Cancer Chemother Pharmacol (1984) 13:22–26

Cancer Chemotherapy and Pharmacology

Antitumor activity of a nitrosourea derivative, CNUA, on murine tumors

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Summary. *The antitumor activity of a new derivative of nitrosourea, 3-[3-(2-chloroethyl)-3-nitrosoureido]-3-deoxy-D-allose (CNUA), against murine tumors was studied. CNUA showed significant antitumor activity against L1210 leukemia, Lewis lung carcinoma, B-16 melanoma and autochthonous lung tumor induced by 1-ethyl-1-nitrosourea. The effect of CNUA, chlorozotocin, and ACNU on the peripheral white blood cell count (WBC) in normal CDF_1 mice was examined. The lowest WBC count occurred 3 days after administration at the therapeutic dose level and the decreased value returned to the normal level 7–14 days following administration of CNUA and chlorozotocin. CNUA also exerted a depressive action on both humoral and cell-mediated immune response to sheep red blood cells determined by the serum hemagglutinin titer, plaque-forming cells in the spleen, and delayed-type hypersensitivity reaction, while the suppression was almost the same or less than that obtained with chlorozotocin when compared at the dose resulting in similar antitumor activity. These findings suggest that the antitumor activity of CNUA was not at all inferior to those of other nitrosoureas. The bone marrow toxicity was moderate and did not last long.*

Introduction

The chloroethyl nitrosoureas, such as CCNU, methyl-CCNU, and BCNU, are an important class of antitumor agents with a wide spectrum of activity in the chemotherapy of experimental and human tumors. However, these drugs produce delayed and cumulative bone marrow toxicity, which limit their clinical usefulness [6, 8].

Chlorozotocin (CZT), which is the 2-chloroethyl analog of the antitumor antibiotic streptozotocin, was synthesized by Johnston et al. [12] in 1974. It has a chloroethyl nitrosourea linked at the C-2 position of the glucose moiety, and showed antitumor activity with less bone marrow toxicity than hydrophobic nitrosoureas [1, 15]. This finding suggested that bone marrow toxicity, which limited the clinical usefulness, was reduced by the addition of a glucose carrier to a nitrosourea moiety. Since then, many derivatives of nitrosourea showing remarkable antitumor activity with reduced toxicity for the bone marrow have been reported, including: 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea (GANU) [2, 7, 11], a C-1-substituted glucose derivative, methyl-6-[[[(2-chloroethyl)-nitrosoamino]-carbonyl]amino]-6-deoxy-α-D-glucopyranoside (MCNU) [17], a C-6-substituted glucose derivative and 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) [3, 14], a pyrimidine analog.

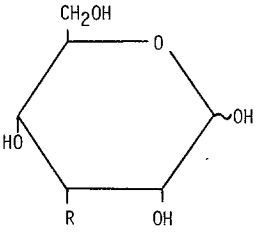

Fig. 1. Structure of CNUA

Recently, we have synthesized a new hydrophilic derivative, 3-[3-(2-chloroethyl)-3-nitrosoureido]-3-deoxy-D-glucopyranose (CNUG), in which a chloroethyl nitrosourea group is linked to the C-3 position of the deoxy-glucose moiety. This compound showed remarkable antitumor effects on various murine tumors with reduced toxicity to the bone marrow [13]. In this paper, we report on the antitumor activity of 3-[3-(2-chloroethyl)-3-nitrosoureido]-3-deoxy-D-allose (CNUA), the stereo isomer of CNUG, against various murine tumors. Its structure is shown in Fig. 1.

Materials and methods

Animals and tumors. Male CDF_1 (BALB/c × DBA/2) mice, BDF_1 (C57BL/6 × DBA/2) mice, and ddY mice weighing 18–25 g and 5–7 weeks of age were obtained from the Shizuoka Agricultural Cooperative Association (Hamamatsu, Japan). The animals were maintained on laboratory diet and water ad libitum.

Lymphoid leukemia L1210, Lewis lung carcinoma, and B-16 melanoma were obtained from Dr Tsukagoshi of the Cancer Chemotherapy Center (Tokyo), and lymphoid leukemia L1210 was maintained by serial passage in DBA/2 mice and transplanted by IP inoculation into CDF_1 mice (1×10^5/mouse); the latter two tumors were maintained in C57BL/6 mice. For tumor transplantation, tumors previously minced finely with scissors and filtered through a 60-mesh stainless steel sieve were inoculated SC into BDF_1 mice (5×10^5 cells/mouse).

