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POTENTIATING ACTION OF 4-VINYLPYRIDOXAL ON INHIBITION OF SERINE TRANSHYDROXYMETHYLASE BY D-CYCLOSERINE AND ITS DIMER

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Abstract—The cooperative inhibition of serine transhydroxymethylase (EC 2.1.2.1) in spleen tissue of mice after intraperitoneal injections of various vitamin B_6 antimetabolites was studied. The data obtained reveal that pyridoxal azine, when injected alone in a dose of 75 mg/kg of body weight, produced moderate inhibition of serine transhydroxymethylase, but, when injected in the same dose 1 hr prior to D-cycloserine (500 mg/kg of body weight) injection, exerted a slight potentiating effect on the enzyme inhibition produced by D-cycloserine. In similar experiments, pyridoxal monomethylhydrazone in a dose of 7.5 mg/kg of body weight did not inhibit the activity of serine transhydroxymethylase and did not produce a potentiating effect on inhibition of the enzyme by D-cycloserine. 4-Vinylpyridoxal (4-vinyl-4-deformylpyridoxal), injected into mice in a dose of 0.5 to 1.0 mg/kg of body weight, does not decrease the activity of serine transhydroxymethylase in spleen tissue, but when injected 1 hr prior to injective of D-cycloserine or its dimer, it potentiates (unlike pyridoxal monomethylhydrazone) the inhibitory effects of both D-cycloserine and its dimer, producing more extensive and more prolonged inhibition of the enzyme. If D-cycloserine or its dimer is injected into mice in doses of 500 mg/kg of body weight, the potentiating effect of 4-vinylpyridoxal approaches 200–250 per cent or more, depending on the time after the injection of the antimetabolite. Thus, it is of interest to study further the chemotherapeutic action of moderate doses of D-cycloserine or its dimer in combination with 4-vinylpyridoxal.

Serine transhydroxymethylase is a key pyridoxal-P-dependent * enzyme participating in the biosynthesis of 5,10-CH_2H_4-folate from H_4-folate in mammalian tissue, and it may be considered a target enzyme in developing new antitumor antimetabolites [1–5]. It was shown previously that the antibiotic D-cycloserine, which is a vitamin B_6 antagonist, or the dimer of D-cycloserine, when administered parenterally into animals, produced a decrease in the activity of serine transhydroxymethylase in neoplasms and possessed chemotherapeutic activity against experimental leukemia [1, 2, 6, 7]. In a number of cases, when patients with acute lymphoblastic or myeloblastic leukemia were given prolonged intravenous injections of high doses of D-cycloserine, temporary partial or complete clinical and haematological remission was observed. Clinically, however, the antileukemic action of D-cycloserine and the degree of its target-enzyme inhibition in leukemic cells at maximal therapeutic doses were only moderate [8, 9]. Hence, it is of chemotherapeutic interest to develop more effective means to inhibit serine transhydroxymethylase in tumor cells.

Preliminary studies demonstrated that the stability *in vitro* of enzyme–inhibitor [E.I.] complexes of serine transhydroxymethylase with D-cycloserine or its dimer, as well as the degree and duration of the inhibition of the target-enzyme in tissues *in vivo*, is dependent on the concentration of pyridoxal-P [6, 7]. Thus, it is of interest to investigate the possible influence of vitamin B_6 antagonists in producing a decrease in the content of pyridoxal-P in tissues, as well as on the degree and duration of serine transhydroxymethylase inhibition by D-cycloserine or its dimer.

In this paper, we report the effects of pyridoxal azine, pyridoxal monomethylhydrazone, and 4-vinylpyridoxal on the inhibition of serine transhydroxymethylase in mouse spleen by D-cycloserine or its dimer. Pyridoxal azine [10, 11] and other substituted pyridoxal hydrazones [11] manifest the properties of inhibitors of pyridoxal phosphokinase (EC 2.7.1.35); this enzyme, as well as pyridoxol-P oxidase (EC 1.4.3.5), plays a key role in the biosynthesis of pyridoxal-P in mammalian tissues [5, 12]. 4-Vinylpyridoxal is one of the most powerful antagonists of vitamin B_6 [13–15], and in experiments *in vitro* is a very effective inhibitor of pyridoxal phosphokinase [16]. Since it is also a substrate of pyridoxal phosphokinase, 4-vinylpyridoxal is phosphorylated by the enzyme in the presence of ATP and the product formed, 4-vinylpyridoxal-P, is a potent inhibitor of pyridoxin-P oxidase, competing with pyridoxol-P [16]. It has been shown also that 4-vinylpyridoxal-P may interact selectively with some pyridoxal-requiring apoenzymes and produce irreversible or relatively strong [E.I.] complexes with apo-L-glutamate decarboxylase (EC 4-1-1-15) [17], apo-aspartate aminotransferase (EC 2.6.1.1) [18], and apo-L-arginine decarboxylase (EC 4.1.1.19), as determined by Prof. E. E. Snell and cited in Ref. 16.

