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Mechanistic Studies of a Novel Antitumor Drug, α-1,3,5-Triglycidyl-s-Triazinetrione

Antitumor and Cytotoxic Effects

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SUMMARY

A triepoxy compound, α-1,3,5-triglycidyl-s-triazinetrione (α-TGT, NSC 296934), has recently been shown to be a highly effective antineoplastic drug with a wide spectrum of activity. We have initiated mechanistic studies on the action of this novel antitumor drug. The mean survival time of mice receiving L1210 leukemia cell transplants was significantly prolonged by injection of a single dose of α-TGT or by treatment of the leukemic cells *in vitro* with a much lower dose of the drug before transplantation. α-TGT seems to have a selective toxicity toward neoplastic cells, the LD_{50} being 1 order of magnitude lower for neoplastic L1210 cells than for non-neoplastic BALB/c-3T3 cells. The carcinogenic potency of α-TGT is at least 20-fold weaker than that of benzo[*a*]pyrene, aflatoxin B_1, and mitomycin as evaluated by the Inductest. Furthermore, *Escherichia coli* strains defective in UV repair function were much more sensitive to the drug than were the nondefective strains, suggesting that DNA may be the target of drug action. Although the alkylation of a model compound, 4-(*p*-nitrobenzyl)pyridine, by α-TGT was observed with a measurable rate, no *in vitro* interactions between α-TGT and DNA or its components could be detected under physiological conditions by using a variety of biochemical and physicochemical techniques. These findings suggest that α-TGT may not be a DNA cross-linker. Possible mechanisms of the drug action are discussed.

INTRODUCTION

The new antitumor drugs, 1,3,5-triglycidyl-s-triazinetriones, were originally synthesized by Budnowski (1). Atassi *et al.* (2, 3) have initiated studies on the antitumor properties of the α- and β-stereoisomers of TGT[3] on various transplantable mouse tumor systems. Although the two stereoisomers displayed a high therapeutic activity against P388 and L1210 leukemias, α-TGT was superior to the β-isomer in prolonging the life-spans of treated animals and in inducing long-term survival. Moreover, α-TGT also demonstrated antitumor effects against advanced L1210 leukemia, Lewis lung carcinoma, and a subline of P388 leukemia resistant to cyclophosphamide. Spreafico *et al.* (4) extended the initial experiments to a larger series of mainly solid tumors and showed that treatment with α-TGT can produce clear reductions in tumor growth resulting in significant increases in life-span and, in certain systems, in consistent proportions of long-term survivors. Therefore, α-TGT appears to have a high level of antineoplastic effectiveness and a wide spectrum of activity. Recently, pharmacokinetic studies (5) and Phase I clinical trials (6, 7) with α-TGT have been performed.

TGT has a novel structural characteristic. It is a unique triepoxy derivative (Fig. 1), and can be resolved by fractional crystallization into α- and β-stereoisomers having the configuration (*R,R,S/S,S,R*) and (*R,R,R/S,S,S*), respectively (1). The other triepoxy derivatives reported to have antitumor activity are the two diterpenoid triepoxides, triptolide and tripdiolide (8). In addition, there are antitumor drugs such as DAG (9–11) which possess two epoxy groups. Studies of DAG in cultured Yeshida sarcoma cells (11) suggested that DAG could form inter-

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[3] The abbreviations used are: TGT, 1,3,5-triglycidyl-s-triazinetrione; α-TGT, the α-stereoisomer of TGT; DAG, dianhydrogalactitol; NBP, 4-(*p*-nitrobenzyl)pyridine; dG, deoxyguanosine; 7-MeG, 7-methyl- guanosine; MEM, minimal essential medium; HPLC, high-performance liquid chromatography.

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strand cross-linking in DNA, and its inhibition of DNA synthesis may be related to the delayed formation of DNA interstrand cross-links. Whether α-TGT will work in a similar manner is not known. We have therefore initiated studies on the mechanism of action of this novel antitumor drug.

In this report, we first confirmed animal screening tests showing that α-TGT possesses antitumor activity by not only the *in vivo* method used earlier (3, 4) but also an *in vitro-in vivo* study with mice. The cytotoxicity of the drug in bacterial and animal cells, both normal and neoplastic, was examined. Furthermore, the alkylating activity of α-TGT, its potential carcinogenicity as measured by the Inductest (12, 13), and its interaction with DNA were studied by various biochemical and physicochemical techniques.

MATERIALS AND METHODS

Chemicals. α-TGT (NSC 296934) was a gift of Drs. P. Dumont and G. Atassi of Institut Jules Bordet (Bruxelles, Belgium). ^{14}C-Labeled α-TGT (20 μCi/mg) was custom-made by Amersham, and was a gift of Dr. Atassi. The solubility of the drug in water is 0.75 mg/ml (2.61 mM). All solutions of the drug were made fresh before use. NBP was a product of Aldrich Chemical Company (Milwaukee, Wisc.). Calf thymus DNA, ethidium bromide, dG, and 7-MeG were purchased from Sigma Chemical Company (St. Louis, Mo.). PM_2 DNA was prepared as described by Butour *et al.* (14), and bacteriophage λplac5 DNA was obtained by the procedure of Yamamoto *et al.* (15). Bio-Gel P-6 and trypan blue dye were products of Bio-Rad Laboratories (Richmond, Calif.) and Eastman Kodak Company (Rochester, N. Y.), respectively.

