Title
Modulation of 6-thioguanine induced cell toxicity and differentiation by guanine in S-49 and HL-60 cells

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MODULATION OF 6-THIOGUANINE INDUCED CELL TOXICITY

AND DIFFERENTIATION BY GUANINE IN S-49 AND HL-60 CELLS

by

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DISSERTATION

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To my dear parents and my wife Linda

for their endless love, support, and encouragement.

ABSTRACT

The mechanism of 6-TG toxicity has been attributed to its incorporation into DNA. The presence of guanine *in vivo* is expected to modify the action of 6-TG by either interfering with 6-TG metabolism or its incorporation into nucleic acids. The effects of guanine on 6-TG metabolism and cell toxicity were therefore studied in mouse lymphoma S-49 cells. The results showed that while guanine does not affect the accumulation of 6-TGMP, it does eliminate the formation of 6-dTGTP. Guanine also competes effectively with 6-TG for incorporation into DNA and RNA, but fails to prevent the growth inhibitory effect of 6-TG. Therefore, mechanisms other than 6-TG incorporation into DNA and RNA contribute to 6-TG toxicity in S-49 cells.

6-TG is able to induce cell differentiation in human promyelocytic HL-60 cells at low concentrations. Coadministration of guanine with 6-TG decreases the accumulation of 6-TGMP and suppresses of cell differentiation; therefore, differentiation apparently depends on 6-TGMP formation at low 6-TG concentrations. 6-TGMP is a poor substrate of GMP kinase with a low V_{max} value and k_m = 384 μM. It also behaves as a noncompetitive inhibitor of GMP for 5'-nucleotidase, acid phosphatase, and alkaline phosphatase and again represents a poor substrate for these enzymes studied. Thus, high concentrations of 6-TGMP can be reached in cells that in turn inhibit several other enzymes involved in guanosine nucleotide biosynthesis, such as PRPP amidotransferase, HGPRTase, and IMP dehydrogenase, thus causing guanine starvation. Therefore, guanine starvation induced by 6-TG treatment may play an important role in regulating cell differentiation.

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CHAPTER I.

GENERAL INTRODUCTION

1. Cancer chemotherapy - Antimetabolite

Several modes of therapy are currently used to treat neoplastic disease, including surgery, radiotherapy, and chemotherapy (1). The common goal of these modalities is to eliminate or restrain the growth of cancerous tissue at the least possible cost to the health of normal host tissue. From the standpoint of chemotherapy, an obvious approach to the problem is to exploit whatever biochemical differences exist between normal and tumor tissue, since all cancer cells are transformed and have a biological commitment to continued replication, which is manifest in the need for more metabolic resources to fulfill this replication process (2). Therefore, an agent that induces a metabolic perturbation in cancer cells, e.g., nucleic acid synthesis, might be expected to have a greater toxic effect on cancer cells than host cells. Advances have been made in showing the role of antimetabolite in determining anticancer action. These antimetabolites can either inhibit activities of key enzymes in de novo and/or salvage pathway, or become incorporated into nucleic acids and cause subsequent impairment of nucleic acids. However, the toxicities associated with these agents are often severe, indicating that more detailed studies of the biochemical mechanisms of antimetabolites are needed.

2. Clinical Uses of 6-Thioguanine

6-Thioguanine (6-TG) (Fig. I-1) is of particular value in the treatment of acute myelogenous leukemia (AML) when given with Ara-C (26-33). Clinical studies on AML performed by Clarkson et al. gave Ara-C

