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Antitumor Activity and Mechanism of Action of 6-Thio-3-deazaguanine[1]

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ABSTRACT

6-Thio-3-deazaguanine (TDG), a relatively new purine antimetabolite, exhibits significant antitumor activity against a variety of experimental animal tumor models including C_3H mammary adenocarcinoma, Lewis lung carcinoma, adenocarcinoma 755, and leukemias L1210 and P388. However, the drug was ineffective against 3-deazaguanine-resistant L1210 (both *in vitro* and *in vivo*) and CEM cells (*in vitro*). The resistant cells appear to lack HGPRTase activity because the extracts from these cell lines failed to convert hypoxanthine to IMP. These data indicate that TDG needs to be activated by hypoxanthine guanine phosphoribosyltransferase prior to its growth inhibitory effects. Cytotoxicity of TDG was completely reversed by hypoxanthine and inosine. TDG inhibited the synthesis of DNA and RNA equally and effectively, whereas the inhibition of protein synthesis required a prolonged drug exposure and appears to be a consequence of the inhibition of DNA and RNA synthesis. Data from these studies suggest that TDG is an effective antitumor agent, and its spectrum of antitumor activity and mechanism of action appears to be different from that of 3-deazaguanine.

INTRODUCTION

The clinical usefulness of purine antimetabolites TG[3] (see Fig. 1) and 6-mercaptopurine for the treatment of acute leukemias is well documented (1–5). These analogues require metabolic conversion to the nucleotide level by the purine salvage enzyme HGPRT, in order to exert cytotoxicity (6–9). Thus, the activated purine antagonists inhibit several enzymes involved in *de novo* purine biosynthesis (10–12). The mechanism of cytotoxicity of TG is complex and is not completely understood. However, it is believed that a major effect is due to its incorporation into DNA resulting in aberrant template functions (13–15). Similarly, MP nucleotide is known to be converted to TG nucleotides intracellularly and thus may also exert its antitumor activity through a thioguanylate incorporation mechanism (16, 17).

DG, a relatively new purine antagonist with marked growth inhibitory effects against experimental animal tumor models (18–20), is currently undergoing phase I clinical evaluation (see Fig. 1). It is also anabolized by HGPRT, and the DG nucleotides inhibit DNA synthesis and are also incorporated into nucleic acids (21–24). The incorporation of DG nucleotides into DNA caused an irreversible G_2-M cell cycle traverse block in CEM cells which may be the basis for its cytotoxicity (22).

The synthesis of TDG was reported by Rousseau *et al.* (25), but the biological studies with the compound have not been described. Thiopurines and DG appear to have a similar mode of action and activation process but exhibit a different spectrum of antitumor activity. It was, therefore, reasoned that TDG may also be activated by HGPRT and its metabolites may exert different and unique biological effects. In this communication, we report the antitumor activities of this analogue against a panel of experimental animal tumors. A number of *in vitro* studies to determine its preliminary mode of action are also described. A brief report of these data has been presented (26).

MATERIALS AND METHODS

Drugs and Chemicals. TDG was synthesized according to the reported literature procedure (25). Hypoxanthine and inosine were purchased from Aldrich Chemical Co., Milwaukee, WI. [^{3}H]Thymidine (82 Ci/mmol), [^{3}H]leucine (147 Ci/mmol), and [8-^{14}C]-hypoxanthione (45 mCi/mmol) were purchased from New England Nuclear Co., Boston, MA. [^{3}H]Uridine (19 Ci/mmol) was obtained from Amersham Co., Arlington Heights, IL. The drugs were dissolved in sterile phosphate-buffered saline with nontoxic amounts of dimethyl sulfoxide (0.10% or less). The final concentration and purity of TDG solution was determined by thin layer chromatography and UV spectrophotometric analysis.

