INHIBITION OF DNA SYNTHESIS IN MURINE TUMOR CELLS BY GELDANAMYCIN, AN ANTIBIOTIC OF THE BENZOQUINOID ANSAMYCIN GROUP

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The mechanism of action of geldanamycin, a benzoquinoid ansamycin, was investigated with murine lymphoblastoma L5178Y cells. The agent inhibited the cell growth at concentrations over 0.01 μg/ml. The antibiotic blocked DNA synthesis more markedly than RNA and protein syntheses. Mitosis was not significantly affected by the drug in the cells synchronized with demecolcine (Colcemid). The antibiotic did not interfere with *in vitro* assembly of tubulin.

In the synchronized cells, strong inhibition of DNA synthesis was observed when geldanamycin was introduced into the culture prior to S phase of the cell cycle. The degree of inhibition was stronger with prolongation of incubation period and with increase of DNA synthesis rate. The results suggested that initiation of DNA synthesis or S phase is affected by the drug. DNA degradation was not significantly induced *in vivo* by the antibiotic.

Geldanamycin blocked DNA polymerase α more markedly than β and γ. The degree of inhibition depended upon concentrations of enzyme but not upon those of template, suggesting a drug-enzyme interaction. IC_{50} for DNA polymerase α was 10 μg/ml and for DNA polymerase β 100 μg/ml at low concentrations of enzyme. The inhibition of DNA polymerase α by the antibiotic was non-competitive and *Ki* was 20 μM.

The mechanism of action of naphthoquinoid ansamycins, *i.e.* rifamycins and streptovaricins, has been extensively investigated (*cf.* reviews[1,2]). The antibiotics bind to RNA polymerase of prokaryotes, and inhibit the transcription process. Retrovirus reverse transcriptase and eukaryotic DNA polymerase are blocked by lipophilic rifamycin derivatives[3,4]. Although the mechanism of action of naphthoquinoid ansamycins has been well established, the molecular mechanism of action of benzoquinoid ansamycins remains to be determined.

Geldanamycin (GDM), a benzoquinoid ansamycin, shows strong cytotoxicity against tumor cells and protozoa, and weak antimicrobial activity.[5] RNA polymerase of *E. coli* is not significantly affected by the antibiotic[2], but retrovirus reverse transcriptase is inhibited at high drug concentrations[6,7].

We have studied the mechanism of action of GDM and found that the antibiotic is a potent inhibitor of DNA synthesis in cultured cells of murine lymphoblastoma L5178Y, and DNA polymerase, derived from Ehrlich carcinoma, is affected at high drug concentrations. RNA synthesis is not significantly blocked by GDM. The results are presented in this publication.

Materials and Methods

Chemicals

Geldanamycin was generously given by Prof. N. ŌTAKE, Institute of Applied Microbiology, University of Tokyo, and aphidicolin by Dr. M. ŌHASHI, Department of Biochemistry, Tokyo Metropolitan Institute of Gerontology, Tokyo. [^{3}H-Methyl]dTTP (41 Ci/mmole), [5,6-^{3}H]uridine (45.9 Ci/

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mmole), [^{3}H-methyl]thymidine (20 Ci/mmole), and L-[3-^{3}H]alanine (82.7 Ci/mmole) were products of New England Nuclear, Boston, Mass. Deoxyribonucleoside triphosphates were purchased from Sigma Chemical Co., St. Louis, Missouri, oligo(dT)-poly(rA) from Collaborative Research Inc., Waltham, Mass., and salmon sperm DNA from Calbiochem, Calif. Other reagents were of the highest grade commercially available.

Cells

Murine lymphoblastoma L5178Y cells were cultured in FISHER's medium supplemented with 10% horse serum, in test tubes for observation of cell growth or in Nunclon plates (Delta Inc.) for incorporation of labeled precursors. Ehrlich ascites carcinoma cells at logarithmic phase of growth were used for preparation of DNA polymerase.

