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3-(Tetraacetyl Glucopyranos-2-yl)-1-(2-chloroethyl)-1-nitrosourea, an Antitumor Agent with Modified Bone Marrow Toxicity

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SUMMARY

3-(Tetraacetyl glucopyranose-2-yl)-1-(2-chloroethyl)-1-nitrosourea (GCNU) is an aminoglucose chloroethylnitrosourea structurally related to the nonmyelosuppressive but diabetogenic antitumor agent, streptozotocin. GCNU is active against L1210, producing a greater than 100% increase in life-span at a 10% lethal dose without an accompanying leukopenia. In contrast, treatment with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea or 1,3-bis(2-chloroethyl)-1-nitrosourea at doses of comparable overall toxicity produced a sustained 60 to 75% reduction in peripheral white blood cell counts. Twenty-four hr after administration, GCNU inhibited DNA synthesis in L1210 and gastrointestinal mucosa but spared the bone marrow. In contrast, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea nonselectively inhibited all three tissues.

Nitrosoureas and nitrosamines that have a R—N(NO)—$(CH_2)_{1-2}$ H_1 but not a chloroethyl end group depress hepatic nicotinamide adenine dinucleotide concentrations, the proposed mechanism of diabetogenicity of streptozotocin. GCNU, a chloroethylnitrosourea, does not depress nicotinamide adenine dinucleotide and is not diabetogenic in the mouse.

These structure-activity studies suggest that the addition of an aminoglucose carrier to a cytotoxic nitrosourea moiety can selectively reduce bone marrow toxicity while retaining antitumor activity.

INTRODUCTION

The treatment limiting toxicity of the principal nitrosourea anticancer agents in clinical use, BCNU[2] and CCNU, is myelosuppression (2, 3). In previous structure-activity studies with nitrosourea antitumor agents, specific chemical features have been identified that modify toxicity. The placement of an aminoglucose carrier on the N-3 position of 1-methyl-1-nitrosourea, a bone marrow toxin in animals, results in the formation of a nonmyelosuppressive but diabetogenic antitumor agent streptozotocin (10). An additional observation was the identification of a class of nitrosoureas and nitrosamine compounds with a R—N(NO)—$(CH_2)_{1-2}H$ end group that depresses pyridine nucleotide concentrations in liver, the proposed mechanism of diabetogenicity of streptozotocin (8, 9). Nitrosoureas possessing a chloroethyl end group, represented by BCNU and CCNU, were shown not to affect NAD concentrations (8).

The subject of this report is the preliminary investigation of a newly synthesized aminoglucose nitrosourea containing a chloroethyl end group, GCNU (Chart 1). The toxicity and biochemical activity of this compound is compared with previously studied nitrosourea antitumor agents in an attempt to confirm the importance of the aminoglucose carrier in modifying bone marrow toxicity. The importance of NAD depression for nitrosourea-related diabetogenic activity is also examined.

MATERIALS AND METHODS

Male C57BL/6 × DBA/2 F_1 mice, weighing 18 to 25 g and maintained on Purina laboratory chow pellets and water *ad libitum*, were used for all studies. GCNU and CCNU [CCNU (NSC 79037)] were suspended in an aqueous solution of 5.0% polyethoxylated vegetable oil and 5.0% ethanol (Antara Chemicals, New York, N. Y.). BCNU (NSC 409962) was dissolved in 10% ethanol in water. Streptozotocin (NSC 85998) was dissolved in 0.005 M citrate buffer, pH 4.6. All drugs were administered i.p. at a volume of 1 ml/100 g body weight.