Antitumor activity was evaluated by the increase in life-span (ILS): $(T/C - 1) \times 100\%$, where T is the median

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Table 1. Effect of drugs tested on L1210 leukemia

Expt no.	System[a]	Drug	Dose range (mg/kg/day)	Optimal dose (mg/kg/day)	ILS[b] (%)	60-Day[c] survivors
1	IP–IP	CNUA	70–10	40	–	7/7
	D_1	CZT	50– 5	25	129	4/7
		ACNU	60–10	35	–	7/7
2	IP–IP	CNUA	20– 5	14	–	7/7
	D_{1-5}	CZT	16– 4	8	213	6/7
		ACNU	20– 4	16	263	6/7
3	IP–IV	CNUA	60–10	40	214	6/7
	D_1	CZT	60– 5	30	107	5/7
		ACNU	60–10	30	357	6/7
4	IP–PO	CNUA	300–50	200	250	6/7
	D_1	CZT	200–50	200	13	0/7
		ACNU	120–22.5	45	213	5/7

[a] L1210 cells (1×10^5/mouse) were inoculated IP into CDF_1 mice on day 0. Drugs were given IP, IV, or PO on day 1 (D_1) or daily from days 1 to 5 (D_{1-5})
[b] Median survival of controls was 7 days in Expts 1 and 3 and 8 days in Expts 2 and 4. Data refer to dead mice only
[c] No. of 60-day survivors at optimal dose/no. treated

survival in days (MSD) of the treated group and C is the MSD of the control group. Survival of mice was scored 60 days after inoculation of tumors, and mice remaining alive at this time were considered as cured.

Lung tumors were developed according to the method previously described [18]. Briefly, pregnant ddY mice were each given a single IP injection of 100 mg/kg of 1-ethyl-1-nitrosourea (ENU) in water on day 16 or 17 of gestation. Five weeks after birth, five mice were sacrificed to confirm the development of lung tumors, and the rest of the mice were used for the chemotherapeutic study. They were sacrificed 9 weeks after birth, and the size of the tumors observed on the lung surface under a dissecting microscope was measured.

Drugs. CNUA and chlorozotocin were synthesized in our laboratory. ACNU was commercially available. They were dissolved in sterile physiological saline and administered at a volume of 0.1 ml/10 g body weight.

Peripheral white blood cell (WBC) count. Male CDF_1 mice, in groups of six each, were each given a single IP injection of one of the drugs. Blood (5 μl) was withdrawn from the caudal vein periodically, and the WBC were counted with a micro-cell counter (TOA model CC-108).

Antibody titration. Mice were immunized by IP injection with 5×10^8 sheep red blood cells (SRBC). Four days later, blood was obtained by cardiac puncture. Serum (50 μl) was serially diluted in veronal-buffered saline containing 0.1% human serum albumin in a microtiter plate. After dilution, 50 μl of the above buffer containing 1×10^8 SRBC/ml was added. The mean titer was expressed in $\log_2$ of the highest dilution of serum showing complete hemagglutination.

Antibody-forming cells. Mice were immunized as described above; 5 days later the mice were sacrificed, and hemolytic plaque-forming cells (PFC) in their spleens were assayed according to the method of Cunnigham and Szenberg [5].

Delayed-type hypersensitivity. Mice were immunized SC with 5% SRBC in a 0.1 ml volume. Four days later, 0.02 ml of a 20% suspension of SRBC was injected into the rear footpad. Footpad thickness was measured with a dial thickness gauge 24 h after challenge.

Results

Effect on L1210 leukemia

The antitumor activity of CNUA, CZT, and ACNU on L1210 leukemia is shown in Table 1. Following a single IP injection of CNUA at a dose of 40 mg/kg or 50 mg/kg, seven of seven mice survived for more than 60 days. Daily injections of CNUA for 5 successive days at doses of 10 or 14 mg/kg/day were also effective. All mice survived for more than 60 days. Treatment with 40 mg/kg IV produced 214% ILS, and six of seven mice survived for more than 60 days. When CNUA was administered PO at a dose of 200 mg/kg, six of seven mice survived for more than 60 days. CNUA and ACNU showed similar antitumor effects following IP, IV, and PO administration. In contrast, CZT administered PO was less effective against L1210 leukemia.