Bacterial strains, media, and cell culture. The wild-type *Escherichia coli* strain used was GY 4015, an Amp^r derivative of C 600 (12), which was a gift from Dr. Otsuji, Kyushi University (Fukuoka, Japan). The mutant strains of *E. coli*, BEM 11 (*tolC*) (16), BE 1186 (*uvrA ruv tolC*) (17), GY 5027 [*envA uvrB*(λ)], GY 5021 (*envA uvrB*$^+$), and GY 5026 (*envA uvrB*$^-$), were gifts of Drs. Moreau and Devoret, Gif-sur-Yvette, France.

Bacterial media and plates were prepared according to ref. 12. The LB medium contained 5 g of Difco yeast extract, 10 g of Difco tryptone, and 10 g of NaCl in 1 liter of water. The LB plate contained 15 g of Biomar agar in 1 liter of LB medium. The LBE medium was the LB medium supplemented with 0.2% glucose, 0.16 mM $MgSO_4$, 1.9 mM citric acid, 11.4 mM K_2HPO_4, and 11.4 mM $NaNH_4HPO_4$. The soft agar contained 7.5 g of Difco agar in 1 liter of water. The BT medium contained 8 g of peptone, 5 g of Difco tryptone, and 5 g of NaCl in 1 liter of water. The GT medium contained 12 g of Biomar agar in 1 liter of BT medium. The GT-amp medium was the GT medium supplemented with D-ampicillin (10 μg/ml).

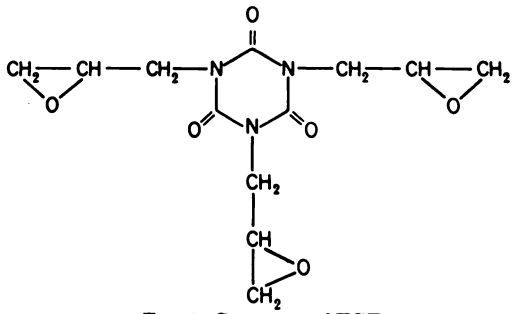

FIG. 1. *Structure of TGT*

L1210 leukemia cells were grown in suspension culture containing RPMI 1640 medium supplemented with L-glutamine (Flow Laboratories, Rockville, Md.) plus 10% fetal calf serum (GIBCO), penicillin (1×10^5 units/liter), and streptomycin (100 mg/liter). The cells were routinely passaged by dilution of cells in fresh medium every 2nd or 3rd day to a density of 1×10^5 cells/ml. Cultures were incubated in Corning tissue-culture flasks at 37° under 5% CO_2-air in a CO_2 incubator (National, Heinicke Instruments, Hollywood, Fla.).

BALB/c-3T3 cells were obtained from Larisa Rudenko of State University of New York at Stony Brook and were grown in monolayer culture containing MEM with Earle's salts and L-glutamine (Flow Laboratories) plus 10% fetal calf serum.

Animal studies. Approximately 2-month-old, female DBA/2 mice weighing 20–22 g were used in animal studies.

For the *in vivo* experiments, 30 mice which received L1210 cells (1×10^5 cells/ml) i.p. 1 day before drug treatment were divided into two groups. The control group received 0.2 ml of the medium alone, while the sample group was treated with 0.2 ml of α-TGT (5 mg/ml) in a single 50 mg/kg dose. The life-spans of these two groups of mice were compared.

For the combined *in vitro-in vivo* experiments, 18 mice divided into 3 groups were used for the evaluation of increase in life-span by the drug treatment. The α-TGT solution (0.1 ml of 0.2 mg/ml or 0.4 mg/ml in water) mixed with 0.9 ml of medium was incubated with 1 ml of ascitic L1210 cells (4×10^7 cells/ml) at 37° for 1 hr. The control solution contained no drug. An aliquot (0.3 ml) of the above mixture was injected i.p. into each mouse. The survival times of the three groups of animals treated with α-TGT (0, 10, or 20 μg/ml) were recorded.

Toxicity of α-TGT in animal cells. An aliquot (3 ml) of L1210 cells (1×10^5 cells/ml) which was mixed with 0.1 ml of α-TGT stock solutions (final concentrations 0.01–100 μg/ml) was incubated at 37° in a CO_2 incubator for 24 hr. After incubation, the cell suspension (0.5 ml) was diluted with 1.5 ml of RPMI medium, and an aliquot (0.5 ml) of cell suspension was then mixed with 0.5 ml of trypan blue dye solution (2 ml of 4.5% NaCl and 8 ml of 1.2% trypan blue dye in water) before being injected into the cell counter (6300A Cytograf) or counted in a hemocytometer. The percentage of dead cells was measured as compared with that of controls without drug treatment at zero time.