Cell Cultures. L1210 murine leukemia cells were obtained from American Type Culture Collection, Rockville, MD, and maintained as a suspension culture in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 50 μM 2-mercaptoethanol, and antibiotics (penicillin and streptomycin) in a humidified 5% CO_2 atmosphere at 37°C. Human leukemic lymphoblasts (CCRF-CEM cells, T-cell origin, hereafter called CEM) were obtained from Dr. Herbert Lazarus of this university. These cells were grown in Eagle's minimal essential medium for suspension cultures containing 10% FCS and antibiotics. L1210/DG and CEM/DG cells were obtained by subculturing the cells in the presence of serially increasing drug concentrations. The resistant cells grew in the presence of 5000 μM DG without any apparent change in their growth pattern.[4]

Cytotoxicity Studies. The cytotoxic effect of TDG was evaluated by treating the appropriate cells in triplicate with various drug concentrations and determining the growth of treated and untreated cells by suspension cultures and colony-forming assays. Suspension cultures (2.5×10^5 cells/ml) of L1210, L1210/DG, CEM, and CEM/DG cells in the logarithmic growth phase were exposed to TDG (0.1 to 1000 μM) for 48 h under standard culturing conditions. Cell counts were determined by hemocytometer, and the cell viability was assessed by trypan blue dye exclusion (0.4% in unbuffered 0.9% NaCl). The percentage of control growth was determined as

$$\% \text{ of control growth} = \frac{\text{Final treated cell no.} - \text{initial cell no.}}{\text{Final control cell no.} - \text{initial cell no.}} \times 100$$

Only viable cells were considered for the calculations. Soft agar clonogenic assays were carried out in a single layer of 0.3% soft agar. L1210 and L1210/DG cells exposed to TDG (0.1 to 1,000 μM) were plated in 35- x 10-mm Petri dishes (10,000 to 15,000 cells/plate) using RPMI 1640 media supplemented with 20% FCS, 50 μM 2-mercaptoethanol, and antibiotics. After 7 days of incubation at 37°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2, colonies (>30 cells) were counted from triplicates of the control and treated plates under an inverted microscope at ×100. Plating efficiency for these experiments ranged between 45 and 57%.

Assay of HGPRTase Activity. DG-resistant and -sensitive L1210 and CEM cells (50×10^6 cells/experiment) were harvested, washed, diluted 10-fold with distilled water (0.5 ml), and heated at 100°C for 1 min (27). Supernatants were collected and the heating process was repeated once more with the precipitates. Combined supernatants were lyophilized, and the residue obtained was taken up in 1 ml of 10 mM Tris/HCl buffer (pH 7.4) and was used to determine the enzyme activity.

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[3] The abbreviations used are: TG, 6-thioguanine; TDG, 6-thio-3-deazaguanine; DG, 3-deazaguanine; L1210/DG, 3-deazaguanine-resistant L1210 cells; CEM/DG, 3-deazaguanine-resistant CEM cells; % ILS, a percentage increase in life span; TWI, tumor weight inhibition; FCS, fetal calf serum; HGPRT, hypoxanthine guanine phosphoribosyltransferase.

[4] A. M. Mian, S. Furusawa, and A. Krishan. Studies on the mechanism of cytotoxicity of 3-deazaguanine, submitted for publication.

TDG DG

Fig. 1. Structures of TDG and DG.

The enzyme assay (27, 28) mixture in a final volume of 400 μl contained 50 μmol Tris/HCl, 4 μmol $MgCl_2$, 70 μmol phosphoribosylpyrophosphate 80 nmol [8-^{14}C]hypoxanthine (45 mCi/mmol), and 100 μl of crude HGPRT extract. After 15 min at 37°C the reaction was stopped by immersing the tubes in boiling water for 5 min. Assay mixture (20 μl) was spotted on cellulose thin layer and overspotted with a mixture of cold IMP and hypoxanthine to facilitate the localization. Chromatograms were developed in an *n*-butanol:glacial acetic acid:water (5:2:3, v/v) solvent system. The developed thin layer chromatograms were cut into one-half-inch wide strips and counted for radioactivity.

For quantitation, the formation of IMP from [^{14}C]hypoxanthine was taken as a direct measure of HGPRTase activity present in the extracts of sensitive and resistant cells. Data from the repeated assays (in duplicate) show that 24.1 ± 2.3% (SD) of IMP was formed in the extracts of sensitive cells with 73.5 ± 4.8% of radioactivity being recovered as unchanged hypoxanthine base. On the other hand, less than 2% radioactivity was found to be associated with IMP in the incubation mixture of resistant cell extracts, while most of the counts (94.6 ± 3.7%) were still in the area of hypoxanthine. From these results, it can be inferred that the DG-resistant cells lack HGPRTase activity.

