

Antitumor Activity of Tetraacetylglucosamine Mustard¹

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SUMMARY

1,3,4,6-Tetra-*O*-acetyl-2-(di-2-chloroethyl)amino-2-deoxy-D-glucopyranose is active against L1210 leukemia, giving over 100% increased life-span at optimal dose. Against P388 leukemia, it gives 200% increased life-span with long-term survivors. The compound is most active when given i.p., but shows some activity when given s.c. and p.o., and is more potent (therapeutic and toxic effect) than mechlorethamine on both a molar and a mg basis. Of importance, the schedule dependency for the administration of 1,3,4,6-tetra-*O*-acetyl-2-(di-2-chloroethyl)amino-2-deoxy-D-glucopyranose in L1210 leukemia differs from most alkylating agents in that it is best given by multiple daily injections rather than as a single large injection on Day 1. This characteristic can be attributed to the aminoglucose moiety.

INTRODUCTION

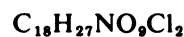
Because of the report by Schein *et al.* (7) of modification of bone marrow toxicity by the addition of an aminoglucose moiety to CCNU³ (NSC 79037) to form GCNU (NSC 114460), we were led to prepare and study a simple aminoglucose mustard and compare it with HN2. The formula of TGM is shown in Chart 1, where it is compared with some related drugs that will be considered in the discussion.

MATERIALS AND METHODS

TGM was synthesized by the following modification of the method of Vargha *et al.* (8). To an aqueous solution (176 ml) containing ethylene oxide (99.0 g, 89 ml), cooled on an ice bath, was added tetra-*O*-acetyl-D-glucosamine (19.65 g,

56.6 mmole). The reaction mixture was kept at 0 to 5° for 12 days, then was evaporated to dryness under reduced pressure. The liquid residue was mixed with 30 ml of methanol:ether (1:9) and chilled in a refrigerator overnight. The deposited white crystals were collected by filtration and dried with the following yield: 9.0 g (36%) of 1,3,4,6-tetra-*O*-acetyl-2-(di-2-hydroxyethyl)amino-2-deoxy-D-glucopyranose; m.p., 130–132° [literature (8), 131–132°].

To a solution of this compound (4.0 g, 9.1 mmole) in chloroform (32 ml) and pyridine (3 ml) at 0°, thionyl chloride (4 ml) was added. This mixture was boiled for 5 hr. The solution was evaporated to dryness under reduced pressure. The residue was mixed with ice water (30 ml), and the aqueous extract was discarded; this procedure was repeated several times. The undissolved material was crystallized from ethanol, yield: 3.33 g (77%); m.p. 102–103° [literature (8), 103–104°]. Elemental analysis of the compound was performed.



Calculated:	C 45.77, H 5.76, N 2.96
Found:	C 45.70, H 5.80, N 2.96

TGM was dissolved in absolute ethanol in a concentration of 10 mg/ml. This solution was diluted 1:20 to 1:100 with 0.9% NaCl solution for animal treatment. In the higher concentrations, a fine precipitate could be observed. Shelf-stored crystalline TGM was stable for over 1 year, and the refrigerated ethanol solution was stable over 3 months as evidenced by unchanged infrared and nuclear magnetic resonance spectra and unchanged melting point, and as demonstrated by the ability to prolong life-span in animals bearing L1210 leukemia. In regard to stability, the drug was more convenient to use than HN2, which was studied for comparative purposes. The standard commercial preparation of HN2 (Merck, Sharp & Dohme, West Point, Pa.) was used in these experiments and was mixed fresh each day it was used. Drugs were administered in volumes of 0.1 to 1.0 ml/injection.

L1210 leukemia was maintained in our laboratory by weekly i.p. passage of 10⁵ L1210 cells into DBA/2 mice. P388 leukemia was maintained by weekly i.p. passage of 10⁶ P388 cells into DBA/2 mice. C57BL/6 × DBA/2 (hereafter called B6D2F₁) female mice were used for the test animals in both cases. L1210 cells (10⁵) or 10⁶ P388 cells were inoculated i.p. into each test animal; life-span was recorded.

For LD₁₀ and LD₅₀ determinations, incremental logarithmic doses of medication were given i.p. to groups of 10 B6D2F₁ female mice individually weighed for calculation of

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³ The abbreviations used are: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; GCNU, 3-(tetraacetyl glucopyranose-2-yl)-1-(2-chloroethyl)-1-nitrosourea; HN2, mechlorethamine (nitrogen mustard); TGM, 1,3,4,6-tetra-*O*-acetyl-2-(di-2-chloroethyl)amino-2-deoxy-D-glucopyranose; LD₁₀, 10% lethal dose; LD₅₀, 50% lethal dose; STZ, streptozotocin; TIC mustard, 5-[3,3-bis (2-chloroethyl)-1-triazeno] imidazole-4-carboxamide; DTIC, 5-(3,3 dimethyl-1-triazeno) imidazole-4-carboxamide.