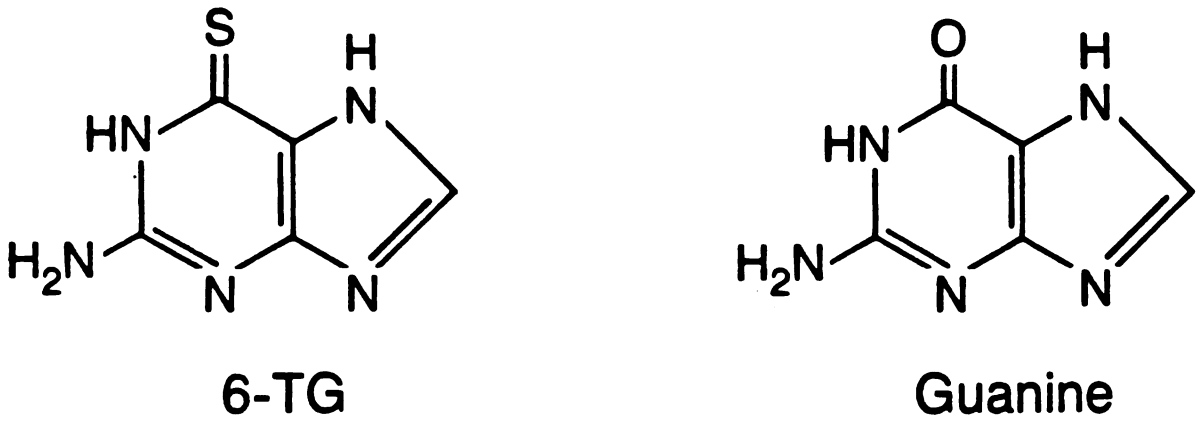

Fig. I-1. Chemical structure of 6-TG and guanine.

3.0 mg/kg intravenously supplemented with 6-TG 2.5 mg/kg orally every 12 hours (26,32). The results showed that 56% of the 88 patients had complete remissions and the median duration of remission was 10 months. Administration of Ara-C at a daily dose of 100 mg/m^2 in a one-hour infusion together with oral dose of 6-TG at a dose of 2.5 mg/kg/day was used by Carey et al. (33). The achievement of 41 responses out of 84 patients is similar to the results reported by Clarkson. Other cytotoxic drugs such as adriamycin, daunorubicin, etc., have also been used quite often in combination with 6-TG and Ara-C to treat AML (28-31). Table I-1 summarizes the results from these studies.

Use of 6-TG against solid tumors was not successful (34-37). Horton et al. gave 6-TG orally at 1 mg/kg/day continuously to patients with advanced colorectal carcinoma. They noted two complete and two partial responses among 54 patients (36). Presant et al. gave 6-TG orally on a single day at high dose, and intravenously at high dose, and noted 5 responses in 60 patients with solid tumors, and 5 responses of 21 patients with colorectal carcinoma (37). Britell et al. studied 6-TG given intravenously every 21 days at 700-1000 mg/m^2 to 29 patients with advanced colorectal carcinoma, and only one achieved a partial regression (34). Konits et al. noted no measurable regressions in 19 patients with advanced colorectal carcinoma when treated with a single dose of intravenous 6-TG, ranging from 700-1200 mg/m^2 every 3 weeks (35). These groups concluded that a high dose of 6-TG given intravenously intermittently had little activity in colorectal cancer and discouraged further use of this treatment.

On the other hand, treatment of polycythemia rubra vera (PRV) with 6-TG looks promising. Milligen et al. administered daily doses of 40-

Table I-1. Combinational chemotherapy of 6-TG with other agents.

Agent 1	Agent 2	Agent 3	Disease	Remission Rate	Reference
6-TG	Ara-C		AML	40-65%	26,27,32,33
6-TG	Ara-C	adriamycin	AML (+RDA)	--	29,31
6-TG	Ara-C	daunorubicin	AML	>70%	28,30
6-TG			PRV	89%	38

Abbreviations: AML = adult myelogenous leukemia; RDA = refractory dysmyelopoietic anemia; PRV = polycythemia rubra vera; 6-TG = 6-thioguanine; ARA-C = cytosine arabinoside.

160 mg on alternate weeks according to individual response (38). Of 27 patients 24 responded to treatment satisfactorily. Thus, as an antimetabolite, 6-TG therapy in PRV is of particular interest in view of its advantages of a) being less oncogenic than alkylating agents (which are usually used in the treatment of PRV) and b) possessing a rapid onset of action.

3. Pharmacokinetics and Metabolism of 6-Thioguanine

The pharmacokinetics of 6-TG have been investigated primarily in the dog (3) and man (6). The pharmacokinetics were found to differ when two different radiolabeled tracers were used (Table I-2). When [^{35}S]6-TG was used, it tended to have a longer terminal half life, smaller Vd, and clearance than did [8-^{14}C]6-TG. However, 5-hour cumulative urinary excretion values did not differ very significantly between these two tracers.