BALB/c-3T3 cells (5×10^5 cells/ml) in MEM medium were mixed with α-TGT (0.01–100 μg/ml) and incubated for 24 hr at 37° in a CO_2 incubator. The cells were mixed with an equal volume of 1.0% trypan blue solution before placing them in a hemocytometer for counting the number of dead cells. Control experiments without drug treatment were carried out for calculating the percentage of dead cells.

Toxicity of α-TGT in bacterial cells. E. coli (strains BEM 11 and BE 1186) cells were grown in LB medium

up to A_{650} = 0.9–1.0. The cells were diluted to a density of 1×10^4 cells/ml in 2 ml of medium and incubated with α-TGT at a final concentration of 0–25 μg/ml for 1 day at 37°. The A_{650} readings of cell suspensions were recorded after incubation.

To measure the number of cells surviving after α-TGT treatment, an overnight culture of *E. coli* cells (BEM 11 or ME 1186) was inoculated at 3 ml of LB medium and grown at 37° to A_{650} = 0.9. The cells were harvested and resuspended in 3 ml of 0.1 M Tris-HCl (pH 7) containing 10 mM $MgSO_4$ (minimal medium), and diluted with the same buffer to give a suspension of 5×10^3 cells/ml. One milliliter of this cell suspension was incubated with various concentrations of α-TGT at 37° for 6 and 21 hr, an aliquot (0.1 ml) of which was poured onto an LB plate after mixing with 2.5 ml of soft agar (kept at 47°). Following an incubation at 37° overnight, the surviving cells per plate were counted.

Similar experiments with *E. coli* strains GY 5021 and GY 5026 were also performed where LBE medium instead of LB medium was used for bacterial growth.

Inductest. The procedure of Moreau and Devoret (13) was used for the Inductest. An overnight culture of *E. coli* (GY 5027) cells was inoculated in 3 ml of the minimal medium. The cells were grown to A_{650} = 0.8, harvested, and diluted as described in the previous section. Aliquots (1 ml) of cell suspension (4–5 × 10^3 cells/ml) were incubated with different concentrations of α-TGT (0–15 μg/ml) at 37° for 6 or 21 hr. Wild-type *E. coli* cells (GY 4015) were grown at 37° overnight. A mixture of *E. coli* GY 5027-drug suspension (0.1 ml), *E. coli* GY 4015 overnight culture (0.2 ml), and soft agar (2.5 mi) was poured onto GT-amp plates. After incubation for 14 hr at 37°, the number of plaques (or infective centers) on the plate was counted. The control experiment was carried out with the known antitumor drug, mitomycin C, at 2 μg/ml concentration.

Alkylation of NBP by α-TGT. The alkylating activity of α-TGT was measured by the method of Wheeler *et al.* (18) with some modifications. Buffer solutions at various pH values were made up as follows: pH 1.2 and 2.4, HCl-KCl; pH 3.0, 4.0, and 6.0, Na_2HPO_4-citric acid; pH 5.0, acetic acid-sodium acetate; pH 7.0 and 8.0, NaH_2PO_4-Na_2HPO_4; pH 9.2 and 10.5, Na_2CO_3-$NaHCO_3$. The drug and NBP were first dissolved in acetone and then the buffer was added (the final acetone content was 20%) to yield final concentrations of drug and NBP of 3.3×10^{-4} M and 2.2×10^{-3}M, respectively. After the mixture was incubated at 37° for various time periods, a 1-ml aliquot of the reaction mixture was withdrawan and mixed with 0.8 ml of acetone and 2 ml ethyl acetate; the pH was adjusted to 11 by the addition of 0.8–0.9 ml of 1 N NaOH solution. The solution was stirred vigorously with a Vortex mixer and allowed to stand at room temperature for few minutes until two layers were separated. The upper organic layer (pink or purple color) was pipetted out and its A_{540} readings were measured in a spectrophotometer.

In vitro binding of α-TGT with DNA. Calf thymus DNA (1 μg/ml) was incubated with ^{14}C-labeled α-TGT (1–10 μg/ml) in 0.05 M sodium phosphate buffer (pH 7.5) at 37° for 24 hr. The mixture was applied to a Bio-Gel P-6 column (1 × 12 cm), and 0.5-ml fractions were collected. The A_{260} reading and radioactivity of each fraction were measured.

In vitro interaction of α-TGT with DNA or its components. The interaction of α-TGT with DNA components such as dG and 7-MeG was studied fluorimetrically by the method of Hemminki (19). The reaction mixture (2.4 ml), containing 0.33 mM α-TGT and 2.3 mM dG or 7-MeG in 50 mM sodium phosphate buffer (pH 7.5) and 30% ethanol, was incubated at 32° for various time periods (30 min; 1, 3, 5, 7, and 24 hr). An aliquot (0.4 ml) of the reaction mixture was withdrawn, mixed with 0.6 ml of water, and extracted with 2 ml of ethyl acetate. The organic phase was removed and the water phase was mixed with 2 ml of 30% methanol. The methanol-water mixture was examined in a fluorescence spectrophotometer. The control was the same reaction mixture containing 7-MeG alone. Both excitation spectra ($\lambda_{emission}$ = 400 nm) and emission spectra ($\lambda_{excitation}$ = 300 nm) were measured. The reaction products from 7- and 24-hr incubations were also analyzed by HPLC (Waters Associates, Milford, Mass.), using a μBondapak C_{18} column and a solvent system of 0.1 M ammonium acetate (pH 4.5) and methanol/0.1 M ammonium acetate (pH 4.5) (50:50). Both UV (at 254 and 313 nm) and fluorescence ($\lambda_{excitation}$ = 300 nm and $\lambda_{emission}$ = 389 nm) detections were employed.