Incorporation of Labeled Precursors. Drug effects on DNA, RNA, and protein synthesis in L1210 and L1210/DG cells were determined by measuring the incorporation of [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H] leucine into cellular acid-precipitable material. The L1210 and L1210/DG cells in log phase (1 × 10^6 cells/ml) were incubated with TDG (10 to 100 μM) for 4 h. Cultures were pulse labeled with 1.0 μCi/ml of [^{3}H] thymidine, [^{3}H]uridine, or [^{3}H]leucine for 1 h. Cells were washed with cold phosphate-buffered saline, centrifuged, and the cell pellets extracted in cold 10% trichloroacetic acid. The precipitates were dissolved in 0.3 N NaOH, neutralized, and resuspended in scintillation fluid (Aqualyte; J. T. Baker Chemical Co., Phillipsburg, NJ). Triplicate samples were counted in an LS-7000 liquid scintillation counter (Beckman Instruments). Typical counts per 10^6 cells were 51,833 ± 2,672 for [^{3}H]thymidine, 35,349 ± 2,039 for [^{3}H]uridine, and 3,938 ± 201 for [^{3}H]leucine.

Animal Studies. Female DBA/2, C57BL/6 × DBA/2 F_1, and C3H mice, with an initial body weight of 20 to 22 g, obtained from The Jackson Laboratory, Bar Harbor, ME, and Charles River Breeding Laboratories, Wilmington, MA, were used throughout the studies. The animals were housed in plastic cages under standard laboratory conditions with free access to food and water. P388 leukemia, Lewis lung carcinoma, and B16 melanoma cells were obtained from American Type Culture Collection. Mammary adenocarcinoma 755 cells were kindly supplied by Dr. Sheldon Greer, Department of Microbiology of this university. Tumors were maintained by serial transplants in appropriate hosts and were used for antitumor testing in accordance with the test protocols described by the Drug Evaluation Branch of the National Cancer Institute. Mammary adenocarcinoma 16/C was obtained through the courtesy of Dr. T. Corbett (Michigan Cancer Foundation, Detroit, MI) and was maintained in C3H female mice according to the published method (28). The mean survival time for the ascitic tumors was measured in days, and from this the % ILS was calculated according to the equation

$$\% \text{ ILS} = 100 \times \frac{T - c}{c}$$

where t is the mean survival time of the treated group and c is the mean survival time of the control group. In all tumor systems >25% ILS was considered necessary to demonstrate activity. The antitumor activities in mammary adenocarcinomas 16/C, 755, or Lewis lung carcinoma were determined from the TWI on specific days (as shown in the tables) after tumor transplantation. Tumor weights (mg) were estimated by measuring length (l) and width (w) of each tumor with a vernier caliper (mm) and using the formula

$$\frac{l \times w^2}{2}$$

Changes in body weight were examined as an indicator of drug toxicity.

RESULTS

Cytotoxic Effects. The cytotoxic effects of TDG on log phase cultures of L1210, L1210/DG, CEM, and CEM/DG cells are shown in Table 1. TDG inhibited the growth of sensitive L1210 and CEM cells in a dose-dependent manner. The 50% infective doses of 0.8 and 1 μM were observed for L1210 and CEM cells, respectively, as compared to 1000 and 700 μM in L1210/DG and CEM/DG cells for this drug. These data clearly demonstrate the cross-resistance for TDG in L1210/DG and CEM/DG cells. In a separate set of experiments, similar results in L1210 and L1210/DG were also observed in a soft-agar colony-forming assay.

Fig. 2 illustrates that hypoxanthine or inosine when added simultaneously and in equimolar concentrations protected L1210 cells from the cytotoxic effects of TDG; however, lower concentration of hypoxanthine or inosine (10 μM) were only partly effective in reversing the cytotoxic effects of TDG. At the concentrations used in these experiments, neither hypoxanthine nor inosine alone had any effect on the viability of L1210 cells, but, the studies using guanine and guanosine to affect reversal were complicated as both of these natural derivatives exhibited toxicity at 100 μM concentrations (results not shown).