Effect on Lewis lung carcinoma

Table 2 shows the antitumor effect of CNUA on Lewis lung carcinoma. When mice were given a single IV injection of CNUA at a dose of 40 mg/kg on day 1, five of seven survived for more than 60 days. When drugs were administered IV on day 7 after implantation of the tumor, an injection of CNUA or ACNU at a dose of 30 mg/kg gave an ILS value of 71%. On the other hand, injection of CZT caused no prolongation of the life-span of the treated mice. The effect of CNUA on Lewis lung carcinoma implanted IV was investigated. The survival time of individual animals in the nontreated group was 18–23 days, and tumor growth was observed only in the lung macroscopically. As shown in Table 2, five or all of seven mice were cured at a dose of 20 or 30 mg/kg, respectively.

Effect on B-16 melanoma

As shown in Table 3, a single injection of 30 mg/kg of CNUA yielded an ILS value of 158%, and five of seven mice survived for more than 60 days. The antitumor activity of CNUA was

Table 2. Effect on Lewis lung carcinoma

Expt no.	System[a]	Drug	Dose range (mg/kg/day)	Optimal dose (mg/kg/day)	ILS[b] (%)	60-Day survivors
1	SC–IV	CNUA	50– 5	40	42	5/7
	D_1	CZT	40– 5	20	33	1/7
		ACNU	50–10	30	31	5/7
2	SC–IV	CNUA	50–20	30	71	0/7
	D_7	CZT	40–10	20	0	0/7
		ACNU	50–20	30	71	0/7
3	IV–IV	CNUA	50–20	30	–	7/7
	D_1	CZT	40–10	20	63	6/7
		ACNU	40–10	30	–	7/7

[a] Lewis lung carcinoma cells (5×10^5/mouse) were inoculated SC into BDF_1 mice on day 0
[b] Median survival of control was 26 days in Expt 1, 24 days in Expt 2, and 19 days in Expt 3. Data refer to dead mice only

Table 3. Effect on B-16 melanoma

Expt no.	System[a]	Drug	Dose range (mg/kg/day)	Optimal dose (mg/kg/day)	ILS[b] (%)	60-Day survivors
1	SC–IV	CNUA	50– 10	30	158	5/7
	D_1	CZT	40– 10	30	132	1/7
		ACNU	60– 10	40	68	5/7
2	SC–PO	CNUA	300–100	200	58	3/7
	D_1	CZT	200– 75	200	17	0/7
		ACNU	90– 30	45	42	3/7

[a] B-16 melanoma cells (5×10^5/mouse) were inoculated SC into BDF_1 mice on day 0
[b] Median survival of controls was 19 days in Expt 1 and 24 days in Expt 2

almost the same as that of ACNU. A single oral administration of CNUA on day 1 was also effective against B-16 melanoma, and treatment with 300 mg/kg was curative for four of seven mice.

Effect on autochthonous lung tumors

The antitumor activity of CNUA, ACNU, and CZT on autochthonous lung tumors induced by ENU was investigated. Five weeks after birth, mice with lung tumors were treated IV or PO with several treatment schedules, and they were sacrificed 9 weeks after birth. The diameter of the tumors observed on the surface of the lung were measured under a dissecting microscope, and since the tumor nodules were almost round, the area of the tumor was calculated from the diameter. Table 4 shows the effect of CNUA administered IV or PO once a week for 4 weeks. When CNUA was administered IV at a dose of 80 mg/kg, the number of tumors observed on the lung surface was 31.0 and the total tumor size was 5.9 mm^2, while the corresponding data in the controls were 46.1 and 20.3 mm^2, respectively. The inhibition ratio for the number of tumors was 33% and that for the size of the tumors was 61%. When CNUA was administered PO by the treatment schedule shown in Table 4, a 30%–60% inhibition of the mean number of tumors and a 50%–70% inhibition of total tumor size was observed at doses of 200–400 mg/kg. Administration of ACNU at a dose of 120 mg/kg gave a 35% inhibition for numbers of tumors and 60% for tumor size. CNUA and ACNU showed almost equal efficacy against autochthonous lung tumors. On the other hand, administration of CZT produced inhibition of tumor growth but no statistically significant inhibition of the number of tumors was observed.