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dose. The results were plotted on a logarithmic probability graph after 20 days of observation for deaths. LD₁₀ and LD₅₀ values were read from the straight line of best fit.

For pathological study, tumor-inoculated or drug control animals received 3 dose schedules of either TGM or HN2 and were sacrificed on Day 7 or 12 after treatment. The dose range extended from just below optimal dose to above the LD₅₀ dose. Tumor control and control animals were also studied. Samples of various organs were fixed in 10%

buffered formalin. In addition, for bone marrow evaluation, a segment of vertebral bone and the entire sternum were initially fixed in 10% buffered formalin and later were decalcified in equal volumes of 20% sodium citrate and 45% formic acid, then were sectioned to 5 μm, stained either by hematoxylin and eosin or Giemsa, and studied by light microscopy.

RESULTS

Toxicity. Lethal doses of TGM or HN2 killed mice in 7 to 8 days (range, 4 to 10). The LD₁₀ and LD₅₀ values for TGM by i.p. administration were determined to be 2.4 and 3 mg/kg, respectively, and for HN2·HCl, 3.6 and 4.1 mg/kg. Since TGM is a larger molecule (M.W., 472.3; HN2·HCl, 192.5), the aminoglucose derivative is approximately 3.5 times more toxic on a molar basis.

The most striking pathological changes in animals that received lethal doses of TGM (4 mg/kg) were observed in the bone marrow. These changes included marked diminution of hemopoietic cells, extensive dilation of intramarrow capillaries, with focal to diffuse intramarrow hemorrhage. None of the other groups of animals demonstrated marrow changes to this extent, although a similar but much milder change was observed in animals that received 2 mg of TGM per kg or 4.5 or 6 mg doses of HN2·HCl per kg. Animals treated with TGM, 1.25 mg/kg, daily for 8 days or HN2·HCl, 1.5 mg/kg, on Day 1 (therapeutic doses of medication slightly below optimal doses) showed only minimal marrow changes or no change at all at Day 12 after tumor inoculation.

Mice receiving optimal treatment schedules of TGM lost 13 to 25% of their body weight by Day 12. This degree of weight loss was greater than that seen in mice receiving optimal doses of HN2.

Antitumor Activity. On a daily times-8 treatment schedule, the administration of TGM by i.p. injection gave 100% increased life-span in L1210 leukemia (Table 1). The

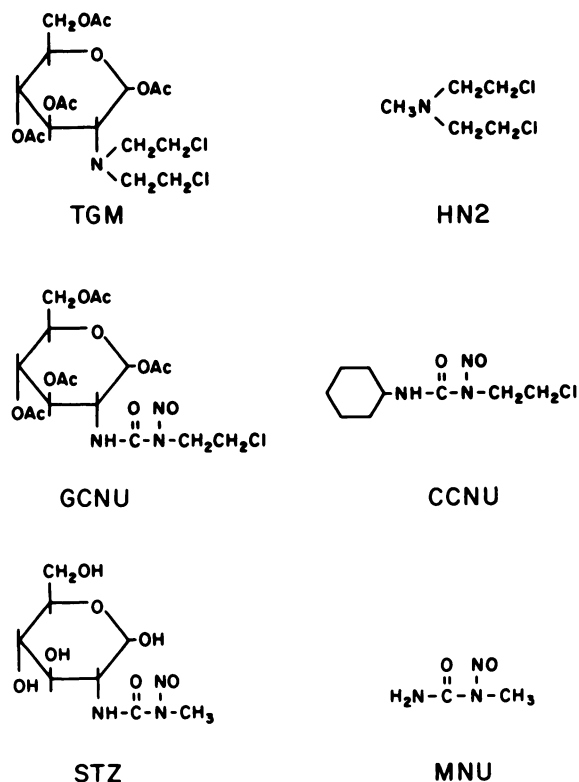


Chart 1. Comparative chemical structures of TGM, HN2, GCNU, CCNU, STZ, and 1-methyl-1-nitrosourea (MNU).

Table 1

Activity of TGM against L1210 leukemia^a daily versus twice-daily administration

An analysis of variance comparing the 5 groups of daily times-8 treatment with the 2 groups of twice daily times-8 treatment results in a F value of 4.38 $p < 0.05$.