Survival studies of antitumor activity were conducted in mice given inoculations i.p. of a 10^5 cell suspension of L1210, with drug treatment on the 2nd day of tumor growth. Percentage of increase in life-span was calculated by the ratio of survival time in days of treated *versus* control group according to the Cancer Chemotherapy National Service Center protocol (Ref. 6, p. 3). Investigation of the effect of treatment on DNA synthesis was carried out with mice receiving the same L1210 tumor inoculum. On the 6th day of tumor growth the animals were given GCNU (20 mg/kg), CCNU (50 mg/kg), or the vegetable oil ethanol vehicle i.p. and sacrificed at 8, 24, 48, or 72 hr after

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[2] The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; GCNU, 3-(tetraacetyl glucopyranose-2-yl)-1-(2-chloroethyl)-1-nitrosourea; LD_{10}, 10% lethal dose; LD_{20}, 20% lethal dose,

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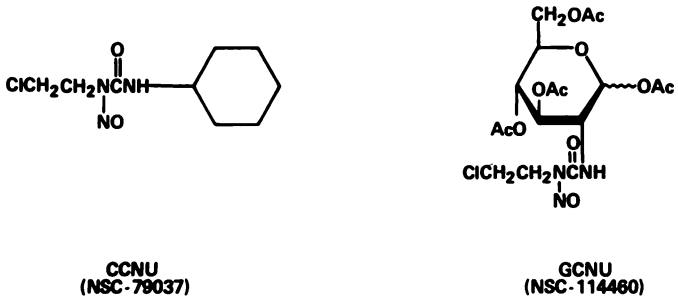
treatment. One hr prior to sacrifice the mice were given injections of 100 μCi thymidine-^{3}H (specific activity, 1.9 Ci/mmole; New England Nuclear, Boston, Mass.) i.p. Animals were killed by cervical traction and the ascitic tumor was aspirated from the abdominal cavity into 0.0067 M phosphate-buffered 0.85% NaCl solution, pH 7.4, at 4°. The duodenum of each animal was split lengthwise and the mucosa was scraped from the muscularis and placed into 0.0067 M phosphate-buffered 0.85% NaCl solution. Both tibias were removed and the bone marrow was expressed with 0.0067 M phosphate-buffered 0.85% NaCl solution. We have previously determined that the bone marrows of mice examined after 6 days of i.p. L1210 growth are not replaced by tumor (11). The samples of ascites, duodenal mucosa, and bone marrow from 3 mice in each tumor group were pooled. The DNA content of each pooled specimen was extracted by a modification of the method of Schneider (7) and a 0.5-ml aliquot of the final supernatant was added to 15 ml of Aquasol (New England Nuclear) and counted in a Packard Tri-Carb liquid scintillation spectrometer. An additional 0.5-ml aliquot was used for the measurement of DNA by the method of Burton (1). Results are expressed as cpm/μg DNA.



CCNU (NSC-79037) GCNU (NSC-114460)

Chart 1. Comparison of the chemical structures of CCNU and GCNU.

WBC suspended in Isoton (Scientific Products, Washington, D. C.) were counted in a Model F Coulter counter after treatment with Zap-Isoton (Coulter Diagnostics, Inc., Hialeah, Fla.).

Blood glucose determinations in mice eating *ad libitum* were performed using the method of Kingsley and Getchell (4).

The NAD content of liver was assayed enzymatically using alcohol dehydrogenase (Sigma Chemical Co., St. Louis, Mo. (5).

RESULTS

Lethality and WBC Count Depression in Normal BDF$_1$ Mice. Animals were given single doses of GCNU ranging from 10 to 50 mg/kg ip. Lethality was recorded and peripheral WBC counts were serially measured over a 30-day period and compared with those obtained with an LD_{10-20} of BCNU or CCNU (Table 1). All deaths occurred within Days 5 to 14 after treatment. An LD_{10} of GCNU (15 mg/kg) produced no leukopenia. At a 40% lethal dose of 25 mg/kg there was a reduction in mean WBC count from 12,740/cu mm for control to 9,529/cu mm at Day 5 posttreatment with a rapid return to pretreatment levels. GCNU at a 100% lethal dose of 50 mg/kg produced definite leukopenia (5117/cu mm) at Day 4 with all animals dying on Day 5 or 6.