The biotransformation of 6-TG follows the pathways shown on Fig. I-2 (4). Urine samples from both dogs and men were analyzed after intravenous administration of 6-TG and the following metabolites were found in the samples: unchanged 6-TG, 6-thiouric acid, 6-thioxamine, S-methyl-6-thioguanine, and inorganic sulfate. 6-TG apparently undergoes cleavage at the C-S bond <u>in vivo</u>, forming metabolites which may be incorporated readily into endogenous purine and sulfate pools. Thus, in the case of [^{35}S]6-TG, the purine base was not radioactive and therefore [^{35}S] sulfate was the only cleavage product detected. When the terminal plasma $t_{1/2}$ of [^{35}S] in the dog after [^{35}S]6-TG administration was compared with that of $Na_2{}^{35}SO_4$ administration, values were found be

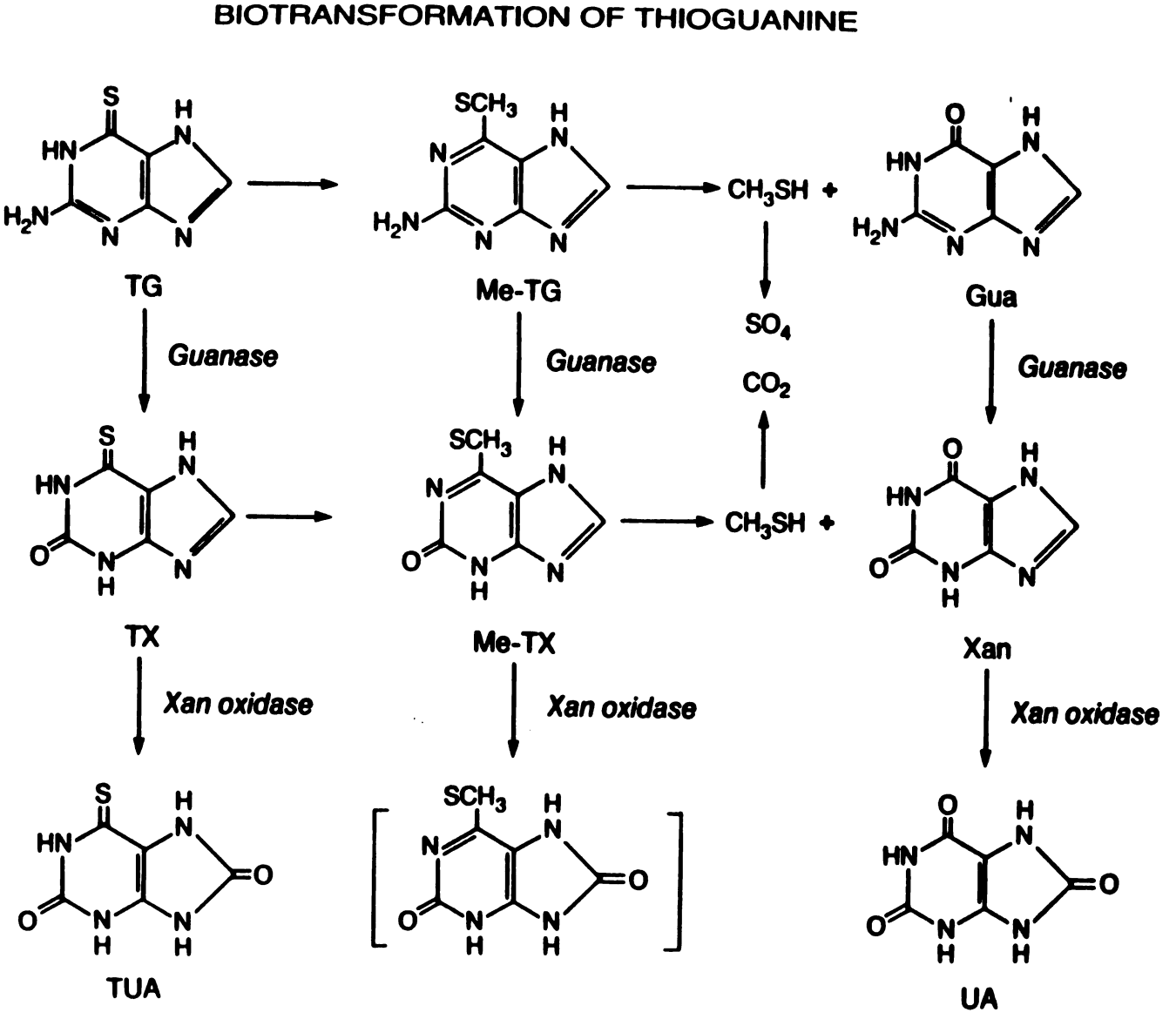

Fig. I-2. Metabolism of thioguanine (4).

Table I-2. Pharmacokinetics of 6-TG in dogs and humans. Average plasma disappearance and cumulative urinary excretion of total radioactivity after I.V. administration was measured.