Experiment	TGM daily dose ^b (mg/kg)	TGM life-span (mean ± S.E. of 8 animals)	Control life-span (mean ± S.E. of 8 animals)	Increased life-span ^c (%)	Median life-span (treated/control)
1	1.25	17.9 ± 1.4	8.1 ± 0.1	120	17.5/8
2	1.25	16.5 ± 0.9	7.9 ± 0.3	110	17/8
3	1.5	17.1 ± 0.9	8.6 ± 0.4	99	17/8
4	1.33	17.6 ± 1.7	9.3 ± 0.3	91	17.5/9
5	1.67	16.8 ± 1.0	8.4 ± 0.7	100	16.5/8
5	1 (×2)	21.6 ± 2.7 ^d	8.4 ± 0.7	157 ^d	20/8
6	1 (×2)	18.5 ± 2.0	9.0 ± 0.7	106	18/8

^a L1210 cells (10⁶) were injected i.p. on Day 0; treatment was started on Day 1 for the B6D2F₁ female mice.

^b Drug given i.p. for 8 days. For single daily administration (1st 5 lines) only the best results of 2 to 4 different dose levels for each experiment is given. For twice-daily administration (last 2 lines), only 1 dose level was used with injections given 3 hr apart.

^c Percentage increased life-span treated/controls calculated as follows: (life-span (treated) - life-span (controls) × 100)/life-span (controls).

^d Includes 1 survivor counted as a 40-day death for the calculation.

Table 2
Activity of TGM against P388 leukemia
P388 (10^6) cells were given i.p. Day 0 to 8 B6D2F₁ female mice per group.

Group	Treatment schedule ^a	100-day survivors/ no. treated	Life-span ^a (days)	Increased life-span ^b (%)
Control	No treatment	0/8	10.6 ± 0.2 ^c	0
TGM	1 mg/kg, QD×8 ^d	0/8	31.1 ± 10.9	193
TGM	1.5 mg/kg, QD×8	3/8	32.6 ± 4.8	207
Cyclophosphamide	150 mg/kg, Day 1	5/8	41.0 ± 8.5	286

^a All drugs were given i.p. starting 24 hr after tumor inoculation; only animals that died were included in the result.

^b Percentage increased life-span as calculated for Table 1; only animals that died were included in the result.

^c Mean ± S.E.

^d QD×8, every day, times 8.

optimal dose was approximately 1.5 mg/kg. A dose of 2 mg/kg/day was toxic unless the dose was divided into two 1-mg injections 3 hr apart, in which case a result superior to the single daily injection was achieved. These results indicate that more frequent daily injections may further improve efficacy.

Against P388 leukemia on a daily times-8 treatment schedule, TGM, 1.5 mg/kg/injection by i.p. administration, produced a 207% increase in life-span with 3 of 8 long-term survivors (Table 2). No other treatment schedules have been tested. HN2 has given 66% increased life-span at 3.1 mg/kg (optimal dose) on Day 1 after P388 injection (11).

Schedule Dependency. Results for 3 schedules of treatment of L1210 leukemia with TGM and HN2 are shown in Chart 2. For TGM, the every-4-days, times-3 (Days 1, 5, and 9) schedule (intermittent) was not significantly different from the daily schedule. However, the single-dose injection was clearly less effective than the intermittent and daily regimens. The 2.25-mg/kg optimal dose for both the single injection and the intermittent schedules was in the LD₅₀ range. Recovery permitting repeated dosing apparently occurred within 4 days, while lethal injections usually produced death at Day 7 or 8. In contrast to TGM, the optimal schedule for HN2 treatment was a single dose on Day 1.

The total cumulative dose of TGM and HN2·HCl administered to animals receiving the drugs at optimal dose on a daily schedule was 5.3 and 2.4 times that of animals receiving the respective drugs at optimal dose on a single-dose schedule.

Route Dependency. Of 3 routes of administration investigated, the i.p. route gave the best and most reproducible results. TGM was weakly active, when given either s.c. or p.o. Optimal dosage by these routes was higher than achieved by the i.p. route, and antitumor activity was usually seen at a dose level of 2 to 3 mg/kg/day. Optimal doses generally have produced 10 to 20% increased life-span when given p.o. and a 15 to 30% increased life-span when given s.c.