* Abbreviations: pyridoxal-P, pyridoxal 5′-phosphate; 4-vinylpyridoxal (4-VPAL), 4-vinyl-4-deformylpyridoxal; 4-vinylpyridoxal-P, 4-vinyl-4-deformylpyridoxal 5′-phosphate; H_4-folate, tetrahydrofolate; D-cycloserine dimer, *cis*-2,5-$(NH_2OCH_2)_2$-piperazine-3,6-dione (diketopiperazine of β-amino-oxy-D-alanine).

EXPERIMENTAL PROCEDURES

Animals. Approximately 170 random-bred healthy male white mice weighing 22–25 g, and 20 male leukemic mice of BALB/c strain, weighing 20–22 g, were used. The mice were fed a standard stock diet and were given water *ad lib.* BALB/c mice inoculated with standard doses of Friend leukemia virus 15 days prior to the experiment [15] had, at the time of the experiment, a significantly higher spleen weight (about 1.2 to 1.7 g) than did healthy mice (about 0.2 g).

Neutral solutions of antimetabolites in volumes of 0.25 to 0.50 ml of 0.9% NaCl were injected into the mice intraperitoneally. Pyridoxal azine hydrochloride (75 mg/kg of body weight), pyridoxal monomethylhydrazone (7.5 mg/kg of body weight) and 4-vinylpyridoxal (1.0 mg/kg of body weight) were used in maximal doses, such that a single injection into a mouse did not produce an appreciable acute toxic effect. Mice of the control group were injected intraperitoneally with appropriate volumes of 0.9% NaCl. Some details of the experiments are provided in Tables 1 and 2 and in Fig. 1.

Assay of serine transhydroxymethylase in spleen tissue. At specified time intervals after injection of saline (control) or test compound, the mice were decapitated and the spleens were removed, chilled, rinsed, blotted, weighed, and homogenized with approximately 7 vol. of ice-cold 0.1 M potassium phosphate, pH 7.8, containing 0.1 M β-mercaptoethanol. The activity of serine transhydroxymethylase in freshly prepared homogenates of tissue has been measured in the system coupled with 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5), as described by Bukin and Sergeev [19]. The reaction mixture contained 0.25 ml of crude tissue homogenate (28.5 to 35.7 mg tissue), 5 μmoles L-serine, 1.5 μmoles H_4-folate, 1.0 μmole NADP, and 10 μ moles β-mercaptoethanol in 2.25 ml of 0.1 potassium phosphate, pH 7.8. The reaction was started by adding tissue homogenate. Incubation was carried out at 37° for 10 min, and was stopped by adding 1 ml of 45% $HClO_4$ to the mixture. After centrifugation, the amounts of 5,10-CH-H_4-folate in the $HClO_4$ extracts were determined by measuring $E_{355\ nm}$ and using a molar extinction coefficient of 2.2×10^4 liters $\times$ mol^{-1} $\times$ cm^{-1} for the substance [20]. The formation of 1 nmole of 5,10-CH-H_4-folate from 1 nmole of 5,10-CH_2-H_4-folate in 1 min at standard conditions was taken as 1 unit of serine transhydroxymethylase