Subject	Radiolabel (6-TG)	Dose (mg/m^2)	$t_{1/2}$		Vd (ml/kg)	Clearance (ml/kg/min)	Urinary Excretion (% Dose)
			Initial (min)	Terminal (hr)			
Dogs	[^{35}S]6-TG	124	4	4.6±0.5	370±27	0.7	47±0.6
Dogs	[8-^{14}C]6-TG	138	6	1.9±0.1	520±41	4.8	40±2.3
Humans	[^{35}S]6-TG	125	40	28.9±0.8	148±0.7	0.74±0.07	54±5.3
Humans	[8-^{14}C]6-TG	125	55	3.1±0.1	262±0.1	4.77±0.41	43±6.4

similar (4.6 h vs. 5.3 h) (3). It is possible that sulfate may largely contribute to plasma [^{35}S] activity after 6-TG administration during the terminal phase. On the other hand, compared with [^{35}S]6-TG, the utilization of labeled purines derived from the metabolism of [8-^{14}C]6-TG could readily account for the shorter half life and the higher urinary excretion of radioactivity. Moreover, the relatively high total clearance of ^{14}C-labeled metabolites is similar to that of urate in the dog (3).

4. Mode of Action of 6-TG

6-TG has been shown to inhibit de novo purine synthesis resulting from inhibition of several enzymes involved in purine biosynthesis (17-23). The consequences of the incorporation of 6-TG residues into RNA have also been investigated (13-16,94). Kwan et al. showed that 6-TG is readily incorporated into all of the major species of RNA; at the same time, protein synthesis was also inhibited (13). Carrico and Sartorelli, using 6-TG containing poly(A)$^+$RNA from 6-TG treated regenerating rat liver, demonstrated that 6-TG interferes with the synthesis of certain proteins by depressing the synthesis of their mRNA when it is given before the onset of the S phase (15,16). Gray and Rachmeler reported that the incorporation of 6-TG into tRNA resulted in alteration of the acceptor activity of these molecules for their amino acids (94). Incorporation of 6-TGuo into RNA has been reported to cause inhibition of the maturation process of rRNA (14,78), and has been suggested to be related to the growth-inhibiting effect of this drug. However, these mechanisms cannot fully explain the cell toxicity of

6-TG, and recent reports have accumulated much evidence to show that incorporation of 6-TG into DNA is closely related to the cell toxicity of this drug (8-12).

In support of this hypothesis, Nelson et al. showed that a partial protection of human epidermoid carcinoma H.Ep.2 cells from the cytotoxicity of 6-TG could be achieved by coadministration with 1 mM thymidine, which specifically inhibits the synthesis of DNA (12). In addition, the presence of potent DNA synthesis inhibitors such as ara C, hydroxyurea (HU), or 5-fluorodeoxyuridine (FUdR) has been demonstrated to antagonize the cytotoxicity of 6-TG to HeLa and L1210 cells in culture (100,101).

Examination of chromatin structure following 6-TG treatment has reinforced the idea that the incorporation of 6-TG residues into DNA may form the basis of its cytotoxic action. Maybaum and Mandel used the premature chromosome condensation technique, which permits the visual inspection of interphase chromatin, to show that specific and drastic morphological changes in the chromosomes of CHO cells in the G_2 phase of the cell cycle occur after exposure to 6-TG, and this change appears as unilateral chromatid damage and gross chromosome disruption at higher 6-TG concentrations. The appearance of unilateral chromatid damage paralleled the appearance of 6-TG-induced cell toxicity in terms of exposure time and dose. In addition, the disruption of G_2 prematurely condensed chromosomes is consistent with the 6-TG-induced G_2 arrest in this and other systems (9,24,25), It was therefore concluded that this damage is closely involved in the cytotoxicity (9,11). The observed effect on G_2 chromatin structure presumably resulted from a physical alteration in DNA imposed by the presence of thioguanylate in DNA.

In an effort to further study this effect, Christie *et al.* examined the effects of 6-TG incorporation on DNA structure by the alkaline elution technique. The results obtained suggested that DNA strand breaks are produced, which correlate with the incorporation of 6-TG into DNA and subsequent cell toxicity (8).

5. Background of mycophenolic acid—an IMP dehydrogenase inhibitor

IMP dehydrogenase (IMP: NAD oxidoreductase, EC 1.2.1.14) catalyzes the conversion of IMP to XMP, and it represents a key enzyme in the biosynthesis of guanine nucleotides. The activity of IMP dehydrogenase is positively linked to cellular transformation and tumor progression (2); therefore, this enzyme represents a promising target of cancer chemotherapy (2,44).

