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Oncolytic activity and mechanism of action of a novel L-cysteine derivative, L-cysteine, ethyl ester, *S*-(*N*-methylcarbamate) monohydrochloride*

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Summary. A study on the oncolytic activity of the L-cysteine derivative L-cysteine, ethyl ester, *S*-(*N*-methylcarbamate) monohydrochloride (NSC 303861), revealed that the drug caused complete regression of the MX-1 human mammary tumor xenograft. The compound also exhibited moderate antitumor activity against murine leukemia P388 (T/C value of 169% at a daily dose of 400 mg/kg) and against M5076 sarcoma (T/C value of 135% at a daily dose of 600 mg/kg). The drug was inactive against B16 melanoma, Lewis lung, colon 38 and CD_8F_1 mammary carcinomas. The compound exhibited significant cytotoxicity against hepatoma 3924A cells in culture (LC_{50} = 6 μM). Studies on the mechanism of action revealed that the cytotoxicity of the drug could be partially abrogated by protecting hepatoma 3924A cells in culture with L-glutamine. At 6 h after an injection of the compound (400 mg/kg) into rats bearing hepatoma 3924A, the pools of L-glutamine and L-glutamate in the tumor decreased to 33% and 71%, respectively, of control levels; the drug selectively inhibited the activities of L-glutamine-requiring enzymes of purine nucleotide biosynthesis, amidophosphoribosyltransferase, FGAM synthase, and GMP synthase, to 21%, 1%, and 69%, respectively, without significantly altering the activities of pyrimidine biosynthetic enzymes, carbamoylphosphate synthase II and CTP synthase. Measurement of the nucleotide concentrations further corroborated the actions of the drug on the purine nucleotide biosynthetic enzyme activities. Drug injection (400 mg/kg) in the hepatoma 3924A-bearing rats reduced the concentrations of IMP in the tumor to 52%, those of total adenylates to 52%, those of total guanylates to 57%, and those of NAD to 73%, without significantly perturbing the pyrimidine nucleotide pools. Studies on the mechanism of action of the L-cysteine derivative suggested that the compound behaved as an L-glutamine antagonist, selectively acting on the enzymes of purine nucleotide biosynthesis.

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Abbreviations: HPLC, high-pressure liquid chromatography; PRPP, 5-phosphoribosyl-1-pyrophosphate; FGAM, formylglycinamidine ribonucleotide; FGAR, formylglycinamide ribonucleotide; AIR, aminoimidazole ribonucleotide; TCA, trichloroacetic acid; acivicin, L-(α*S*, 5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid

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Introduction

L-Cysteine derivative (L-cysteine, ethyl ester, *S*-(*N*-methylcarbamate) monohydrochloride; NSC 303861) (Fig. 1) was synthesized by Hasegawa and Kotani [8, 9]. The present studies were aimed at determining the antitumor activity and the mechanism of action of the compound.

Materials and methods

L-Cysteine, *S*-(*N*-methylcarbamate) monohydrochloride was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI) (Bethesda, Md). HPLC apparatus was purchased from Waters Associates (Milford, Mass). All chemicals were of the highest quality obtainable.

Evaluation of in vivo antitumor activity. Antitumor testing was conducted through the screening program of the Drug Evaluation Branch, DTP, DCT, NCI, according to published protocols [5, 7, 14] in mice, with the tumor lines obtained from the Animal Genetics and Production Branch, DTP, DCT, NCI.

Cytotoxicity studies with hepatoma 3924A. Hepatoma 3924A cells were grown in exponential phase in McCoy's 5A medium supplemented with 2 m*M* L-glutamine and 10% dialyzed fetal bovine serum at 37° C in an atmosphere comprising 95% air and 5% CO_2. Cytotoxicity of L-cysteine derivative was determined as previously described [13]. In short, hepatoma cells (500 cells) were exposed to the antimetabolite (1–100 μM) or saline continuously for 7 days, and then the number of colonies was determined as previously detailed [13].

To examine the effect of L-glutamine in protecting hepatoma 3924A cells from the cytotoxicity of L-cysteine derivative, cells in logarithmic

Table 1. Antitumor activity of L-cysteine derivative against murine tumors and human tumor xenografts in vivo