The possible cross-links of DNA by α-TGT were examined by the method of Lee and Morgan (20) and Morgan *et al.* (21) under various conditions. DNA strands were separated by alkaline and heat treatment of the reaction mixture. For a typical reaction, the reaction mixture (1 ml) containing DNA (1 μg/ml) (calf thymus, λplac5, or PM_2) and α-TGT (1 or 10 μg/ml) in 50 mM sodium phosphate buffer (pH 7.0), was incubated at 31°–32° for various time intervals. An alqiuot (40 μl) of the reaction solution was withdrawn and mixed with 4 ml of "pH 12" buffer (21) [20 mM K_3PO_4, 0.2 mM EDTA, and ethidium bromide (1 μg/ml)]. The solution was divided into two portions, one of which was heated at 96° for 2 min and allowed to stand at 25° for 2 min while the other was kept at 25°. The fluorescence intensity of the reaction mixture at 610 nm was measured with excitation at 525 nm.

Electron microscopic study of the interaction of α-TGT with DNA. The electron microscopic analyses of DNA with and without TGT treatment were performed by Dr. E. Delain at the Institut Gustave-Roussy. The reaction mixture (100 μl), consisting of nicked PM_2 DNA (64 μg/ml) and α-TGT (1, 10, or 100 μg/ml) in 0.01 M Tris-HCl (pH 8.0) and 2 mM EDTA (TE buffer), was incubated at 37° overnight. In a similar experiment with covalently closed-circular PM_2 DNA, the reaction solution (60 μl) contained DNA (75 μg/ml) and α-TGT (1, 10, or 100 μg/ml) in TE buffer plus 0.13 M NaCl.

Measurement of DNA melting profiles in the presence and absence of α-TGT. Calf thymus DNA (5 ml, 70 μg/ml) was dialyzed overnight against 500 ml of sodium cacodylate buffer (10 mM NaCl, 5 mM sodium cacodylate (pH 6.5), and 0.1 mM EDTA). The reaction mixture, containing dialyzed calf thymus DNA (61 μg/ml) and drug (10 or 100 μg/ml), was incubated at 37° for 6 hr. The DNA melting profiles of the sample solution and the

control (without drug) were then measured. A_{260} readings of the reaction mixture for every 2°–3° increment in temperature were recorded. The temperature of the water bath was thermostated, and varied from 30° to 80°.

RESULTS

Antitumor activity. To confirm the initial *in vivo* report on the antineoplastic activity of α-TGT (2–4), we first examined the effect of this drug on the survival times of mice receiving L1210 mouse leukemia cell transplants by two different procedures: an *in vivo* experiment and a combined *in vitro-in vivo* experiment. For the *in vivo* experiment, L1210 cells were injected i.p. into 30 mice (10^5 cells/ml per mouse); one-half of the mice received a single dose of α-TGT (50 mg/kg) 1 day after the leukemia cell transplantation. The survival times of these mice as compared with those of the other half without drug treatment are given in Table 1. In the combined *in vitro-in vivo* experiment, the L1210 ascitic cells (4×10^7 cells/ml) were first treated with or without drug (0, 10, or 20 μg/ml) before the i.p. injection of these cells into three groups of six mice each, and the survival times of the mice in each group were compared (Table 2). The results from the above two experiments showed a significant prolongation of the mean survival time with drug treatment both *in vivo* and *in vitro*.

Toxicity in animal cells. Both normal (BALB/c-3T3) and neoplastic (L1210) culture cells were used to examine the toxicity of α-TGT in animal cells. The cells (1×10^5 cells/ml) were incubated with various concentrations of the drug for 24 hr, and the cells killed by the drug were counted in a cell counter or a hemocytometer after trypan blue treatment. As shown in Fig. 2, the concentration of drug required for killing 50% of BALB/c-3T3 cells (30 μg/ml) was about 1 order of magnitude higher than that required for L1210 cells (3 μg/ml).

Toxicity in bacteria. The toxicity of α-TGT in bacteria was investigated by using various mutant strains of *E. coli* (BE 1186, BEM 11, GY 5021, and GY 5026) with two different methods. In the first method, the growth of *E. coli* was measured by absorbance at 650 nm of the growth medium. Figure 3 shows that the growth of certain strains of *E. coli* (BEM 11 versus BE 1186) was inhibited by the presence of various concentrations of α-TGT in the growth media. *E. coli* strain BEM 11 is a *tolC* mutant (16) defective in membrane permeability, while strain BE 1186 (*uvrA ruv tolC*) (17) is defective in UV repair in addition to having the *tolC* mutation. As shown in Fig. 3, strain BE 1186 was much more sensitive to drug inhibition than was strain BEM 11. The concentrations required for 50% inhibition of growth were 4 μg/ml for BE 1186 and 100 μg/ml for BEM 11.