Incorporation of Labeled Precursors into L5178Y Cells

The cells at early logarithmic phase of growth, in 0.2 ml of culture, were distributed into wells of Nunclon plates and were labeled with [^{3}H]thymidine (1 μCi/ml), [^{3}H]uridine (1 μCi/ml) or [^{3}H]alanine (10 μCi/ml) for 4 hours at 37°C in a CO_2 incubator. The cells were then trapped on glass fiber disks (GF/C, Whatman), and washed once with phosphate-buffered saline (PBS), twice with 5% trichloroacetic acid (TCA) and once with 1% acetic acid. The radioactivity was determined in a scintillation counter.

Purification of DNA Polymerases

DNA polymerases α and γ were purified from Ehrlich ascites tumor cells following the method of MATSUKAGE *et al.*[8,9] The cell extract was subjected to ammonium sulfate fractionation, and the fraction of 25~60% saturation was further purified on successive column chromatography with DEAE-cellulose, phosphocellulose and hydroxyapatite. DNA polymerases α and γ were separated on hydroxyapatite column.

DNA polymerase β was prepared by a modified procedure of YAMAGUCHI *et al*[10]. The fraction of 25~80% saturation of ammonium sulfate was dialyzed against buffer (0.5 M KCl, 10% glycerol, tris-HCl, pH 7.6, 20 mM, ethylenediaminetetraacetic acid (EDTA) 0.1 mM, dithiothreitol 1 mM) and was applied to Sephadex G-200 column. The fractions of DNA polymerase β, dependent on activated DNA and resistant to aphidicolin, were pooled, and further purified by successive chromatography with DEAE-cellulose and phosphocellulose columns. Aphidicolin is a specific inhibitor of DNA polymerase α[11,12], and the α enzyme, prepared as above, was inactivated over 90% at 2 μg/ml of aphidicolin.

Assay of DNA Polymerases

DNA polymerases α, β and γ were assayed by the method of MATSUKAGE *et al.*[8,9] Activated DNA was prepared by the procedure of SCHLABACH *et al.*[13]

Assay condition (A): Activated DNA was used as a template for DNA polymerases α and β. The reaction mixture, in 25 μl, contained: tris-HCl, pH 7.6, 50 mM, $MgCl_2$ 6 mM, KCl 100 mM, dithiothreitol 1 mM, activated salmon sperm DNA 3 μg, dATP, dCTP and dGTP 0.1 mM each, [^{3}H]dTTP 0.25 μCi, bovine serum albumin 10 μg and DNA polymerase.

Assay condition (B): Oligo(dT)-poly(rA) was used as a template for DNA polymerases β and γ. The reaction mixture, in 25 μl, contained: tris-HCl, pH 7.6, 50 mM, KCl 100 mM, $MnCl_2$ 0.5 mM, oligo(dT)-poly(rA) 1 μg, [^{3}H]dTTP 0.25 μCi, bovine serum albumin 10 μg, and DNA polymerase.

Aphidicolin was dissolved in dimethylsulfoxide (DMSO) and GDM in methanol; the solutions were diluted with H_2O and added into the reaction mixtures. The final concentration of DMSO was 1%, and that of methanol 2%.

GDM or aphidicolin was mixed with the enzyme, and then the other components were added to the mixture.

The reaction of both assay systems was carried out at 30°C for a period indicated in the legend, terminated and washed with 0.5 ml of 5% TCA containing 0.02 mM sodium pyrophosphate, and further washed twice with 1% acetic acid. The radioactivity, collected on Millipore filter (HAWP 304 FO), was determined in a toluene scintillator.

Synchronization of L5178Y Cells

The cells were synchronized by the treatment with Colcemid as described previously[14]. The cells arrested at metaphase divided within 1.5 hours after removal of Colcemid, and entered S phase around 2 hours.

Results

Effects of GDM on Multiplication of L5178Y Cells in Random and Synchronized Cultures

L5178Y cells were grown in FISHER's medium supplemented with 10% horse serum, inoculated at cell density of 7.5×10^5/ml. GDM was introduced into the culture on the day after cell inoculation. The cell number was counted every 24 hours by a Coulter counter. As illustrated in Fig. 1, the cell growth was not significantly affected by GDM for the first 24 hours after the drug addition, but thereafter markedly inhibited at antibiotic concentrations over 0.01 μg/ml.