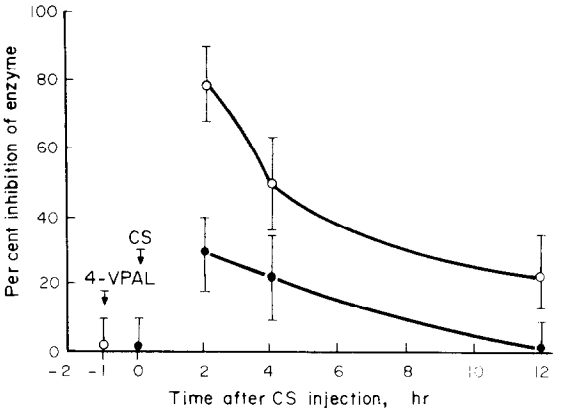

Fig. 1. Effect of 4-vinylpyridoxal (4-VPAL) on the course of inhibition of serine transhydroxymethylase in spleen tissue of mice injected intraperitoneally with D-cycloserine (CS). 4-VPAL was injected into mice intraperitoneally in a dose of 1.0 mg/kg of body weight 1 hr before CS was injected in a dose of 500 mg/kg of body weight. 4-VPAL injected alone did not affect the activity of the enzyme; the activity of the enzyme was in the range of 196 ± 13 to 205 ± 17 units/g of tissue as compared with 202 ± 15 units/g of tissue in the controls. Key: (——●——) inhibition of the enzyme by CS injected alone; and (——○——) inhibition of the enzyme by CS injected in combination with 4-VPAL. Arrows indicate the times of injections of 4-VPAL and CS. Circles and vertical lines represent the average values of enzyme inhibition and their 95% confidence limits ($X \pm tS_{\bar{x}}$); there were five mice in each group.

Table 1. Effects of some pyridoxal analogues, D-cycloserine, and its dimer on the activity of serine transhydroxymethylase of mice spleen tissue *in vitro* *

Antimetabolites	Concn (M)	Enzyme activity (units/g tissue)	Percentage inhibition of the enzyme
Control		207	
Pyridoxal azine	1×10^{-4}	199	4
	5×10^{-4}	190	8
Pyridoxal monomethylhydrazone	1×10^{-4}	197	5
	5×10^{-4}	193	7
4-Vinylpyridoxal	1×10^{-5}	203	2
	1×10^{-4}	201	3
D-Cycloserine	1×10^{-3}	170	18
	5×10^{-3}	68	67
Dimer of D-cycloserine	1×10^{-4}	178	14
	1×10^{-3}	50	76
	5×10^{-3}	39	81

* Activity of the enzyme was detected at standard conditions in the absence of added pyridoxal-P in samples containing 0.2 ml of tissue extract. Prior to the incubation, samples were preincubated 20 min at 0° without antimetabolites (control activity, 100 per cent) or in the presence of antimetabolites in the concentrations indicated. Similar results were obtained in experiments *in vitro* with extracts of mouse liver as a source of the enzyme.

Table 2. Activity of serine transhydroxymethylase in spleen tissue of mice injected intraperitoneally with D-cycloserine or its dimer in combination with pyridoxal azine, pyridoxal monomethylhydrazone, or 4-vinylpyridoxal

Series of experiments	Antimetabolites (doses in mg/kg of body weight)*	Activity of the enzyme and its inhibition: Enzyme activity† (units/g tissue) ($X \pm tS_x$)	A. Percentage inhibition of the enzyme observed in experiment (statistical significance, P)	B. Percentage inhibition of the enzyme assuming simple additive action of the antimetabolites	P‡
I	Control	180 ± 11			
	Pyridoxal azine (75)	146 ± 10	18.9 (< 0.01)		
	Pyridoxal azine (75) + D-cycloserine (500)	72 ± 7	60.0 (< 0.01)	40.6	< 0.01
	D-Cycloserine (500)	141 ± 12	21.7 (< 0.01)		
II	Control	186 ± 10			
	Pyridoxal monomethylhydrazone (7.5)	189 ± 9	−1.6 (> 0.5)		
	Pyridoxal monomethylhydrazone (7.5) + D-cycloserine (500)	147 ± 11	21.0 (< 0.01)	23.6	> 0.1
	D-Cycloserine (500)	139 ± 7	25.2 (< 0.01)		
III	Control	201 ± 7			
	4-Vinylpyridoxal (0.5)	203 ± 12	−1.0 (> 0.5)		
	4-Vinylpyridoxal (0.5) + D-cycloserine (500)	72 ± 11	64.2 (< 0.01)	26.9	< 0.01
	4-Vinylpyridoxal (0.5) + dimer of D-cycloserine (500)	125 ± 12	37.5 (< 0.01)	21.9	< 0.01
	D-Cycloserine (500)	145 ± 8	27.9 (< 0.01)		
	Dimer of D-cycloserine (500)	155 ± 7	22.9 (< 0.01)		
IV	Control	221 ± 20			
	4-Vinylpyridoxal (1.0)	219 ± 15	1.0 (> 0.5)		
	4-Vinylpyridoxal (1.0) + D-cycloserine (500)	55 ± 14	75.1 (< 0.01)	28.6	< 0.01
	D-Cycloserine (500)	160 ± 13	27.6 (< 0.01)		
V	Control	210 ± 16			
	4-Vinylpyridoxal (1.0)	208 ± 10	1.0 (> 0.5)		
	4-Vinylpyridoxal (1.0) + dimer of D-cycloserine (500)	89 ± 16	57.6 (< 0.01)	22.9	< 0.01
	Dimer of D-cycloserine (500)	164 ± 15	21.9 (< 0.01)		
VI	Control§	440 ± 23§			
	4-Vinylpyridoxal (1.0)	425 ± 29	3.5 (> 0.5)		
	4-Vinylpyrodoxal (1.0) + dimer of D-cycloserine (500)	57 ± 32	87.1 (< 0.01)	39.4	< 0.01
	Dimer of D-cycloserine (500)	282 ± 30	35.9 (< 0.01)		