Table 4. Antitumor activity of CNUA against autochthonous lung tumor induced by ENU

	Sample	Total dose (mg/kg)	No. of tumor	Total tumor size (mm^2)
I[a]	Control	–	30.3 ± 7.6[b] (100)[c]	13.6 ± 6.1 (100)
	CNUA	200	20.0 ± 5.6 (66)	6.8 ± 4.4 (50)
		300	20.3 ± 6.5 (67)	6.6 ± 3.4 (49)
		400	11.6 ± 5.6 (38)	3.8 ± 2.6 (28)
	CZT	300	35.5 ± 11.1 (117)	11.0 ± 4.0 (81)
		400	26.1 ± 7.3 (86)	9.6 ± 3.9 (71)
		600	27.7 ± 11.5 (91)	9.4 ± 3.0 (69)
	ACNU	80	24.9 ± 5.5 (82)	6.9 ± 1.9 (51)
		120	19.7 ± 6.8 (65)	5.4 ± 2.8 (40)
		160	18.0 ± 3.7 (59)	4.0 ± 2.7 (29)
II	Control	–	46.1 ± 14.2 (100)	20.3 ± 12.1 (100)
	CNUA	40	40.0 ± 10.2 (87)	10.8 ± 4.2 (53)
		80	31.0 ± 8.8 (67)	5.9 ± 2.4 (29)

[a] I, administered PO; II, administered IV
[b] Means ± SD
[c] No. in parentheses indicates percent of control value in each case

Effect on WBC count

A group of six normal CDF_1 mice received a single IP injection of CNUA, and serial peripheral WBC counts were compared with those of mice that received CZT or ACNU. Each drug was administered IP at the optimal dose for L1210 leukemia. As shown in Fig. 2, the lowest WBC count occurred 3 days

Table 5. Differential effect of CNUA, CZT, and ACNU on peripheral WBC counts at nadir

Sample	Dose (mg/kg)	Lethality[a] (%)	Nadir WBC[b] (% control)	L1210 activity[c]
CNUA	20	0	88	2/7
	30	0	65	6/7
	40	0	49	7/7
	60	50	36	2/7
CZT	10	0	81	2/7
	20	0	66	3/7
	30	20	44	4/7
ACNU	10	0	68	0/7
	20	0	54	3/7
	40	0	28	7/7

Drugs were given to CDF_1 mice IP on day 0
[a] % Lethality in normal CDF_1 mouse
[b] The nadir WBC counts occurred 3 days after injection. Mean WBC counts in controls were 7,160 ± 1,100/mm^3
[c] No. of 60-day survivors/no. treated

Fig. 2. Effect on peripheral WBC count in normal CDF_1 mice after a single IP injection at the optimal dose for L1210 leukemia. Mean ± SD WBC counts in controls were 9,970 ± 1,060/mm^3 (day 3), 10,260 ± 2,020/mm^3 (day 7), and 10,280 ± 980/mm^3 (day 14)

Table 6. PFC in mice treated with CNUA and CZT at various times from immunization with SRBC on day 0

Sample	Day of administration	log PFC/spleen (mean ± SD)
Control	–	5.75 ± 0.14
CNUA	−3	4.40 ± 0.26
	0	4.62 ± 0.50
	+3	4.61 ± 0.50
Control	–	5.43 ± 0.31
CZT	−3	3.82 ± 0.16
	0	4.24 ± 0.58
	+3	4.44 ± 0.67

Normal CDF_1 mice received CNUA (40 mg/kg) or CZT (20 mg/kg) IP

Table 7. Effect of CNUA and CZT on immune response

Sample	Dose (mg/kg)	log PFC/spleen	$\log_2$ HA titer	DTH (mm)
Control	–	5.72 ± 0.16[a]	12.8 ± 1.3	1.12 ± 0.04
CNUA	10	5.43 ± 0.31	13.8 ± 1.6	1.06 ± 0.23
	20	4.78 ± 0.22	10.2 ± 2.1	0.95 ± 0.24
	40	3.83 ± 0.20	4.2 ± 2.3	0.42 ± 0.22
CZT	5	5.19 ± 0.65	10.9 ± 3.1	0.75 ± 0.18
	10	4.57 ± 0.86	8.4 ± 3.1	0.57 ± 0.18
	20	4.03 ± 0.22	6.4 ± 2.8	0.48 ± 0.13