The cytotoxicity of α-TGT in bacteria can also be determined by the measurement of the survival colonies of cells after drug treatment. *E. coli* cells were incubated with various concentrations of the drug at 37° for 22 hr and then replated on an LB plate to count the surviving colonies. The concentrations of α-TGT required to achieve 50% survival of the control (without the drug

TABLE 1

In vivo study of the effect of α-TGT on the life-spans of mice receiving L1210 leukemia transplants

Thirty mice received L1210 leukemia cells (1×10^5 cells/ml) i.p. 1 day before drug treatment. The control group (15 mice) received 0.2 ml of the medium (i.p.), while the sample group (15 mice) was treated with 0.2 ml of α-TGT (5 mg/ml) in a single 50 mg/kg dose.

Days after treatment	% Cumulative mortality	
	Control	Drug-treated (50 mg/kg)
8	6	
9	40	
10	73	
11	86	6
12	93	26
13	100	60
14		73
15		86
16		93
20		100
Mean survival time (days)	9.9 ± 1.3	13.7 ± 2.2
Treated/control (%)	100	138

TABLE 2

Combined in vitro-in vivo study of the effect of α-TGT on the life-spans of mice receiving L1210 leukemia transplants

α-TGT solution (0, 10, or 20 μg/ml in water) was incubated with L1210 leukemia cells (4×10^7 cells/ml) at 37° for 1 hr. An aliquot (0.3 ml) of α-TGT-treated cells was injected (i.p.) to each mouse of three six-mouse groups.

Days after treatment	% Cumulative mortality with drug concentration of		
	0 μg/ml	10 μg/ml	20 μg/ml
7	33		
8	83		
9	100	83	
10		100	16
11			66
12			83
13			100
Mean survival time (days)	7.8 ± 0.7	9.2 ± 0.4	11.3 ± 1.0
Treated/control (%)	100	117	144

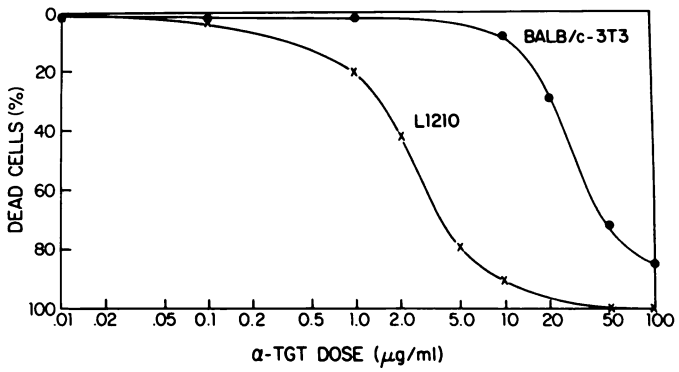

FIG. 2. *Cytoxicity of α-TGT in normal (BALB/c-3T3) and neoplastic (L1210) cultured cells*

Cells (1×10^5/ml) were incubated with different concentrations of α-TGT at 37° for 24 hr. The percentage of dead cells was measured in a cell counter or hemocytometer after trypan blue treatment of the cells.

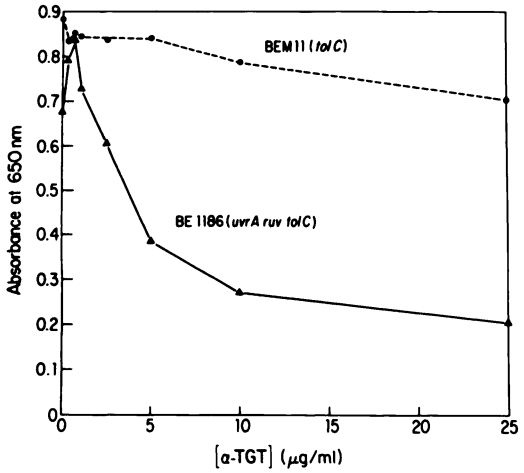

FIG. 3. *Cytoxicity of α-TGT in bacterial cells as measured by its effect on cell growth*

Escherichia coli cells (1×10^4/ml) were incubated with different concentrations of α-TGT in LB medium at 37° for 1 day. The A_{650} readings of the cell suspension were measured as an indication of cell growth.

treatment) were 4 μg/ml for strain BE 1186 (Fig. 4A) and >80 μg/ml for strain BEM 11 (data not shown) in agreement with the results obtained by the first method. The effect of the drug was proportional to the length of incubation time: incubation of BE 1186 cells for 6 hr required a drug concentration of 10 μg/ml instead of 4 μg/ml to achieve 50% survival of the controls (Fig. 4A). Similar results were obtained with *E. coli* strain GY 5021 (*envA uvr* B^+) and GY 5026 (*envA uvrB*$^-$). The drug concentrations required for 50% survival of controls were 15 μg/ml for strain GY 5026 (defective in UV repair) and 100 μg/ml for strain GY 5021 (normal UV repair function) (Fig. 4B).