The WBC count obtained using a 40% lethal dose of GCNU (25 mg/kg) were simultaneously compared with those obtained using an LD_{10} of BCNU (30 mg/kg) and an LD_{20} of CCNU (50 mg/kg). With the latter 2 agents a major degree of leukopenia was produced by the 5th posttreatment day, mean WBC count being 3610 and 1144/cu mm for BCNU and CCNU, respectively, with a

Table 1

Peripheral WBC counts and survival in normal BDF mice after treatment with GCNU as compared with BCNU or CCNU

Treatment	Dose (mg/kg, i.p.)	Mean and range of WBC/cu mm for 10 mice[a] on Day 5	15	30	% of mice surviving 30 days after treatment
Vehicle[b]		12,740 (7,500-17,700)	14,700 (7,500-20,300)	13,490 (8,500-19,200)	100
GCNU	10	10,956 (8,700-13,600)	12,770 (9,500-14,500)	12,070 (8,100-16,200)	100
	15	11,244 (8,000-14,500)	11,350 (7,900-18,600)	13,978 (8,300-21,300)	90
	20	11,740 (7,800-15,000)	12,030 (9,100-15,400)	12,340 (10,000-18,400)	30
	25	9,529 (7,500-13,200)	11,050 (8,000-14,500)	14,914 (5,800-26,800)	60
	50	5,117[c] (4,600-5,800)			0
BCNU	30	3,610 (1,900-4,800)	6,411 (4,700-9,900)	8,400 (6,000-11,400)	90
CCNU	50	1,144 (500-2,100)	2,614 (1,800-3,400)	8,300 (5,700-11,500)	80

[a] Mean WBC/cu mm for 30 normal untreated BDF$_1$ mice: 12,473 (7,900 to 20,000).
[b] Five % polyethoxylated vegetable oil with 5.0% ethanol, in water.
[c] Day 4 counts; all animals were dead by Days 5 to 6.

gradual but incomplete return toward normal counts by Day 30.

Antitumor Activity of GCNU against i.p. L1210. An LD_{10} of GCNU (15 mg/kg), administered as a single dose on Day 2 after tumor inoculation, produced a 137% increase in life-span compared to vehicle-treated controls. At this dose there were four 30-day survivors. Larger single doses were less effective but did produce one to two 30-day survivors in animals that survived the acute toxicity inherent at these dose levels (Table 2).

Effect of GCNU on *in Vivo* DNA Synthesis. The effect of *in vivo* treatment with GCNU on DNA synthesis of L1210, duodenal mucosa, and bone marrow was studied using a dose in excess of the LD_{10} (20 mg/kg). The results were directly compared with those obtained using an LD_{20} of CCNU in the same experiments. Both drugs produced a major reduction in DNA synthesis of L1210 by 24 hr, an effect that was sustained through the 72-hr test period of the study (Chart 2). The action of GCNU and CCNU was essentially identical on duodenal mucosa, with a maximal inhibition of DNA synthesis to 17% of control at 24 hr postinjection. This was followed by a return toward control

Table 2

Survival of BDF_1 mice inoculated i.p. with 10^5 L1210 cells

Treatment[a]	Dose (mg/kg, i.p.)	Mean survival time (days)	30-day survivors (%)
Vehicle[b]		8.4	
GCNU	10	11.7	
	15	20.1	40
	20	14.4	20
	25	12.6	10
	30	9.6	10

[a] Day 2 only.
[b] Five % polyethoxylated vegetable oil w th 5.0% ethanol.