* Indicated doses of the pyridoxal analogues were injected into mice 3 hr before killing. D-Cycloserine and its dimer were injected into mice 1 hr after injections of pyridoxal analogues (2 hr before killing). Mice of control groups were injected with 0.25 to 0.5 ml of 0.9% NaCl 3 hr before killing.

† Average values of enzyme activity ± 95 per cent confidence limits ($\bar{X} \pm tS_{\bar{x}}$) are indicated; there were four to five mice in each group.

‡ Probability that the difference between A and B occurred by chance (Student's t-test).

§ Mice with Friend birus leukemia; the content of pyridoxal-P in spleen tissue is about 0.6 μg/g of wet weight, as compared with 0.9 μg/g of wet weight in healthy mice[5].

activity. The activity of the enzyme was expressed in units per g of fresh spleen tissue. The precision of the method in ± 3.5 per cent. The activity of 5,10-methylenetetrahydrofolate dehydrogenase in spleen tissue does not limit the rate of formation of 5,10-CH-H_4-folate from 5,10-CH_2-H_4-folate. Under standard conditions, the amounts of 5,10-CH-H_4-folate formed in the reaction mixture were proportional to the content of the tissue in the mixture from 20 to 40 mg, and to incubation time from 5 to 15 min.

In some experiments, the activity of the enzyme was determined in extracts of mouse spleen or liver tissues. In those experiments, 12.5% (w/v) tissue homogenates were centrifuged for 30 min at 4×10^4 *g* at 2–4°, and a 0.2 ml supernatant fraction (spleen tissue) or a 0.1-ml supernatant fraction (liver tissue) was taken for the assay.

Antimetabolites and reagents. Pyridoxal azine hydrochloride [21], pyridoxal monomethylhydrazone [22], 4-vinylpyridoxal [14, 16], and *cis*-2,5-(NH_2OCH_2)$_2$-piperazine-3,6-dione (dimer of D-cycloserine [23] were synthesized by the methods indicated. D-Cycloserine (free base) was obtained from the Soc. Farmaceutici Italia, Milan, Italy; before the experiment, D-cycloserine was purified by the appropriate method [24]. L-Serine was obtained from CalBiochem, La Jolla, CA, U.S.A. NADP was obtained from Lowson, London, England. H_4-folate (fresh solutions) was prepared before use by reducing H_2-folate with $NaBH_4$ [19]. Other chemicals were cp-grade or analytical-grade U.S.S.R. preparations.

RESULTS AND DISCUSSION

In preliminary experiments *in vitro*, we found that pyridoxal azine, pyridoxal monomethylhydrazone, or 4-vinylpyridoxal failed to decrease the activity of serine transhydroxymethylase in mouse spleen extracts if added, 20 min before the start of the reaction, to the reaction mixture in final concentrations of 1×10^{-5}–1×10^{-4} M (Table 1). The concentrations of pyridoxal analogues used in these experiments were much higher than those that might be obtained in tissues of mice injected with maximal subtoxic doses of the antimetabolites. On the other hand, D-cycloserine and its dimer, in *in vitro* experiments (Table 1), exerted a significant decrease in serine transhydroxymethylase activity in concentrations which are comparable to those that may be expected in tissues of mice injected with moderate nontoxic doses of the antimetabolites.

