

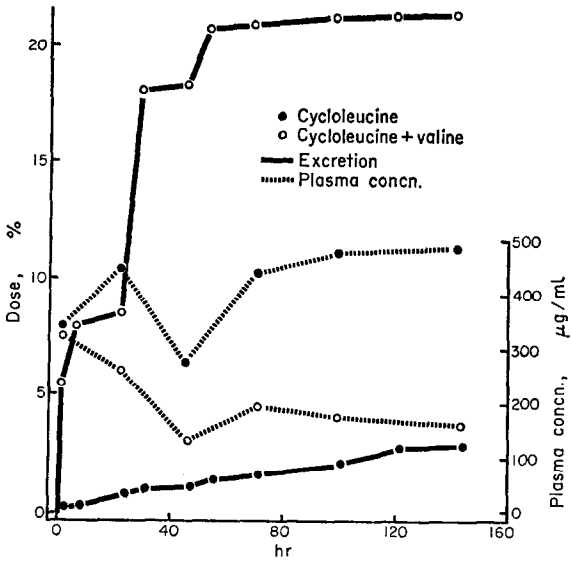

FIG. 2. Plasma concentration and renal excretion of cycloleucine in mice given a single dose of cycloleucine followed by daily doses of L-valine. Two groups of female mice were given a single intraperitoneal dose of 400 mg/kg of cycloleucine at 0 hr. One group received three daily 2000 mg/kg doses of L-valine at 0, 24 and 48 hr; the other received an equivalent amount of saline at the same time intervals. Four mice in each group were sacrificed at intervals to obtain blood for the estimation of cycloleucine concentrations in plasma.

TABLE 2. EFFECT OF SEVERAL AMINO ACIDS ON THE RENAL EXCRETION OF CYCLOLEUCINE BY MALE MICE*

Amino acid†	Cumulative cycloleucine excretion (% of dose) Time after cycloleucine dose (hr) 0–24	24–48	48–72	72–96
Saline (control)‡	0	0·9	2·3	
L-Valine§		7·4	17·6	21·3
L-Leucine§		4·0	15·6	17·6
L-Isoleucine‡	5·2	6·2	11·8	
L-Alanine‡	0·3	0·5	1·3	
L-Ornithine§		0·5	1·9	3·5
L-Lysine§		1·0	2·0	3·7
L-Arginine§		1·0	1·7	2·9
Glycine§		1·2	2·4	4·5

* All mice including controls received a single 400 mg/kg intraperitoneal dose of cycloleucine at 0 time.

† All amino acids were administered intraperitoneally at a dose equivalent to 2000 mg/kg of L-valine.

‡ Administered at 0, 24 and 48 hr after the cycloleucine dose.

§ These amino acids administered at 24, 48 and 72 hr after the cycloleucine dose.

TABLE 3. EFFECT OF BRANCHED CHAIN AMINO ACIDS ON RENAL EXCRETION AND TOXICITY OF CYCLOLEUCINE*

Amino acid	Cumulative cycloleucine excretion (% of dose)† Amino acid treatment				
	Pretreat	First	Second	Third	No. of deaths‡
Control (saline)	0·4	0·8	1·2	1·7	13
L-Leucine	0·5	2·9	12·8	16·5	9
L-Isoleucine	0·4	10·4	17·9	26·0	5
L-Valine	0·3	11·7	22·7	27·0	4

* All mice (males) including controls were given a single 400 mg/kg dose of cycloleucine. Three daily doses (equivalent to 2000 mg/kg of L-valine) of the indicated amino acids were then given beginning 24 hr after the dose of cycloleucine.

† Urine was collected for the 24-hr intervals after cycloleucine and each of the three treatments with the amino acids.

‡ Number of deaths per group of 15 mice in 28-day period after the cycloleucine dose.

^{14}C-Cycloleucine was not bound to rat plasma in equilibrium dialysis according to the method of Keen.[16]

The results presented in this report clearly establish that massive doses of L-valine increase cycloleucine excretion, with a concomitant decrease in the concentration of cycloleucine in the blood and tissues. Thus, it appears that the reduced toxicity of cycloleucine in mice receiving a single lethal dose of the cyclic amino acid followed by several large doses of L-valine is due, at least in part, to the valine-induced increase in the renal excretion of cycloleucine.