Inductest. An antitumor drug may also be a carcinogen. To test this possibility, we employed the Inductest developed by Devoret and his co-workers (12, 13) based on the lysogenic induction of prophage λ in *E. coli*. The tester bacteria used were *E. coli* GY 5027 [*envA uvrB* (λ)]. The cell suspension (4×10^3 cells/ml), incubated with different concentrations of drug for 6 or 21 hr, was then mixed with wild-type *E. coli* (GY 4015) cells and plated. After incubating the plates at 37° overnight, the number of infective centers per plate as a function of drug concentration was determined (Fig. 5). The potency of α-TGT to induce prophage λ was at least 20-fold less than the reference inducer and DNA cross-linker mitomycin C (13) at a drug concentration of 2 μg/ml after 21 hr of incubation.

Alkylating activity of α-TGT. The alkylating activity of α-TGT was first studied by its reaction with a model compound, NBP, in buffer with varying values pH (1.2–10.5) at 37° for various times. The reaction product was measured colorimetrically at 540 nm. The rate constants (k) and half-lives ($t_{1/2}$) of the alkylation reactions were calculated according to the equations: K (min^{-1}) = $d[\ln(C/C_0)]/dt$ and $t_{1/2} = \ln 2/k$, respectively. C/C_0 is the fraction of drug remaining unreacted at various times. The plot of $\ln(C/C_0)$ versus t at various pH values, being linear, indicated that the reactions were first-order. The rate constants and $T_{1/2}$ values calculated at t = 5 hr are listed in Table 3. Figure 6 shows the pH dependence of log k for the alkylating reactions with optimal pH at 7.0.

The alkylation of α-TGT to the components of DNA was studied by the method of Hemminki (19), with modifications. The concentrations of reactants were lowered to approximate the physiological conditions. The drug (0.33 mM) was allowed to react with 7 M excess of dG 7-MeG, and the reaction mixture was analyzed by both fluorescence spectrometry and HPLC. The control reaction mixture containing 7-MeG without drug gave 100% fluorescence, whereas the fluorescence of the sample solution (drug and dG) did not increase as the incubation time increased from 30 min to 7 hr, suggesting that there was no interaction occurring between drug and dG. After 7 and 24 hr of incubation, the reaction mixtures were further subjected to HPLC analysis. No significant reduction of the original compound leak was registered, and no new leak appeared.

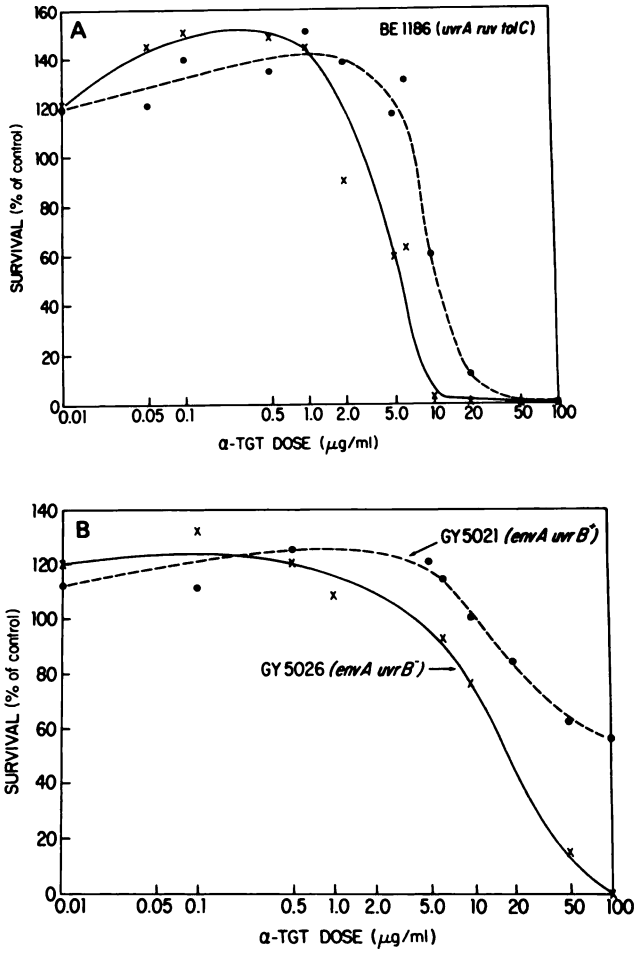

FIG. 4. *Cytoxicity of α-TGT in bacterial cells as measured by cell survival colonies*

A. *Escherichia coli* (BE 1186) cells (5×10^3/ml), after incubation with various concentrations of α-TGT at 37° for 6 hr (●– – –●) or 22 hr (×——×), were replated on LB plates. The survival colonies of each plate were counted.

B. Same procedure as A, except that *E. coli* strains GY 5021 (*envA uvrB*$^+$) and GY 5026 (*envA uvrB*$^-$) were used instead of BE 1186. Cells were incubated at 37° for 6 hr.