Effects on Macromolecular Synthesis. The effects of TDG on DNA, RNA, and protein precursor incorporation were measured by pulse labeling the control and drug-treated sensitive and DG-resistant L1210 cultures with appropriate ^{3}H-labeled precursors. Data in Table 2 show that TDG at 10 and 100 μM concentrations almost completely inhibited the incorporation of [^{3}H]thymidine and [^{3}H]uridine into DNA and RNA, respectively, in L1210 cells. Under similar experimental conditions, the protein synthesis was only inhibited by 40 and 50%, respectively. In DG-resistant L1210 cells, no inhibition of protein and RNA synthesis was observed at either drug concentration, but the higher TDG concentration (100 μM) was partially (30%) inhibitory to DNA synthesis. In an effort to see time-dependent inhibition of macromolecular synthesis, L1210 cells were exposed to TDG (10 μM) for various intervals of time prior to the addition of radioactive precursors. Data in Fig. 3 show that the

Table 1 *Cytotoxic effects of TDG in leukemic lymphoblasts*

Drug concentration (μm)	Suspension cultures[a]				Colony-forming assay[b]	
	L1210	L1210/DG	CEM	CEM/DG	L1210/0	L1210/DG
0.1	80	99	88	96	85	100
1	43	107	54	97	63	97
10	10	114	20	99	40	92
100	5	85	18	77	15	65
1000	0	54	4	34	0	50

[a] Cultures in triplicate were treated with TDG for 48 h, and trypan blue dye-excluding cells were counted in a hemacytometer. Number of dye-excluding cells in treated populations is expressed as a percentage of untreated controls.

[b] Number of colonies (>30 cells) after 7 days of incubation is expressed as a percentage of control.

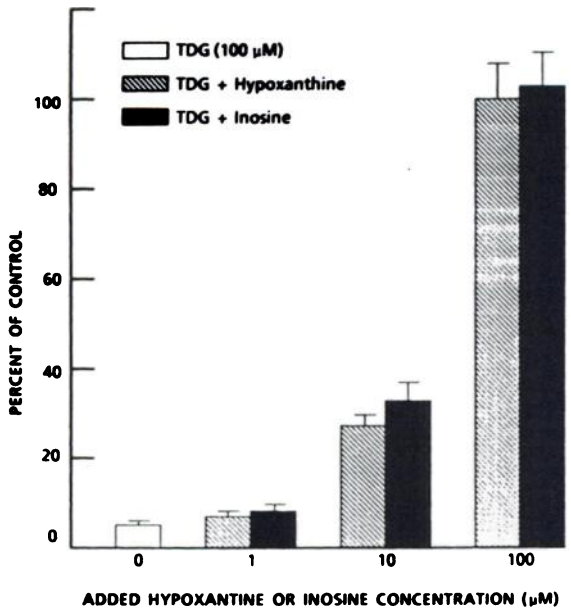

Fig. 2. Reversal of cytotoxic effects of TDG. Cultures of L1210 cells in triplicate were treated with TDG alone, TDG hypoxanthine, or TDG and inosine for 48 h. Cell counts were determined and the results are expressed as a percentage of untreated controls. Values are means of triplicate assays ± SD (*bars*).

Table 2 *Effects of TDG on the incorporation of radiolabeled precursors into L1210 and L1210/DG macromolecues*

Cells (1×10^6 cells/ml) were incubated with TDG for 4 h followed by 1 h of pulse labeling with (1 μCi/ml) of [^{3}H]thymidine, [^{3}H]uridine or [^{3}H]leucine. Results represent mean values for triplicate assays and are expressed as a percentage of untreated controls. SDs were less than 10%.

Drug concentration (μm)	Precursor incorporation (%)					
	[^{3}H]Thymidine		[^{3}H]Uridine		[^{3}H]Leucine	
	L1210	L1210/DG	L1210	L1210/DG	L1210	L1210/DG
10	16	91	17	110	58	109
100	11	69	14	102	48	98