Tumor system	Treatment route and schedule	Control mice:		Drug-treated mice[a]:	
		Survival (days)	Final tumor weight (mg)	Optimal dose (mg/kg per day)	Response (% T/C)
Murine tumors:					
i.p. L1210 leukemia	i.p. qd, days 1–9	8.7		400	132
i.p. M5076 sarcoma	i.p. q4d, days 1–13	25.8		600	135
i.p. P388 leukemia	i.p. qd, days 1–5	10.0		400	165
	i.p. qd, days 1–9	11.1		444	169
i.p. B16 melanoma	i.p. qd, days 1–9	18.4			Inactive
i.v. Lewis lung carcinoma	i.p. qd, days 1–9	18.0			Inactive
s.c. CD_8F_1 mammary carcinoma	i.p. qd, day 25		724		Inactive
s.c. Colon adenocarcinoma 38	i.p. q7d, days 2, 9		1,040		Inactive
Human tumor xenografts:					
src MX-1 mammary tumor	s.c. q4d, days 1–9		13.48	990	–65[b]
src CX-1 colon tumor	s.c. q4d, days 1–13		4.34		Inactive
src LX-1 lung tumor	s.c. q4d, days 1–9		8.80		Inactive

Experimental details are presented in Materials and methods

[a] Responses were confirmed at least once

[b] Tumor regression was based on initial and final tumor weights in the test group (100% tumor regression in 2/6 mice)

phase were adapted to grow in 0.25 m*M* L-glutamine by decreasing the concentration of L-glutamine from 2 to 0.25 m*M* over a period of 30 generations. Cells were incubated with the drug (6 μM) or saline in an atmosphere comprising 95% air and 5% CO_2 in the presence of L-glutamine (0.25, 0.5, 1.5, or 3.0 m*M*) for 7 days, then the colony-forming ability was determined [13].

Measurement of L-*glutamate and* L-*glutamine pools.* Male ACI rats (180–200 g, Harlan Industries, Cumberland, Ind) were transplanted s.c. with hepatoma 3924A cells (2×10^7 cells). At 10–15 days after tumor inoculation, rats were injected i.p. with L-cysteine derivative (200 or 400 mg/kg) or saline; 6 h later, tumors were removed and freeze-clamped within 0 to 2 s, ground into fine powder under liquid nitrogen, and extracted with cold 10% perchloric acid [21]. The supernatants were neutralized with KOH and centrifuged, and L-glutamate concentrations were determined by using L-glutamate dehydrogenase from bovine liver [2]. Supernatants were first treated with purified L-glutaminase from *E. coli* (Sigma Chemical Co., St. Louis, Mo) prior to the determination of glutamate content using L-glutamate dehydrogenase from bovine liver [2, 14].

Measurement of nucleotide pools. The procedures for hepatoma 3924A implantation, injection of L-cysteine derivative, and freeze-clamping were as detailed above. Freeze-clamped powder was extracted with cold 10% TCA and neutralized with tri-*n*-octylamine in freon, and an aliquot was used for quantitation of nucleotide pools on a Partisil 10-SAX column of a Waters HPLC unit (Waters Associates) as previously described [11].

Studies on activities of L-*glutamine-utilizing enzymes of purine and pyrimidine nucleotide biosynthesis.* L-Glutamine-utilizing enzyme activities were measured in hepatoma 3924A after an in vivo dose of the compound. Hepatoma cells were implanted into ACI rats, and drug was injected i.p. as described above; 6 h later, rats were stunned, decapitated, and exsanguinated. Tumors were quickly excised, placed in beakers embedded in crushed ice, then homogenized (1:4 w/v) in cold 0.25 *M* sucrose containing 20 m*M* TRIS-HCl (pH 7.4) and 1 m*M* EDTA-Na. The homogenates were centrifuged at 100,000 *g* for 30 min at 2°C in a Beckman Model LS-50 centrifuge. The supernatant fluid containing 50–70 mg/ml protein was used for the studies of L-glutamine-utilizing enzymes.

Amidophosphoribosyltransferase (EC 2.4.2.14) activity was assayed as cited elsewhere [15]. FGAM synthase (EC 6.3.5.3) activity was measured as outlined [6]. GMP synthase (EC 6.3.5.2) activity was determined as previously reported [3]. Carbamoyl-phosphate synthase II (EC 6.3.5.5) activity was assayed as outlined [1]. CTP synthase (EC 6.3.4.2) activity was determined as cited elsewhere [12].