Mycophenolic acid (MA) represents a prototypic IMP dehydrogenase inhibitor (38-41,44), with no other biochemical effects noted. It was first isolated in 1896 by Gazio from the broth of *Penicillin glaucum* (95). It shows weak to moderate antitumor, antiviral, antifungal, and immunosuppressive activity (96). It exerts toxic effects upon mammalian cells, primarily *via* IMP dehydrogenase inhibition, which results in the depletion of cellular guanine nucleotides by blocking purine *de novo* synthesis (39-41,44). Guanine nucleotides are required as substrates, activators, or regulators in many pathways of cellular metabolism, including DNA and RNA synthesis. This depletion of guanine nucleotides caused by MA strongly affects thymidine and uridine incorporation into DNA and RNA (38-41,44), and this inhibition is reversible by the exogenous supply of guanine nucleotides *via* the salvage pathway,

indicating that it acts primarily as a pure IMP dehydrogenase inhibitor (44).

6. S-49 Cells

The mouse T-lymphoma (S-49) cell line was first established from mineral oil-treated BALB/c mice (5). These cells contain both TL and Thy-1 antigens, indicating that they are thymic in origin (5,98). The cell line was maintained in suspension in 75-cm^2 Falcon T flasks in a humidified 10% CO_2 incubator at 37°C. All cells were grown in Dulbecco's modified Eagle's medium containing 3.7 g/ℓ $NaHCO_3$ and 4.5 g/ℓ D-glucose, and supplemented with 10% horse serum that had been previously heat-inactivated at 56°C for 30 min. Extensive work on the mechanism of the toxicity of guanosine nucleotide depletion in S-49 cells has been carried out in this laboratory and results have been accumulated over the past few years, so use of this cell line was continued.

7. HL-60 Cells

The human promyelocytic leukemia HL-60 cell line was the first human cell line with distinct myeloid features ever developed (6). This cell line, isolated from the blood of a patient with acute promyelocytic leukemia, proliferates continuously in suspension culture in a humidified 10% CO_2 incubator at 37°C. All cells were grown in RPMI 1640 medium containing 2 g/ℓ $NaHCO_3$ and supplemented with 10% fetal calf

Table I-3. Differentiation inducers of HL-60 cells (7).

Inducer*	Concentration	% Differentiation for Parameter	Time in Culture (days)
Nonphysiological Inducers			
Chemicals			
Dimethyl sulfoxide	155-180 mM	94% morphol; 85% phago; 72% NBT-red	6
Acetamide	150 mM	71% morphol	6
N-methylacetamide	20 mM	68% morphol	6
N,N-Dimethylacetamide	10 mM	73% morphol	6
N-Methylformamide	150 mM	81% morphol	6
N,N-Dimethylformamide	60 mM	84% morphol	6
Piperidone	37.5 mM	78% morphol	6
1-Methyl-2-piperidone	4 mM	65% morphol	6
Triethylene glycol	100 mM	60% morphol	6
Butyric acid	0.6 mM	58% morphol	6
Hexamethylene bisacetamide	2 mM	95% morphol; 97% NBT-red	6
Methotrexate	14 nM	31% morphol; 31% NBT-red	6
2-Ethionine	2 - 3.2 mM	27 - 45% NBT-red; 53% C3	4 - 5
Nucleoside analogs			
6-Thioguanine	3 μM	49% morphol; 45% NBT-red	6
3-Deazauridine	12 μM	90% morphol; 84% NBT-red	6
Pyrazofurin	0.5 μM	60% morphol; 48% NBT-red	6
TCN	2 μM	50% morphol; 48% NBT-red	6
5-Bromodeoxyuridine	16.2. μM	53% morphol; 33.3% C3	5
Physiological Inducers			
Retinoids			
All-*trans*-β-retinoic acid	1 μM	94% morphol; 95% NBT-red	5
13-*cis*-retinoic acid	1 μM	85% NBT-red	5
4-Keto-retinoic acid	1 μM	93% NBT-red	5
4-Hydroxy-retinoic acid	1 μM	87% NBT-red	5
α-Retinoic acid	1 μM	67% NBT-red	5
Vitamin D			
1α.25-Dihydroxycholecalciferol	12 nM	44% morphol	3
Hypoxanthine	5 mM	86% morphol; 90% NBT-red	6
Prostaglandin E_2	1 μM	28% NBT-red	4
Dibutyl cAMP	0.1 mM	33% NBT-red	4

Note: Abbreviations: *morphol*, morphologically mature cells including myelocytes, banded and segmented neutrophils, monocytes, and macrophages; *phago*, phagocytosis; *C3*, C3 receptors; *NBT-red*, nitroblue tetrazolium reduction; *TCN*, 3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentazaacenaphthylene; *cAMP*, cyclic adenosine 3':5'-monophosphate.