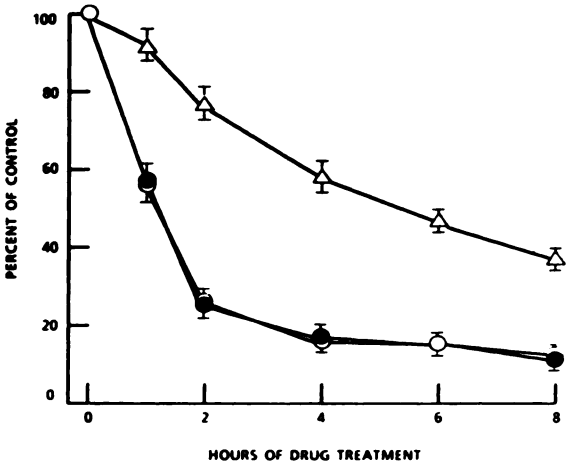

Fig. 3. Effects of TDG on the synthesis of DNA (○), RNA (●), and protein (△) in L1210 cells. Log phase cultures of L1210 cells were exposed to TDG (10 μM) for various intervals of time prior to being pulse labeled with [^{3}H]thymidine, [^{3}H]uridine or [^{3}H]leucine for 1 h. The radioactive counts of triplicate samples were averaged, and the incorporation was expressed as a percentage of untreated controls. Values are means of triplicate assays ± SD (*bars*).

inhibition of DNA and RNA synthesis was time dependent for an initial drug exposure of 2 h and plateaued thereafter over an 8-h observation period. On the other hand, the inhibition of protein synthesis increased slowly and continuously for the duration of the experiment. The results indicate that the protein inhibition may be a consequence of TDG-induced shutdown of DNA and RNA synthesis.

Antitumor Activity. The antitumor activity of TDG was evaluated in a panel of experimental animal tumor models and the results are given in Tables 3 and 4. The drug concentrations used were based upon the demonstrated antitumor activity of DG in similar tumor models (19). Data in Table 3 show that one daily administration (days 1 to 9, i.p.) of 40 and 80 mg/kg of TDG starting on day 1 produced significant inhibition of L1210 (ILS, 44 and 67%) and P388 (ILS, 36 and 52%) leukemias. However, the drug was completely ineffective against L1210/DG tumor. Similarly, the drug appeared to be marginally effective against i.p. implants of B16 melanoma, because the highest drug concentration used (160 mg/kg) produced only 27% ILS. The results of antitumor activity of TDG against s.c. implanted solid tumors are shown in Table 4. In C3H mammary adenocarcinoma 16/C, TDG at 40, 80, and 160 mg/kg when given daily for nine treatments by i.p. route produced TWI values of 55, 62, and 78%, respectively. Similarly, doses of 40 and 80 mg/kg (daily, days 1 to 9) inhibited the growth of adenocarcinoma 755 by 54 and 72%, respectively. Data in Table 4 also show that a similar treatment schedule of TDG (40, 80,

Table 3 *Activity of TDG against murine ascitic tumors*

L1210, L1210/DG cells (1×10^5 cells/mouse), P388 leukemia cells (1×10^6 cells/mouse), or B16 melanoma cells [0.5 ml of tumor brei (Ig in 10 ml phosphate-buffered saline)] were implanted i.p. into female C57BL/6 × DBA/2 mice (6 to 10 mice/group) on day 0; TDG was administered i.p. daily on days 1 to 9 and the control group received phosphate-buffered saline injections.

Tumor	Compound	Dose (mg/kg/day)	Survival time (days) (mean ± SD)	ILS (%)	Body weight change (g)[a]
L1210	Control		10.3 ± 1.2	0	+1.7
	TDG	40	14.8 ± 1.1[b]	44	+2.0
		80	17.2 ± 2.2[b]	67	+0.7
L1210/DG	Control		10.1 ± 1.6	0	+1.5
	TDG	40	11.0 ± 1.1[b]	9	+1.5
		80	10.0 ± 1.0[b]	−1	+1.8
P388	Control		10.3 ± 1.0	0	+1.5
	TDG	40	14.0 ± 1.1[b]	36	+1.8
		80	15.7 ± 2.1[b]	52	+1.1
B16 Melanoma	Control		14.2 ± 0.9	0	+3.0
	TDG	40	15.8 ± 0.4[b]	11	+2.5
		80	16.7 ± 0.9[b]	18	+2.7
		160	18.0 ± 0.8[b]	27	+0.5

[a] The average weight changes on day 5 for L1210, L1210/DG, and P388 but on day 9 for B16 melanoma.
[b] Significantly different from values for controls by Student's *t* test ($P < 0.01$).

Table 4 *Antitumor effect of TDG against various solid tumors*

Tumor fragments (2 to 4 mm) were implanted s.c. into appropriate host (6 to 10 mice/group) on day 0; TDG was administered i.p. daily on days 1 to 9 and antitumor activity was determined on day 12.