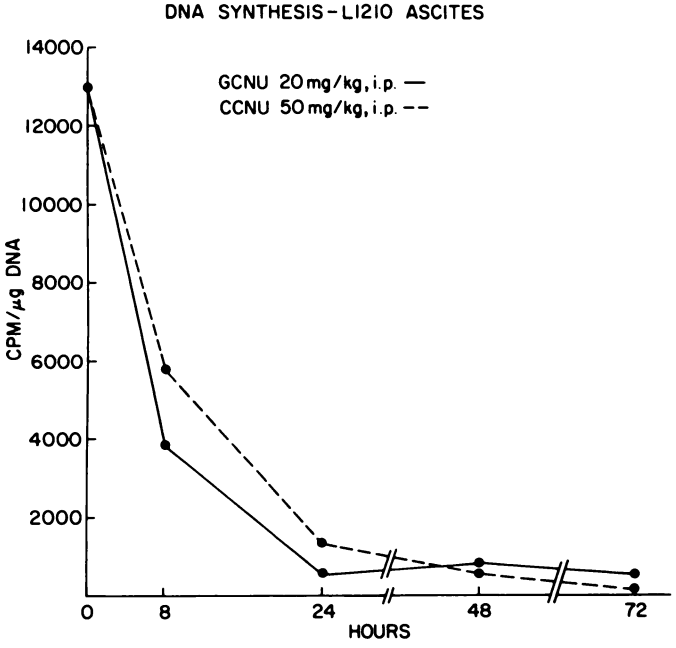


Chart 2. *In vivo* uptake of thymidine-^{3}H into ascitic L1210 after treatment with GCNU or CCNU.

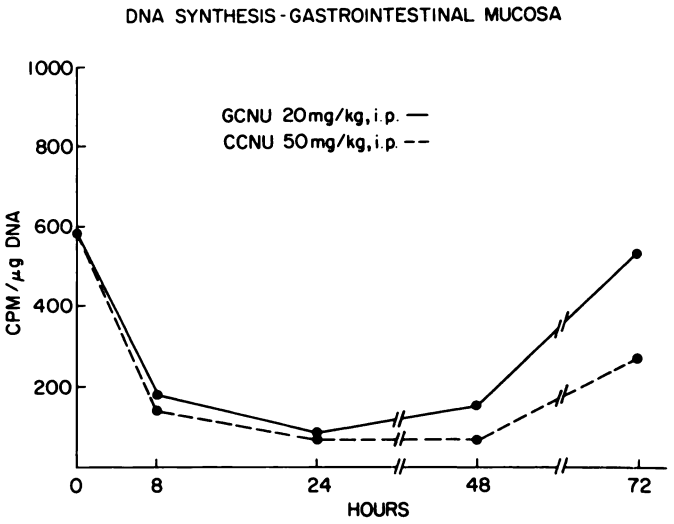


Chart 3. *In vivo* uptake of thymidine-^{3}H into mouse duodenal mucosa after treatment with GCNU or CCNU.

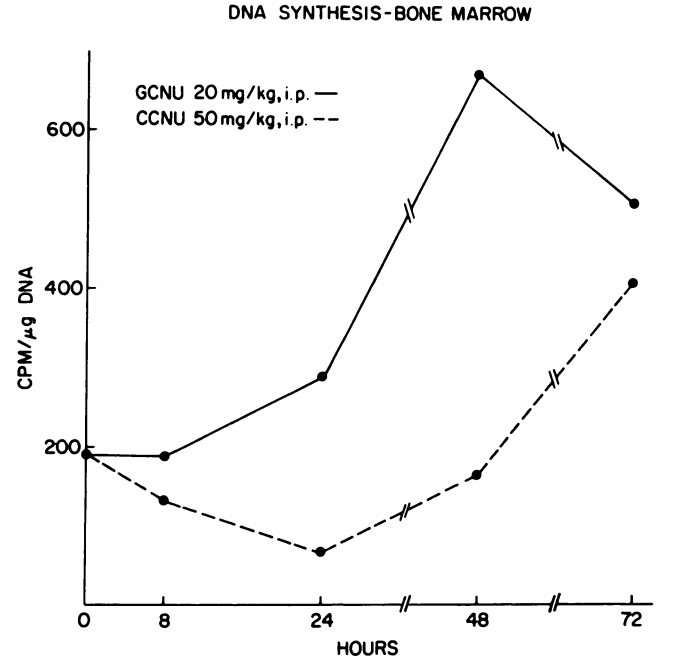


Chart 4. *In vivo* uptake of thymidine-^{3}H into mouse bone marrow after treatment with GCNU or CCNU.

at 72 hr (Chart 3). While a 73% inhibition of DNA synthesis in bone marrow was recorded with CCNU at 24 hr, no depression was recorded with GCNU; both treatments demonstrated a rebound in DNA synthesis, which was maximal at 48 hr with GCNU and at 72 hr with CCNU (Chart 4).