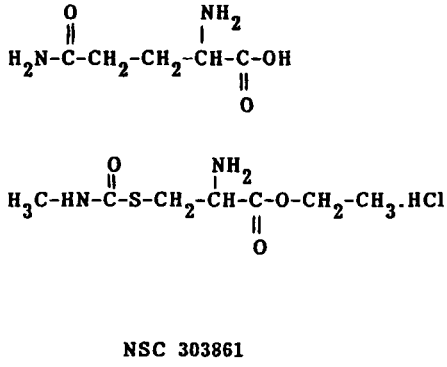
Results and discussion

Antitumor activity in vivo

The therapeutic effectiveness of the L-cysteine derivative against a panel of transplantable murine tumors is documented in Table 1. The antitumor activity of the compound was initially identified in the P388 leukemia prescreening assay [20]. In five experiments, a dose of 400 mg/kg per day given i.p. on days 1–5 produced T/C values ranging from 145%–169%. A dose of 800 mg/kg per day was acutely lethal, and antitumor activity (T/C $\geq$ 120%) was observed at doses as low as 100 mg/kg per day (not shown). Similar activity was observed when the treatment period was extended to 9 days, optimal T/C values of 169% and 170% being achieved in two experiments. L1210 leukemia was less responsive to the antimetabolite, optimal T/C values of 132% and 133% being obtained following daily i.p. administration of 400 mg/kg for 9 days. No activity was observed at one-half the optimal dose. The compound exhibited moderate activity against the i.p. implanted M5076 sarcoma when given on an intermittent schedule but was inactive against B16 melanoma and Lewis lung, colon 38 and CD_8F_1 mammary carcinomas under the experimental conditions used.

The effects of the L-cysteine derivative against three human tumor xenografts indicate that good activity was observed against the MX-1 mammary tumor xenograft but

L-Glutamine



NSC 303861

Fig. 1. Chemical structures of L-glutamine and L-cysteine derivative

Table 2. Effect of L-cysteine derivative on the pools of L-glutamine and L-glutamate in hepatoma 3924A in vivo

Dose of L-cysteine derivative (mg/kg)	Pools: nmol/g (means ± SE):			
	L-Glutamine	% of control	L-Glutamate	% of control
None	890 ± 100	100	1,400 ± 100	100
200	487 ± 90	55*	940 ± 30	67*
400	295 ± 20	33*	990 ± 20	71*

Rats bearing s.c. hepatoma 3924A were injected i.p. with the drug; 6 h later, tumors were removed and freeze-clamped and pools were analyzed as indicated in Materials and methods. Four or more rats were in each experimental group

* Statistically significant according to a comparison of the treated group with the control group ($P < 0.05$)

Table 3. Effect of L-cysteine derivative on the activities of L-glutamine-utilizing enzymes of purine and pyrimidine nucleotide biosynthesis in hepatoma 3924A in vivo

Enzymes	Activity:	
	Control mice (nmol/mg protein per h)	Drug-treated mice (% of control)
Purine:		
Amidophosphoribosyltransferase	31.5 ± 7.9	21*
FGAM synthase	0.2 ± 0.1	1*
GMP synthase	21.1 ± 1.3	69*
Pyrimidine:		
Carbamoyl-phosphate synthase II	61.0 ± 3.0	92
CTP synthase	56.9 ± 1.7	94

Rats bearing s.c. hepatoma 3924A were injected i.p. with the drug (400 mg/kg) and enzyme activities in the tumor were determined 6 h after injection according to the procedure in Materials and methods. Four or more rats were in each experimental group; data represent the means ± SE

* Significantly different from control values ($P < 0.05$)

Table 4. Effect of L-cysteine derivative on the nucleotide pools of hepatoma 3924A in vivo

Nucleotides	Concentration	
	Control mice (nmol/g)	Drug-treated mice (% of control)
IMP	11.0 ± 0.5	52*
Total adenylates	1,817.0 ± 164.7	52*
Total guanylates	385.1 ± 23.9	57*
NAD	292.6 ± 15.5	73*
Total uridylates	502.8 ± 49.6	68
Total cytidylates	164.2 ± 13.6	77

Rats bearing s.c. hepatoma 3924A were injected i.p. with drug (400 mg/kg); 6 h later, tumors were freeze-clamped and nucleotide pools were determined as detailed in Materials and methods. Four or more rats were in each experimental group; data represent the means ± SE

* Significantly different from control values ($P < 0.05$)

none was recorded against either the CX-1 colon or the LX-1 lung tumors (Table 1). Significant inhibition of tumor growth (T/C $\leq$ 10%) was observed over a 4-fold dose range following s.c. administration of the compound on days 1, 5, and 9. Doses of both 900 and 450 mg/kg per day caused tumor regression, with 2/6 neoplasms regressing completely at each dose level. In another experiment (not shown), an 1,800-mg/kg dose given on the same schedule caused lethality prior to completion of therapy, whereas doses of 900 and 450 mg/kg resulted in complete tumor regression in 100% of the mice.

Cytotoxicity studies

The L-cysteine derivative showed potent cytotoxicity in hepatoma 3924A cells in culture. In a clonogenic assay, exposure of these cells to the compound for 1 and 7 days yielded an LC_{50} of 15 and 6 μM, respectively.