Tumor	Host	Dose (mg/kg/day)	Tumor weight (g) (mean ± SD)	TWI (%)
C3H mammary adenocarcinoma	C3H	Control	1.52 ± 0.66	0
		TDG		
		40	0.69 ± 0.45[a]	55
		80	0.58 ± 0.34[a]	62
		160	0.33 ± 0.14[a]	78
Mammary adenocarcinoma 755	C57BL/6 × DBA/2	Control	0.67 ± 0.15	0
		TDG		
		40	0.31 ± 0.13[a]	54
		80	0.19 ± 0.08[a]	72
Lewis lung carcinoma	C57BL/6 × DBA/2	Control	0.76 ± 0.19	0
		TDG		
		40	0.55 ± 0.19[a]	28
		80	0.28 ± 0.15[a]	63
		160	0.20 ± 0.09[a]	74

[a] Significantly different from values for controls by Student's *t* test ($P < 0.01$).

and 160 mg/kg) was inhibitory to Lewis lung carcinoma and gave TWI values of 28, 63, and 74%, respectively.

DISCUSSION

TDG is appreciably more cytotoxic to L1210 and CEM cells than is DG (50% infective dose 0.8 and 1.0 μM *versus* 11 and 80 μM for TDG and DG in L1210 and CEM cells, respectively) both in suspension cultures and in clonogenic assays. However, like DG, it was ineffective against 8-azaguanine-resistant CEM (data not shown) and DG-resistant L1210 and CEM cells (Table 1). Both of the DG-resistant variants have been developed in our laboratory and appear to lack HGPRTase activity, as indicated by their failure to convert radiolabeled hypoxanthine to IMP. The cytotoxic effects of TDG were completely reversed by simultaneous addition of equimolar hypoxanthine or inosine. These results show that TDG, like DG, TG, 6-mercaptopurine, and 8-azaguanine needs to be activated by HGPRTase for its cytotoxic activity.

TDG in experimental animal models exhibited significant tumor growth inhibition against L1210 and P388 leukemias (Table 3). However, TDG was not active against L1210/DG transplants lacking HGPRTase activity, once again underscoring its need to be activated by this enzyme to be effective. In solid tumor models (Table 4), TDG treatments inhibited the growth of C3H mammary 16/C, 755, and Lewis lung adenocarcinomas in a highly significant manner. It is interesting to note that DG was not effective against Lewis lung adenocarcinoma (19) suggesting a different spectrum of antitumor activity for TDG. Activity of this purine antimetabolite against C3H mammary adenocarcinoma is extremely relevant because this tumor is highly metastatic with doubling time of 1.5 to 2 days, and its response positively correlates with most of the clinically used drugs (29). TDG, like DG, was not effective against B16 melanoma (Table 3). In the animal models described, no apparent signs of drug toxicity were observed, eliminating the possibility that the tumor growth inhibition may be due to toxic effects on the host.

In order to determine the mechanism of cytotoxicity of TDG, its effects on the synthesis of DNA, RNA, and protein were studied. TDG was equally effective in inhibiting the synthesis of DNA and RNA in a time-dependent fashion and a maximum inhibition was obtained after 2 h of drug exposure. On the other hand, initially, the effect of TDG on protein synthesis was minimal but increased to 50% inhibition after 4 h of drug exposure at which time the inhibition for the synthesis of both DNA and RNA had plateaued. It is postulated that the inhibition of protein synthesis may be a secondary phenomenon caused as a consequence of the inhibitory effects of TDG on DNA and RNA synthesis. These results, when compared with the effects of DG on macromolecular synthesis, are extremely interesting in the sense that DG has no effect on the synthesis of RNA, and its tumor growth inhibition is associated with its capacity to inhibit DNA synthesis (22, 23, 26, 30), although inhibition of protein synthesis has also been claimed to be the cause for DG cytotoxicity (31). On the other hand, the cytotoxic effect of TDG appears to be due to its inhibition of both DNA and RNA with no apparent direct effect on protein synthesis. In summary, these studies show that TDG is highly effective against transplantable solid tumors, and its spectrum of activity and mode of action is different from DG. Further studies to elucidate the detailed mechanism of action and metabolism of this drug are in progress.

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