In experiments using cells synchronized with Colcemid treatment, GDM significantly blocked the uptake of [^{3}H]thymidine but not the mitosis or cell multiplication at a high concentration of 1 μg/ml (Fig. 2). The results suggested that the target of GDM is the apparatus of DNA synthesis but not that of mitosis. The assumption was also supported by the observation that the *in vitro* assembly of porcine brain tubulin was not significantly affected by GDM (data are not shown).

Effects of GDM on Macromolecular Biosyntheses

The activity of GDM against nucleic acid and protein syntheses was examined by the uptake of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]alanine (see Materials and Methods). As summarized in Table 1, GDM blocked the incorporation of

Fig. 1. Effects of GDM on growth of L5178Y cells.

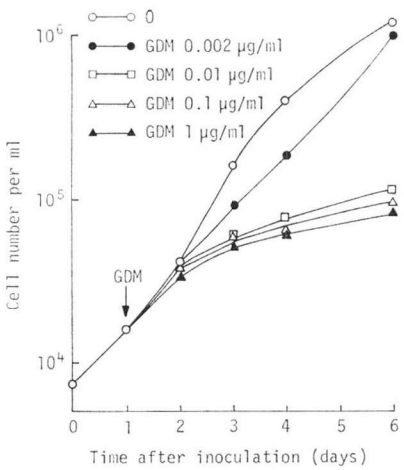
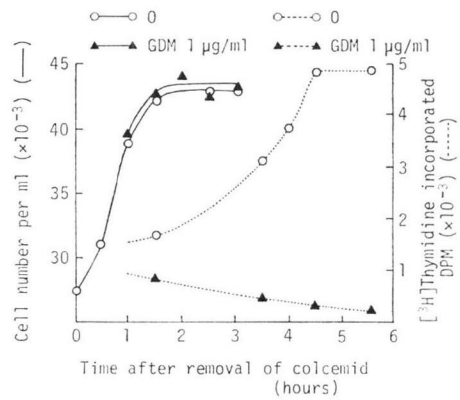


Table 1. Effects of geldanamycin on macromolecular syntheses in murine lymphoblastoma L5178Y cells.

Geldanamycin (μg/ml)	Relative incorporation of		
	[^{3}H]thymidine	[^{3}H]uridine	[^{3}H]alanine
0	100	100	100
0.01	81.2	98.6	103
0.1	52.7	98.9	107
1	46.3	90.3	101

Fig. 2. Effects of GDM on growth of synchronized L5178Y cells.

The cells (1.5×10^4/ml) were cultured for 7 hours with 0.025 μg/ml of Colcemid, which was then removed by washing twice with warmed phosphate-buffered saline. The cells were resuspended in warmed FISHER's medium containing 10% horse serum and 1 μg/ml GDM. The pulse labeling with [^{3}H]thymidine (1 μCi/ml) was carried out for an hour.



[^{3}H]thymidine, and approximately 50% inhibition was observed at a drug concentration of 0.1 μg/ml. The uptake of [^{3}H]uridine and [^{3}H]alanine was hardly affected by GDM. The results suggested that GDM affects DNA synthesis more profoundly than RNA and protein syntheses.

Effects of GDM on DNA Synthesis in Random and Synchronized Cultures of L5178Y Cells

DNA synthesis was more markedly affected by longer incubation with GDM (Fig. 3). The uptake of [^{3}H]thymidine was completely stopped after 8 hours of incubation in the presence of 0.01 ~ 1 μg/ml.

As illustrated in Fig. 4, DNA synthesis was strongly blocked by GDM in synchronized cells. The rate of DNA synthesis was examined by pulse labeling of [^{3}H]thymidine for an hour at indicated points after removal of Colcemid. DNA synthesis in control cells started around 2 hours and continued for about 5 hours; the rate of DNA synthesis increased linearly after Colcemid removal. The degree of inhibition by GDM was getting stronger with enhancement of DNA synthesis rate. Since the over-all rate of DNA synthesis parallels the frequency of initiation of replication[15,16], the results suggested that initiation of replication is affected by GDM.