The structures of L-glutamine and L-cysteine derivative indicated similarities (Fig. 1). If the structural resemblance relates to the drug's activity, L-glutamine should protect cells from the antagonist's cytotoxicity. To test this hypothesis, hepatoma 3924A cells were first adapted to grow in the presence of medium with a low L-glutamine content; after 30 generations, cells were incubated with the antimetabolite (6 μM) and/or L-glutamine (0.5, 1.5, or 3.0 m*M*) and the effect on clonogenicity was determined. At concentrations of 1.5 and 3 m*M*, L-glutamine partially protected the hepatoma cells (56% and 71%, respectively) from the compound's cytotoxicity. This suggested that L-cysteine derivative might be an L-glutamine antagonist. However, the thioester function of the compound could result in non-specific carbamylation, a cytotoxic event that could explain the poor protective potency of L-glutamine [16].

To further clarify the in vivo effect of L-cysteine derivative on L-glutamine and L-glutamate pools, rats bearing hepatoma 3924A were injected with the drug (Table 2). At doses of 200 and 400 mg/kg, the compound significantly decreased L-glutamine concentrations to 55% and 33% of control values, respectively, showing that the drug acted as an antagonist of L-glutamine. L-Glutamate concentrations also significantly decreased to 67% and 71% of control levels at doses of 200 and 400 mg/kg, respectively.

The effect of L-glutamine antagonist on the activities of L-glutamine-utilizing enzymes of purine and pyrimidine nucleotide biosynthesis was examined in rats transplanted with hepatoma 3924A (Table 3). Among the purine synthetic enzymes, drug injection decreased the activities of amidophosphoribosyltransferase, FGAM synthase, and GMP synthase; however, the activities of the L-glutamine-utilizing enzymes of pyrimidine biosynthesis, carbamoyl-phosphate synthase II and CTP synthase, were not reduced.

To determine whether the selective effect of the drug on L-glutamine-utilizing enzymes of nucleotide biosynthesis was reflected in the pools of nucleotides, hepatoma 3924A-bearing rats were injected with the antimetabolite. After 6 h, as indicated in Table 4, the pools of purine nucleotides (IMP, NAD, and total adenylates and guanylates) were significantly reduced, whereas those of pyrimidine nucleotides (total uridylates and cytidylates) were not.

Comparison of biochemical action of the new L-cysteine derivative and the anti-glutamine agent acivicin

Evidence for an anti-glutaminic role for the new L-cysteine derivative is provided by the structure of the compound, which relates to L-glutamine and its protection against the drug's cytotoxicity in hepatoma 3924A cells. The L-cysteine derivative resembles acivicin in its cytotoxic action in L1210 and hepatoma 3924A cells [4, 10, 22]. Differences between the effects of the two compounds involve the depletion of the pools of L-glutamine and L-glutamate by the cysteine derivative, whereas L-glutamine concentrations increase after acivicin treatment [10]. Furthermore, acivicin decreases the activity of all five glutamine-utilizing enzymes of purine and pyrimidine biosynthesis [6, 10, 22], whereas the new derivative selectively inhibited only the enzymes of glutamine use in purine, without affecting those of pyrimidine biosynthesis (Table 3). The selective action of the L-cysteine derivative on glutamine-utilizing enzymes of purine biosynthesis was corroborated by the selective reduction in the concentrations of the purine nucleotides (IMP and the total adenylates and guanylates), with no effect on the pools of uridylates and cytidylates (Table 4).

S-Carbamoyl-L-cysteine exhibits antimicrobial activity [17]. Growth inhibitory activity of the compound in *L. arabinosus* has been partially reversed by L-glutamine [17]. *S*-Carbamoyl-L-cysteine also shows antitumor activity against mammary adenocarcinoma in mice [19]. A series of *S*-(alkyl- and arylcarbamoyl)-L-cysteine derivatives have been prepared and evaluated in a microbial system [18]. *S*-(ethyl-, propyl- and butylcarbamoyl)-Cysteines were inhibitory to the growth of *S. lactis*. These compounds also inhibited L-glutamine use by L-asparagine synthase [18]. *S*-Carbamoyl-L-cysteine and its methyl derivative have inhibited GMP synthase activity in crude bacterial extracts [23].

Novel aspects of the present study

1. This is the first detailed report on the antitumor activity and mechanism of action of an L-cysteine derivative of *S*-carbamoyl cysteine.
2. The compound is shown to be an L-glutamine antagonist.
3. This is the first report in which L-glutamine antagonist selectively inhibited purine nucleotide biosynthesis.
4. The compound is a promising agent for combination with a pyrimidine analog to curtail total nucleic acid biosynthesis.

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