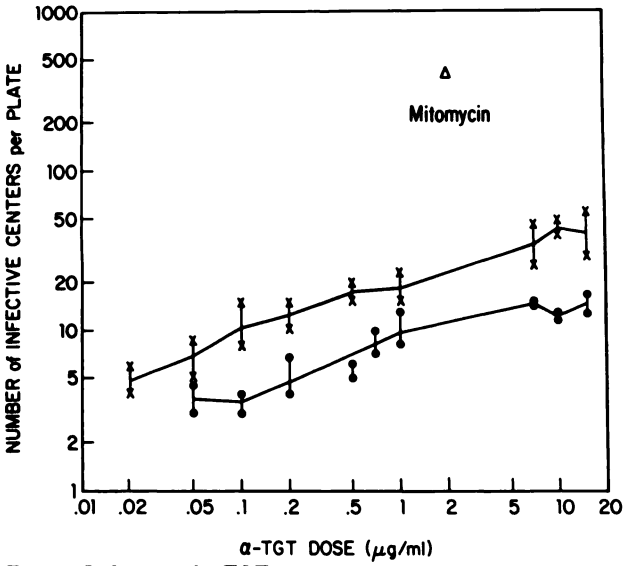

FIG. 5. *Inductest of α-TGT*

Escherichia coli tester strain [GY 5027 (*envA uvrB* (λ), 4 × 10^3 cells/ml], after incubation with different concentrations of α-TGT for 6–21 hr, were mixed with wild-type *E. coli* and replated. The infective centers of each plate were counted. ●——●, 6 hr; ×——×, 21 hr; Δ, mitomycin, 21 hr.

Interaction of α-TGT with DNA in vitro. The possible cross-linking of DNA by α-TGT was examined with various concentrations of α-TGT and DNA (calf thymus, PM_2, or λplac5), using a variety of different methods. After treatment with drug, the DNA was denatured by alkaline or heat treatment, and the fluorescence intensity of the reaction mixture was determined after adding ethidium bromide. Under all conditions examined, the fluorescence of DNA with ethidium bromide remained the same for DNA with or without drug treatment, suggesting that no cross-links of DNA were induced by α-TGT.

The effect of α-TGT on the structure of DNA was also examined by electron microscopy. The results showed that α-TGT did not perturb the shape and length of PM_2 DNA, both nicked and covalently closed-circular forms (data not shown). Furthermore, no alteration of the melting and renaturation profiles of calf thymus DNA was observed in the presence and absence of α-TGT.

TABLE 3

Rate constants and half-lives of the alkylating activity of antitumor drug, α-TGT

The conditions for alkylation reaction of α-TGT and NBP are described under Materials and Methods. The pH dependence of the alkylation reaction is expressed in Fig. 6.

pH	k	$-\log k$	$t_{1/2}$
	min^{-1}		
2.4	3.3×10^{-6}	5.47	3500 hr
3.0	5.0×10^{-5}	4.29	233 hr
4.0	2.1×10^{-4}	3.69	55 hr
5.0	7.4×10^{-4}	3.12	15 hr, 30 min
6.0	4.6×10^{-4}	3.33	24 hr, 53 min
7.0	2.0×10^{-3}	2.70	5 hr, 48 min
8.0	1.0×10^{-3}	2.98	11 hr
9.0	5.3×10^{-5}	3.27	21 hr, 30 min
10.5	2.4×10^{-4}	3.62	48 hr

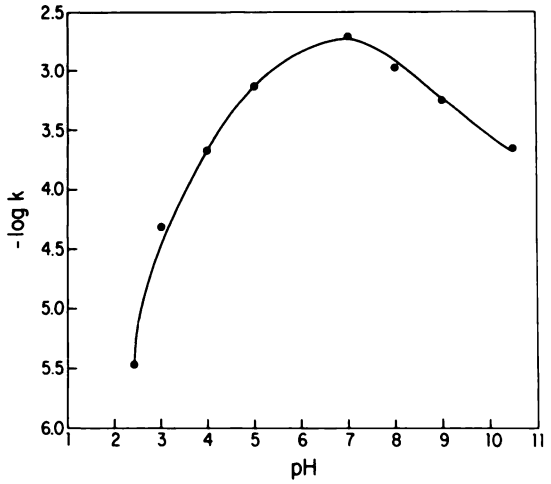

FIG. 6. *pH Dependence of the alkylation reaction of α-TGT and NBP*

The data were taken from Table 3.

In order to ascertain that no significant interaction occurs *in vitro* between α-TGT and DNA, calf thymus DNA (1 μg/ml, single- or double-stranded) was incubated with two concentrations of ^{14}C-labeled drug (1 or 10 μg/ml) for 24 hr at 37° and the reaction mixture was analyzed by a Bio-Gel P-6 column chromatograph. The DNA peak determined by A_{260} readings, which was observed in the void volume of the column, did not coincide with the radioactive peak of the drug, which eluted much later than the DNA peak.