Stronger inhibition of [^{3}H]thymidine pulse labeling was observed when GDM was added to the synchronous culture in G_1 phase of cell cycle or prior to S phase than when added during S phase (Table 2).

For the purpose of studying whether the observed suppression of [^{3}H]thymidine uptake was due to DNA degradation, a chase experiment was performed (Fig. 5). [^{3}H]Thymidine was incorporated into L5178Y cells for 1.5 hours, then excess cold thymidine (2 mM) was introduced to the culture, and the cells were incubated for another 3.5 hours with or without GDM. In the cells without GDM, the increase of [^{3}H]thymidine uptake was abolished after addition of cold thymidine (0/chase). The radioactivity was not significantly affected by the presence of 1 μg/ml of GDM (GDM/chase), showing that DNA degradation was not induced by GDM.

Fig. 3. Effects of GDM on DNA synthesis in L5178Y cells.

[^{3}H]Thymidine was introduced into the culture at time 0. The incorporation of [^{3}H]thymidine is expressed by dpm/4.8×10^4 cells.

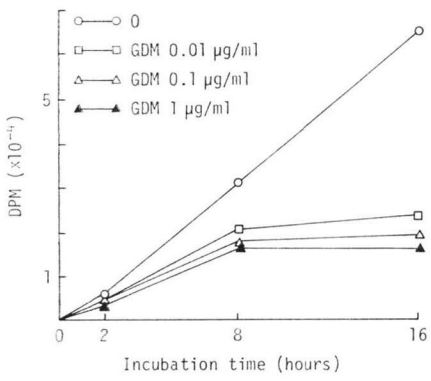


Fig. 4. Effects of GDM on DNA synthesis in synchronized L5178Y cells.

The procedure is presented in the legend of Fig. 2.

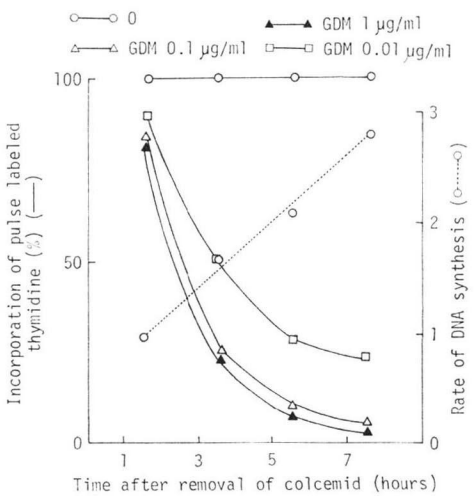


Table 2. Effects of GDM on DNA synthesis in synchronous culture of L5178Y cells: Dependency on cell cycle phase.

Geldanamycin (μg/ml)	Cell cycle phase	
	G_1	S
0	100*	100
1	8.0	57.2.

* The number represents relative incorporation of [^{3}H]thymidine labeled for an hour: 100%= 3,715 dpm/tube. GDM was introduced into the culture immediately (G_1 phase) or 4 hours (S phase) after removal of Colcemid.

Fig. 5. [^{3}H]Thymidine-labeled DNA chase with excess cold thymidine in the presence or absence of GDM.