*Inducers producing approximately a 30% increase in one or more of the maturation parameters: nitroblue tetrazolium reduction, phagocytosis, Fc and C∅ receptors, and cell adhesion within 6 days of culture.

serum, 100 U penicillin, and 100 ng streptomycin. HL-60 may be induced towards either granulocytic or monocytic maturation by exposure to a wide variety of compounds (Table I-3) including dimethyl sulfoxide, retinoic acid, hypoxanthine, butyric acid, and actinomycin D. In recent years the development of this cell line has provided a useful model for studying regulation of both proliferation and differentiation. This can be very important for studies on the treatment of leukemia and leads to an alternative approach to the therapy of certain types of leukemia.

8. Specific Objectives

The specific objectives of this dissertation are as follows:

A. To use S-49 cells as an in vitro model to develop a chemotherapeutic protocol for the treatment of standard tumors by using the combination chemotherapy of IMP dehydrogenase inhibitor with the guanine analog 6-TG.

B. To study the mechanism of 6-TG-induced cell toxicity and differentiation in S-49 and HL-60 cells by differential modulation of guanine on 6-TG metabolism and of the incorporation of 6-TG into nucleic acids.

C. To understand the reasons for the high accumulation of intracellular 6-TGMP levels in S-49 and HL-60 cells by studying the 6-TGMP effect on enzymes that metabolize GMP.

The remainder of this dissertation is divided into three major parts. Chapter II describes the methodological experiments and development and covers most of the studies done on S-49 cells. Chapter III focuses mainly on cell differentiation and compares the differential effect elicited by guanine on 6-TG metabolism in S-49 and HL-60 cells. Chapter IV addresses the manner in which 6-TGMP affects several enzymes involved in the metabolism of GMP.

CHAPTER II.

MODULATION OF 6-THIOGUANINE INDUCED CELL TOXICITY BY GUANINE IN S-49 CELLS

1. Introduction

6-TG, a thio analog of guanine, is widely used for the treatment of human myelogenous leukemia. The mechanism of 6-TG toxicity has been attributed to its incorporation into DNA (8-13), rather than incorporation into RNA (13-16). Based on this hypothesis, any agent that increases (decreases) the incorporation of 6-TG into DNA should theoretically potentiate (antagonize) its toxicity. Our final goal is to develop a chemotherapeutic protocol for the treatment of standard tumor by using the combination of a general purine starvation agent and the toxic guanine analog 6-TG.

Mycophenolic acid (MA) is thought to represent a rather selective IMP dehydrogenase inhibitor that can specifically cause guanine nucleoside starvation in cells (38-41). It is expected that the starvation of endogenous guanine would render the cells more susceptible to the action of 6-TG. Therefore, coadministration of MA and 6-TG was expected to enhance incorporation of 6-TG into DNA of treated cells, and thereby enhance toxicity. However, initial cell growth experiments in mouse lymphoma S-49 cells with varying concentrations of MA plus 6-TG failed to show synergism between these two drugs.

On the other hand, an increased presence of guanine might be expected to modify 6-TG action by either interfering with 6-TG metabolism or its incorporation into nucleic acids. Therefore, coadministration of guanine and 6-TG was expected to decrease incorporation of 6-TG into DNA of treated cells and thereby decrease toxicity. The effects of guanine on 6-TG metabolism and cell toxicity were therefore studied in S-49 cells. The results showed that while guanine did not affect the accumulation of 6-TGMP, it did eliminate the

formation of 6-dTGTP. Guanine also competed effectively with 6-TG for the incorporation into DNA and RNA, but failed to prevent the growth inhibitory effect of 6-TG. Therefore, the growth inhibitory effect is independent of nucleic acid incorportaion in this case.

2. Materials and Methods

2-a. Chemicals and reagents

All chemicals and reagents were of analytical grade. Mycophenolic acid was purchased from Calbiochem Brand Biochemicals (San Diego, CA). 6-TG, guanine, potassium hydroxide, tri-n-octylamine, and ammonium phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). [8-^{14}C]6-TG (56 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA), and was further purified through a C-18 reverse phase column (Alltech Associates, Deerfield, IL) on HPLC before use. Aquasol was purchased from New England Nuclear (Boston, MA). Acetonitrile 1,1,2-trichloro-1,2,2-trifluoromethane and perchloric acid were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ).