The effects of the pyridoxal analogues, injected into mice separately as well as in combination with D-cycloserine or its dimer, on the activity of serine transhydroxymethylase in spleen tissue *in vivo* are summarized in Table 2. Intraperitoneal injections of pyridoxal azine in doses of 75 mg/kg of body weight, 3 hr prior to killing the mice, decreased spleen serine transhydroxymethylase activity by about 19 per cent, on the average. The effect seems to be the result of an indirect action of pyridoxal azine on the enzyme activity. Pyridoxal monomethylhydrazone (7.5 mg/kg of body weight) or 4-vinylpyridoxal (0.5 to 1.0 mg/kg of body weight), injected into mice 3 hr prior to killing, did not decrease the activity of the enzyme in spleen tissue. Intraperitoneal injections of D-cycloserine or its dimer into healthy mice, in doses of 500 mg/kg of body weight 2 hr prior to death, decreased the activity of the enzyme in spleen tissue 22–28 or 22–23 per cent respectively.

In studies of cooperative effects of the antimetabolites on enzyme activity in spleen tissue, the pyridoxal analogues were injected into mice at the doses indicated 1 hr before the injection of D-cycloserine or its dimer, and either of the latter was administered to mice 2 hr before death. In these experiments, it was found that pyridoxal azine, injected into mice prior to D-cycloserine, exerts a slight potentiating effect on the enzyme inhibition produced by D-cycloserine (Table 2, series 1). Pyridoxal monomethylhydrazone injected into mice prior to D-cycloserine did not have any effect on the inhibition of the enzyme by D-cycloserine. In contrast, 4-vinylpyridoxal injected into healthy or leukemic mice in doses of 0.5 to 1.0 mg/kg of body weight prior to injection of D-cycloserine or its dimer significantly potentiates the inhibition of the enzyme by D-cycloserine or its dimer injected alone (see Table 2, series III–VI).

It should be emphasized that 4-vinylpyridoxal not only increases by 2- to 2.5-fold the degree of enzyme inhibition caused by D-cycloserine (for 2–4 hr after D-cycloserine was injected) but also significantly prolongs the period of the enzyme inhibition caused by D-cycloserine (Fig. 1). At 12 hr after the administration of D-cycloserine alone to mice, the activity of serine transhydroxymethylase in spleen tissue became normal, but after combined administration of 4-vinylpyridoxal and D-cycloserine, the activity of the enzyme was still 20–25 per cent lower, as compared with the control (Fig. 1). In similar experiments (data are not included in Fig. 1), it was shown that 4-vinylpyridoxal also prolonged in spleen tissue the duration of the enzyme inhibition by the dimer of D-cycloserine.

Accordingly, the data obtained indicate that 4-vinylpyridoxal, in a relatively low, non-toxic dose of 0.5 mg/kg of body weight, appreciably intensifies the inhibitory effect of D-cycloserine or its dimer on the target-enzyme activity in spleen tissue of mice. It should be noted, however, that 4-vinylpyridoxal potentiates the inhibition of serine transhydroxymethylase caused by D-cycloserine or its dimer only when 4-vinylpyridoxal is administered to mice before the administration of D-cycloserine. This difference may be due to a decrease in pyridoxal-P content in spleen tissue as a result of administering 4-vinylpyridoxal before D-cycloserine. Even so, the possibility of a direct inhibitory action of 4-vinylpyridoxal-P formed *in vivo* on aposerine transhydroxymethylase in spleen tissue cannot be excluded. Previously we found [16] that dissociation of [E.I.] complexes, formed *in vivo* between serine transhydroxymethylase and D-cycloserine or its dimer, is accompanied by the appearance of significant amounts of the apoenzyme.

The molecular mechanism of the potentiating effect of 4-vinylpyridoxal on the inhibition of serine transhydroxymethylase in the spleen of mice *in vivo* by D-cycloserine or its dimer has yet to be determined. Because the extent and duration of the enzyme inhibition in the target tissue of mice with Friend virus leukemia are correlated with the antileukemic action of D-cycloserine or its dimer [6], it will be interesting to study the possible chemotherapeutic activity of moderate doses of D-cycloserine or its dimer in combination

with moderate doses of 4-vinylpyridoxal in experimental leukemia and other experimental neoplasms.

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