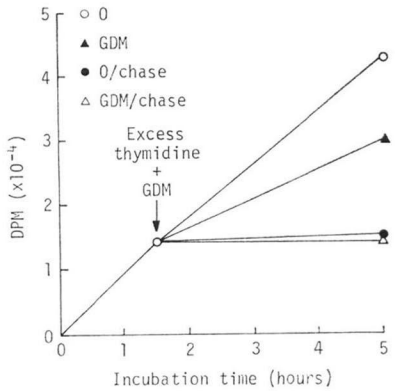
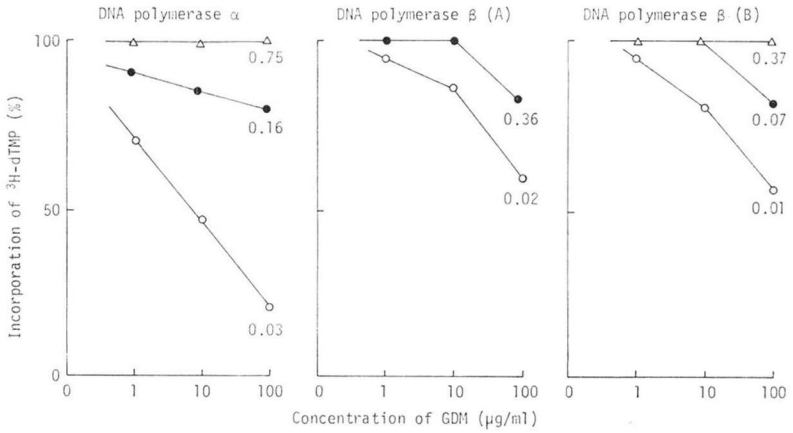


Fig. 6. Effects of GDM on DNA polymerases α and β, derived from Ehrlich ascites tumor cells.
The number represents DNA polymerase units/tube. One unit DNA polymerase was defined as the amount catalyzing the uptake of 1 pmole of [^{3}H]dTMP into DNA in 60 minutes.
DNA polymerase β: (A) reaction with activated DNA. (B) reaction with oligo(dT)-poly(rA).

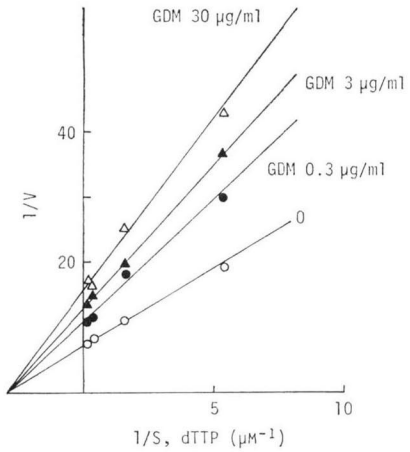


Kinetic studies on the inhibition by GDM of DNA polymerase α were performed, and the LINEWEAVER-BURK plots are presented in Fig. 7. The inhibition was non-competitive as a function of substrate dTTP, and *Ki* was 20 μM.

Effects of GDM on DNA Polymerases α, β and γ, Obtained from Ehrlich Ascites Carcinoma

The results concerning the effect of GDM on DNA polymerases α and β are illustrated in Fig. 6. The extent of inhibition by GDM depended upon concentrations of enzyme, but not upon those of template-primer. Stronger inhibition was observed at low concentrations of enzyme. Approximately 50% inhibition was observed at 10 μg/ml of GDM with 0.03 units of DNA polymerase α. IC_{50} (50% inhibitory concentration) of GDM for DNA polymerase β was found at *ca.* 100 μg/ml, using either activated DNA or oligo(dT)-poly(rA) as a template-primer and 0.01~0.02 units of enzyme, showing that DNA polymerase β was rather resistant to GDM. DNA polymerase γ was resistant to GDM (data are not shown).

Fig. 7. LINEWEAVER-BURK plots of DNA polymerase α reaction with GDM.
The concentration of DNA polymerase α was 0.05 units/tube.



Discussion

In the current experiments, GDM is shown to inhibit DNA synthesis with little effects on RNA and protein syntheses in L5178Y cells.

[^{3}H]Tymidine incorporation slowed down during prolonged incubation with GDM. The profile of DNA synthesis in the presence of GDM (Fig. 3) resembles that of *E. coli* dna_{ts} mutants of initiation type where the cells grow at non-permissive temperature[15], suggesting that GDM decreases the number of cells traversing S phase. DNA synthesis of the cells synchronized with Colcemid was markedly blocked by GDM, when GDM was introduced into the culture before S phase, again suggesting that the initiation of S phase is sensitive to GDM. The assumption was supported by the experiment presented in Fig. 4.

The current results indicate that GDM affects initiation of DNA synthesis or S phase *in vivo* and inhibits DNA polymerase α *in vitro*, suggesting a possible participation of DNA polymerase α in initiation of DNA synthesis.

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