Drugs were administered IP/3 days before immunization of CDF_1 mice with SRBC
[a] Mean ± SD

after administration at a optimal dose for antileukemic activity, CNUA producing a 45% reduction in the mean WBC count on day 3 while a 80% reduction in WBC count was produced when ACNU was administered. Furthermore, CNUA administered at a 50% lethal dose, 60 mg/kg, produced only about a 65% reduction in the minimum WBC count (Table 5). It was observed that the WBC depression caused by the administration of CNUA or CZT recovered to the normal level over 7–14 days following the injection. Following administration of ACNU however, there was still a 32% reduction in the WBC count on day 14.

Effect on immune response

The effects of CNUA and CZT on the hemagglutinin titer, plaque-forming cells, and delayed-type hypersensitivity reaction were examined. To determine the effect of the drugs on the immunization periods, CNUA or CZT was administered IP simultaneously with SRBC 3 days before or after immunization with SRBC. With these administration schedules, relatively marked suppression was observed when drugs were administered 3 days before immunization (Table 6). Next, the relationship between dosage and immunosuppressive activity was determined when mice received the drug 3 days before immunization. As shown in Table 7, pronounced immunosuppression was noted at the maximum dose used of each drug, and this suppression decreased with decreasing dosage.

Discussion

CNUA showed significant antitumor activity against various murine experimental tumors. The majority of mice bearing L1210 leukemia were cured when they received CNUA IV, IP, or PO. CNUA was effective not only against transplantable solid tumors, such as Lewis lung carcinoma and B-16 melanoma, but also against autochthonous lung tumors induced by ENU. CNUA showed antitumor activity similar to that of ACNU and was superior to CZT. The antitumor activity of CNUG, a C-3-substituted glucose derivative, on murine tumors was previously reported; however, this agent did not show any activity following oral administration. The structure of CNUA differs from that of CNUG only in the stereochemical substitution of a nitrosourea, but a difference in antitumor activity on oral administration was observed between CNUA and CNUG. The reason for this discrepancy is not clear at present, but the absorption may differ between glucose and allose.

It has been reported that bone marrow suppression by the nitrosourea group in antitumor agents is often the most important dose-limiting toxicity. Thus, the effect of CNUA on the peripheral WBC count was compared with that of CZT and ACNU. CNUA caused less WBC depression than ACNU and a similar degree of WBC depression to CZT at the therapeutic dose level, and the depression recovered to the normal level within a short period. In addition, in a pathological study of the bone marrow, it was demonstrated that severe lesions of myeloid cells appeared 3 days after administration of ACNU and lasted for 7 days, while with CNUA and CZT lesions of the myeloid cells were also seen on day 3 but remarkable hyperplasia of myeloid cells was observed 7 days after administration (data not shown). It has been reported that only a glucose moiety can selectively reduce bone marrow toxicity of nitrosourea without correlation between myelotoxicity and alkylating activity, carbamoylating activity, water solubility, or log P value [10]. However, CNUA has allose, a stereoisomer of glucose, in this structure, and it is suggested that the allose moiety may also reduce bone marrow toxicity.

Most antitumor agents cause immunodepression of the host as a side-effect. It has been reported that tumor-specific and/or tumor-associated antigens exist in human tumors [9, 16], so that immunodepression of the host by antitumor agents may mean a decrease in the therapeutic effect. Therefore, the degree of selectivity of antitumor agents must also be evaluated with reference to their potential immunosuppressive activity. In the present study, the effect of CNUA on immune response was compared with that of CZT, which was found to have little bone marrow toxicity. It was observed that CNUA and CZT exert a depressive action on both humoral and cell-mediated immune response when administered 3 days before immunization. Cavanna et al. [4] reported that CZT produced immunosuppression when administered before the antigen, and our data are in accord with these observations. However, CNUA depressed the immune responses to almost the same degree CZT or a lesser degree when compared at the dose which resulted in similar antitumor activity against L1210 leukemia under the present experimental conditions. It is considered that CNUA was not at all inferior to other nitrosoureas in antitumor activity, and that its bone marrow toxicity was moderate and did not last long.

Acknowledgement. This work was partially supported by a Grant-in-Aid for Cancer Research from Ministry of Health and Welfare.

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Received September 15, 1983/Accepted January 18, 1984