The precise mechanism by which valine increases renal excretion of cycloleucine has not been determined, but cycloleucine undoubtedly shares a common receptor site with certain closely related natural amino acids. Cycloleucine is not bound to plasma proteins and therefore a mechanism based on its displacement from plasma protein by valine does not explain the observations presented in this communication. Mobilization of cycloleucine from tissues is another possible mechanism of valine–cycloleucine interaction, but this should produce a temporary increase in the plasma concentration of cycloleucine which was not observed. Another possible explanation which we consider the most plausible for our observations is based on a cycloleucine–valine interaction in the kidney tubule. This interpretation takes into account the work of Christensen and Jones[17] as well as the publication of Holtzapple *et al.*[11] The first named authors found that cycloleucine is excreted in the glomerular filtrate and reabsorbed in the proximal tubule, presumably by transport sites operative for certain natural amino acids. Holtzapple *et al.*[11] demonstrated that cycloleucine inhibits the uptake of valine and other amino acids. High concentrations of valine, such as those resulting from frequent massive doses, would be expected to inhibit the uptake of cycloleucine if these two amino acids interact at a common site in the kidney.

Whatever the mechanism is, the interaction seems to be relatively specific since, of the amino acids investigated, only the three neutral branched amino acids increase the excretion of cycloleucine. These are also the only amino acids which afford any protection against the toxic effects of the cyclic amino acid in the mouse. Other natural amino acids have been shown previously to be ineffective in reversing the toxicity of cycloleucine.[13] L-Valine appears to be the most effective antagonist of cycloleucine, followed by L-isoleucine and L-leucine. It is interesting to note that the basic amino acids, which are excreted to the greatest extent in the aminoaciduria induced by cycloleucine,[6,18] had no effect on the excretion of this amino acid under our experimental conditions.

Acknowledgements—We gratefully acknowledge the excellent technical assistance of Mrs. Nancy Martin and Mrs. Jeanne Trainor.

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Biochemical Pharmacology, Vol. 22, pp. 1383–1385. Pergamon Press, 1973. Printed in Great Britain.

Identification of the mononitro derivative of dapsone as a product from an oxidation *in vitro*

(*Received* 12 *July* 1972; *accepted* 1 *December* 1972)

ALTHOUGH the metabolism of the antileprotic drug dapsone, 4,4′-bis (aminophenyl)sulfone, has been studied extensively *in vivo*,[1-3] there appear to have been relatively few studies of its metabolism *in vitro*. However, the oxidation *in vitro* of one of the amino nitrogens of dapsone to form mono-*N*-hydroxydapsone has been reported recently.[4,5] We shall present evidence in this paper to show that dapsone is oxidized to its mononitro derivative in the presence of rat liver microsomal enzyme preparations.

The method of microsome preparation was essentially that of Fouts.[6] Liver microsomes from rats were used unless otherwise indicated. All operations in preparation of microsomes were carried out at 0°. Dapsone, uniformly labeled with ^{14}C, was incubated at a concentration of 5×10^{-4} M with 30 ml of 0·05 M phosphate buffer (pH 7·4) and 10 ml of the microsomal preparation (equivalent to 8 g of the original liver) in a total volume of 50 ml. Mg^{2+} was present at a final concentration of $1{\cdot}8 \times 10^{-4}$ M, nicotinamide at $1{\cdot}2 \times 10^{-4}$ M, NADP at 5×10^{-4} M and glucose 6-phosphate at 5×10^{-4} M. The NADPH generating system was completed with 36 i.u. of glucose 6-phosphate dehydrogenase. Incubations were carried out in 125 ml Erlenmeyer flasks within 6 hr after sacrifice. The flasks were held at 37° for 60 min and swirled at a rate of 100 rev/min.

After incubation, proteins were precipitated with 5 ml of 10% $ZnSO_4$. The pH was adjusted to 7·25 and the mixture centrifuged at 25,000 *g* for 10 min. The aqueous supernatant was extracted with ethyl acetate (3×20 ml). The organic phase contained about 65 per cent of the total radioactivity and was evaporated to dryness at room temperature in a stream of N_2. The residue was dissolved in 0·1 ml methanol and then taken to a final volume of 8 ml with 0·05 N HCl. This solution was extracted with methylene chloride (3×2 ml). Most of the parent compound remained in the aqueous phase, while 35 per cent of the radioactivity went into the organic phase. Thin-layer chromatography of an aliquot of this extract on Silica gel (benzene–ethanol 80:20), revealed two zones of radioactivity, one