DISCUSSION

Studies of α-TGT in murine leukemia and a large series of solid experimental tumors (2–4) have shown that α-TGT is an active antineoplastic agent. The initial *in vivo* studies (2, 3) were performed by inoculating mice with leukemic cells on day 0 and injecting the drug (50 mg/kg dose, i.p.) once a day from day 1 to day 9 or on days 1, 5, 9, 13, and 17, which resulted in prolongation of the mean survival time from 8 (control) to 24 or 21 days, respectively. Our *in vivo* studies indicated that α-TGT can increase the life-span from 10 (control) to 14 days with only one drug treatment (50 mg/kg i.p., day 1) (Table 1). Moreover, we have shown through the combined *in vitro-in vivo* experiments with L1210 cells that α-TGT can exert its antitumor effect *in vitro* (Table 2) when 10 or 20 μg/ml of drug were incubated with leukemia cells (10^5 cells/ml) before injecting the drug (i.p.) into mice.

The toxicity studies of α-TGT in animal cells suggest that neoplastic cells (L1210 leukemia) appear to be more sensitive to the drug than non-neoplastic cells (BALB/c-3T3). The concentration of α-TGT required for killing 50% of L1210 cells was 1 order of magnitude lower than that required for BALB/c-3T3 cells (Fig. 2). It should be pointed out that the toxicity comparison of L1210 versus 3T3 cells is only suggestive, and not necessarily indicative, of differential toxicity. Studies with better combinations of 3T3 and transformed 3T3 cells will be pursued to assess this problem more adequately. However, α-TGT requires a much higher dose to be effective as an antitumor drug in comparison with the other triepoxy

derivatives, triptolide and tripdiolide, which have shown significant antileukemic activity against L1210 and P388 leukemias at a 0.1 mg/kg level, and cytotoxicity (ED_{50}) against KB cell cultures at 10^{-3}–10^{-4} mg/ml (8).

It has been known that carcinostatic substances often display carcinogenic activity, and vice versa (22, 23). Thus, the potential carcinogenic potency of α-TGT was studied by the Inductest (12, 13), which is based on the theory that there exists a basic biological process common to bacterial and animal cells in which chromosomal lesion caused by a carcinogen will induce an "SOS pathway" (13) that may lead to mutagenesis as well as lysogenic induction. Since the efficiency of DNA repair is never absolute, some residual DNA lesions must go through the DNA-replicating machinery. In order to ensure cell survival, SOS functions are induced when the replication fork encounters DNA damage that is likely to cause cell death. The SOS functions are expressed by a set of genes or loci, and the coordinate action of their products in a biochemical pathway is termed the SOS pathway. In the Inductest, α-TGT does induce prophage λ but the potency of induction is at least 20-fold weaker than that of known carcinogens such as benzo [*a*]pyrene, aflatoxin B_1, and the antitumor drug mitomycin C, as evaluated by the Inductest (13). Interpretation of this result is difficult in view of the uncertainties involved in the Inductest and other carcinogen-screening tests. Further studies are needed to establish the carcinogenic potency of α-TGT.

The observation that the bacterial strains defective in the UV repair system are much more sensitive to α-TGT than their nondefective counterparts (Figs. 3 and 4) suggests that the target of drug action may be DNA. This hypothesis appears plausible since α-TGT contains three epoxy groups which are well known for their alkylating capacity, and since the diepoxy drug, DAG, has been shown to function as an alkylating agent (10) which cross-links DNA (11). Although the alkylation of a model compound, NBP, by α-TGT was observed with a measurable rate, no *in vitro* interactions between α-TGT and DNA or its components could be detected under physiological conditions by using a variety of biochemical and physicochemical techniques. Hemminki (19) has observed the alkylation of DNA or dG by epoxide, yet the concentrations of DNA (or dG) and epoxide used were nonphysiological and much higher than those we employed. The unexpected failure to detect any interaction or cross-links of DNA by α-TGT *in vitro* has several implications: (a) α-TGT is inactive *in vitro* and requires metabolic activation for its action, (b) the reactivity of DNA may be different *in vivo* and *in vitro* [e.g., as seen for *cis*-dichlorodiamine-platinum (24)], or (c) the target of the drug may not be DNA. Our preliminary studies[4] have indicated that radioactively labeled α-TGT binds mostly proteins in the nuclei of both BALB/c-3T3 and L1210 cells as analyzed by isopyknic Cs_2SO_4 gradient centrifugation (25). Although the extent of the interaction of carcinogen with DNA is small, the biological consequences of the interaction may be great. Thus, the extensive reaction of carcinogen or drug with protein may render the detection of DNA lesions difficult. MacLeod *et al.* (26, 27) have shown that the vast majority of the binding of benzo[*a*]pyrene to nuclear macromolecules is due to protein rather than to interaction with nucleic acid. It seems that factors other than the intrinsic chemical reactivity of the epoxide group may be important in the interaction of potential ultimate carcinogens or antitumor drugs with biological systems. One such possibility of the interaction of α-TGT with key proteins is the alkylation of enzymes that are involved in the processes of DNA repair and replication. Investigation of various factors that may shed light on the mechanism of α-TGT action is in progress.

[4] F. Y.-H. Wu, unpublished results.

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