2-b. Apparatus

HPLC analysis was performed on a liquid chromatograph (Waters Associates, Milford, MA) equipped with a Rheodyne model 7105 injector, a model 660 solvent programmer, two model M6000-A pumps, and a model LC-15 UV detector (Perkin-Elmer, Norwalk, CT) (254 nm). Cell counts were determined on a model Z_F Coulter counter (Coulter Electronics, Hialeah, FL). Radioactive analyses were performed on a Beckman LS 7800 liquid scintillation counter (Beckman Institute, Palo Alto, CA). The cells

were maintained at 37°C in a model 6300 incubator (National Appliance Co., Portland, OR).

2-c. Cell culture

Mouse T lymphoma S-49 cells were grown in suspension at 37° and 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum. Studies were performed with cells in the logarithmic phase. The growth-inhibition experiments were performed in Costar (24-well) tissue culture plate (Costar, Inc., Cambridge, MA). Two millileters of cells in complete medium (2 X 10^5 cells/ml) were added to the combination of agents to be tested. After 24 h incubation, cell density was measured. Percentage of control growth was defined as described (41):

$$\frac{\text{(Final Treated Density) - (Initial Density)}}{\text{(Final Control Density) - (Initial Density)}}$$

Synergistic effect between drug A and drug B was defined as follows: Percent of control growth (A + B) < percent of control growth (A) X percent of control growth (B). Treatment was considered lethal when the final cell density was less than the initial density after 24 h incubation.

2-d. Chromatographic conditions

Purification of [8-^{14}C]6-TG. Column: Alltech C-18 (25 cm X 46 mm) 10 μ reverse phase column; Solvent: 10 mM $NH_4H_2PO_4$, 1% acetonitrile, pH 3.6; flow rate = 2 ml/min; retention time = 7.8 min.

Analysis of cellular nucleotides pools. Column: Whatman Partisil

10 SAX anion exchange (Whatman Laboratories, Clifton, NJ); Solvent A: 5 mM K citrate, 0.2 M KCl, pH 3.6; solvent B: 0.8 M $NH_4H_2PO_4$, 0.4 M KCL, pH 3.6; flow rate program: isocratic elution with solvent A at 2 ml/min for 5 min followed by a linear gradient, maintaining total flow rate at 2 ml/min, reaching 100% solvent B in 20 min; retention time: 6-TGMP = 4.3 min; GTP = 24 min; 6-dTGTP = 26.5 min.

2-e. [8-^{14}C]6-TG incorporation into DNA and RNA

40 ml of S-49 cells, at a density of 3-4 X 10^5 cells/ml, were incubated at 37°C with 5 μM 6-TG and 0.75 μCi [8-^{14}C]6-TG in the presenc/absence of 200 μM guanine for 3.5 h. After incubation, the cells were harvested by centrifugation. The cell pellet was washed with phosphate buffered saline twice (pH 7.4), then precipitated with 200 μl 0.4 M perchloric acid (PCA). The cell pellet was left to sit in an ice bath for 30 min, then it was centrifuged to separate the supernatant and pellet. The acid soluble supernatant was removed and stored in the freezer for nucleotide pool analysis. The PCA insoluble pellet was washed with water twice and then resuspended in 300 μl of 0.3 N KOH overnight in order to hydrolyze RNA to free ribonucleotides. Acidification with 400 μl of 0.8 M PCA precipitated DNA while free ribonucleotides remained in the supernatant. The supernatant and precipitate were separated by centrifugation, then each was resuspended in 10 ml Aquasol and counted in scintillation counters.

2-f. Preparation of HPLC samples

The acid soluble fraction (200 μl) described in part 2-e was first neutralized with 440 μl of 1,1,2-trichloro-1,2,2-trifluoroethane/tri-n-

octylamine mixture (78/22). After microcentrifugation the aqueous layer was removed and ready to be analyzed as described in part 2-d.

3. Results

3-a. Toxicity of 6-TG on S-49 cells

The growth inhibitory effect of 6-TG on S-49 cells was determined by incubating S-49 cells with varying concentrations of 6-TG for 24 h. The results are shown in Fig. II-1. The 50% growh inhibition value is estimated to be 5 μM. Unless is otherwise specified, this concentration was also used in later experiments.

3-b. Lack of synergistic effect between MA and 6-TG

The IMP dehydrogenase inhibitor MA causes guanine starvation (44,45); hence, incubation of S-49 cells with varying concentrations of MA and 6-TG simultaneously was expected to result in more 6-TG residues incorporated into nucleic acids of S-49 cells because of the lack of competition with endogenous guanosine nucleotide synthesized from the de novo pathway. If 6-TG toxicity is dependent upon its incorporation into nucleic acid, then a synergistic effect may occur between these two agents. Varying concentrations of MA and 6-TG were incubated with S-49 cells for 24 h to test this hypothesis. However, contrary to our expectation, the effect of this on cell growth failed to show synergism between MA and 6-TG (Fig. II-1). The same result was obtained with different incubation times (36 and 48 h) (data not shown).