Comparison of Effects of GCNU and Streptozotocin on Blood Glucose and Liver NAD Concentrations. GCNU was administered to mice at graded doses ranging from 10 to 200 mg/kg. There were no changes in blood glucose concentrations (Table 3). In contrast, streptozotocin administered at an LD_{10} (200 mg/kg) (8) was diabetogenic.

GCNU produced no significant effect on liver NAD concentrations in contrast to the marked depression ob-

Table 3
Blood glucose and hepatic NAD concentrations of BDF_1 mice feeding ad libitum after treatment with GCNU or streptozotocin

Treatment	Dose (mg/kg, i.p.)	Blood glucose[a] (mg/100 ml)[b]	No. of mice	NAD[c] (μmoles/g)[b]	No. of mice
Vehicle[d]		182 ± 12.5	7	0.553 ± 0.053	5
GCNU	200	143 ± 10.4	5	0.597 ± 0.100	5
Streptozotocin in citrate buffer, pH 4.0	200	450 ± 55.2	7	0.124 ± 0.011	5

[a] 2 Days after treatment.
[b] Mean ± S.E.
[c] Four hr after treatment.
[d] Five % polyethoxylated vegetable oil with 5.0% ethanol.

served with streptozotocin by 4 hr after administration (Table 3).

DISCUSSION

The myelosuppressive properties of the cytotoxic chloroethylnitrosourea moiety are reduced by the substitution of an aminoglucose for a cyclohexyl or chloroethyl group. No bone marrow depression was observed at an LD_{10} of GCNU, which was in contrast to the moderate to severe leukopenia observed with comparably toxic doses of BCNU and CCNU. However, this bone marrow-sparing property of GCNU is not absolute. The rebound in bone marrow uptake of thymidine-^{3}H at 48 hr after a dose of 20 mg/kg suggests that GCNU had placed some restraint, although compensated, on DNA synthesis, from which there was a release. Myelosuppression could be produced using 100% lethal doses but never was of sufficient magnitude to be implicated as the principal lethal toxicity. The mechanism by which GCNU selectively spares the bone marrow is not known but is presently undergoing investigation. The full spectrum of quantitative organ system toxicities of GCNU has not been examined but will be evaluated in larger animals in which serial clinical chemical determinations are more readily performed. On the basis of the observed decrease of thymidine-^{3}H into duodenal mucosal DNA, gastrointestinal toxicity is to be anticipated. This study suggests that both the toxicity and antitumor activity of GCNU are mediated, at least in part, through an inhibition of DNA synthesis.

The diabetogenic activity of streptozotocin has been correlated with a depression of pyridine nucleotide concentrations (9). Although a group of nitrosourea and nitrosamine compounds have the capacity to reduce liver NAD concentration, streptozotocin is unique for the presence of glucose in its structure and for its diabetogenicity in animals. It has been proposed that the glucose carrier might facilitate the uptake of streptozotocin into the β-cell of the pancreatic islets of Langerhans (8, 10). The placement of a chloride on the ethyl group of a nitrosourea, as exemplified by BCNU and CCNU, results in a compound that has no effect on NAD concentration (8). Although GCNU closely resembles streptozotocin in chemical structure, it contains a terminal chloride; the observed failure of this compound to decrease liver NAD or produce diabetes in mice was anticipated. These structure-activity relationships would predict that a GCNU analog lacking the terminal chloride would have diabetogenic properties.

The potential importance of this study is the prospect of identifying a new class of nitrosourea antitumor agents with modified bone marrow toxicity. If this relationship of aminoglucose modification of bone marrow toxicity is confirmed in additional animal species and man, the use of such compounds would facilitate treatment of patients with neoplastic disease who have preexisting abnormal bone marrow function or would allow for the more effective use of a nitrosourea agent in combination with anticancer agents possessing more potent myelosuppressive properties.

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