DISCUSSION

A major objective of this work was to determine whether the bone marrow-sparing properties of compounds contain-

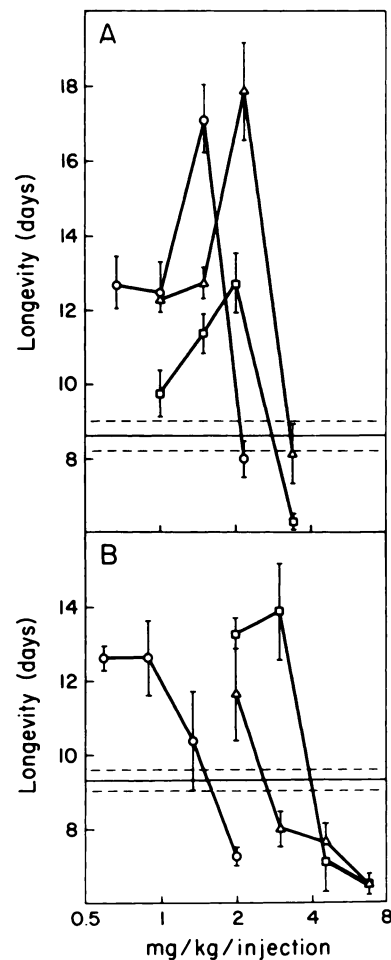


Chart 2. Schedule dependency of (A) TGM, and (B) HN2, in L1210 leukemia. Points, mean life-span ± S.E. of 8 B6D2F₁ female mice. Each curve represents the dose-response for the i.p. treatment schedule indicated: O, drug given on a daily dose schedule for 8 days; Δ, drug given every 4 days times 3 on Days 1, 5, and 9; and □, drug given as a single dose on Day 1. Solid line, mean control life-span; dashed lines, ± S.E. for control life-span. A *t* test comparing the result obtained with TGM, 1.5 mg/kg daily times 8, and TGM, 2.25 mg/kg Day 1, gives a value of 3.86. The means are significantly different, $p < 0.002$. The *t* value comparing TGM, 2.25 mg/kg Days 1, 5, and 9, with TGM, 2.25 mg/kg Day 1 only, is 3.59; $p < 0.003$. The values compared are the optimal responses on the respective curves shown in A.

ing both aminoglucose and nitrosourea moieties such as GCNU and STZ (NSC 85998) could be extended to a compound containing the aminoglucose moiety but without a nitrosourea component. While TGM is marrow toxic, the possibility that other nonnitrosourea-containing aminoglucose derivatives will not be marrow toxic is not excluded.

Enough information has now been accumulated to make certain statements regarding properties attributable to the aminoglucose moiety.

1. Changes in tissue distribution of drug occur. This is evidenced by the loss of ability of compounds containing the aminoglucose structure to cross the blood brain barrier. In support of this, both 1-methyl-1-nitrosourea (NSC 23909) (5) and CCNU (10) readily enter the central nervous system, whereas STZ (1) and GCNU (7) do not.

2. Changes in toxicity occur. There is an increased toxicity of aminoglucose-containing drugs on a molar basis when they are given as a single dose to mice in comparison to a similar parent drug not containing the aminoglucose moiety. For example, the LD₅₀ dose of TGM in mice is 0.0064 mmole/kg, compared with 0.021 mmole/kg for HN2. The LD₅₀ dose of GCNU in mice is lower than CCNU on both a mg/kg and molar basis (7) while STZ is possibly more toxic (6) than 1-methyl-1-nitrosourea (3) on a molar basis in mice. However, the total tolerated cumulative dose of multiple sublethal doses is apparently relatively greater for the aminoglucose compounds. Animals receiving the optimal schedules for TGM treatment reported in this paper received a total of 0.034 mmole/kg, whereas optimal cumulative doses of HN2 were 0.016 to 0.037 mmole/kg, depending on the schedule.

We have been able to demonstrate that TGM has 1 characteristic of STZ, namely, the schedule dependency pattern. The optimal schedule for L1210 treatment with STZ is by daily administration. In fact this is the only schedule that shows significant activity (9). The similar optimal treatment schedule for TGM is unusual for an alkylating agent and can most obviously be attributed to the aminoglucose moiety, since there is no other chemical similarity between STZ and TGM (Chart 1).

Venditti (9) has studied the schedule dependency patterns of a number of drugs in L1210 leukemia, and this type of data can be used in a general way in planning drug schedules for human trial. Drugs that are best given in a single large dose on Day 1, CCNU, cyclophosphamide, and TIC mustard (NSC 82196), have a much higher degree of activity in L1210 leukemia than drugs like STZ, that are best given on a daily schedule, or drugs that are equally as good given on a daily schedule such as DTIC (NSC 45388). Failure to account for the schedule-dependency change occurring with the modification of DTIC to TIC mustard resulted in a direct comparison of activity in L1210 leukemia for these 2 drugs. This led to the assumption that

TIC mustard was clinically superior and, eventually, to disappointing human trials (2). In treatment of L1210 leukemia, TGM is superior to STZ, DTIC (9), and procarbazine (NSC 77213) (4) drugs that are comparable with regard to schedule dependency patterns (9).

TGM offers an attractive option for evaluation of the hypothesis that consideration of schedule dependency patterns can improve on our present ability to evaluate new drugs for human trial.

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