In addition, sequential treatment of S-49 cells with 0.2 μM MA for 8 h to deplete guanine nucleotides followed with 6-TG incubation failed

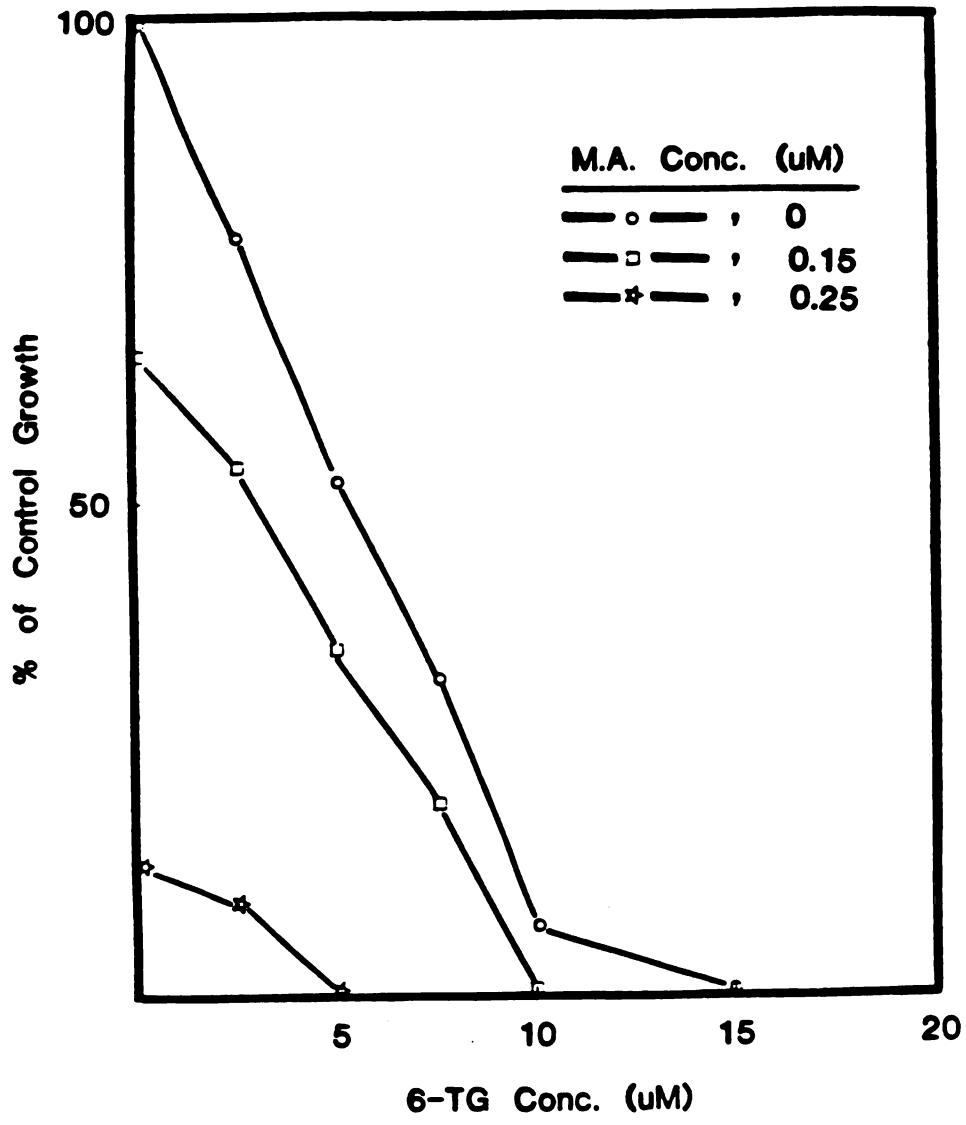

Figure II-1. The combinational growth-inhibitory effect of MA and 6-TG on S-49 cells. This effect was determined by incubating S-49 cells (density 2 X 10^5 cells/ml) with varying concentration of MA and 6-TG for 24 h. Cell density was measured and used to calculate percentage of control growth, as described in Materials and Methods. Each point was assayed in duplicate samples from one representative experiment. Similar results were obtained in two independent experiments.

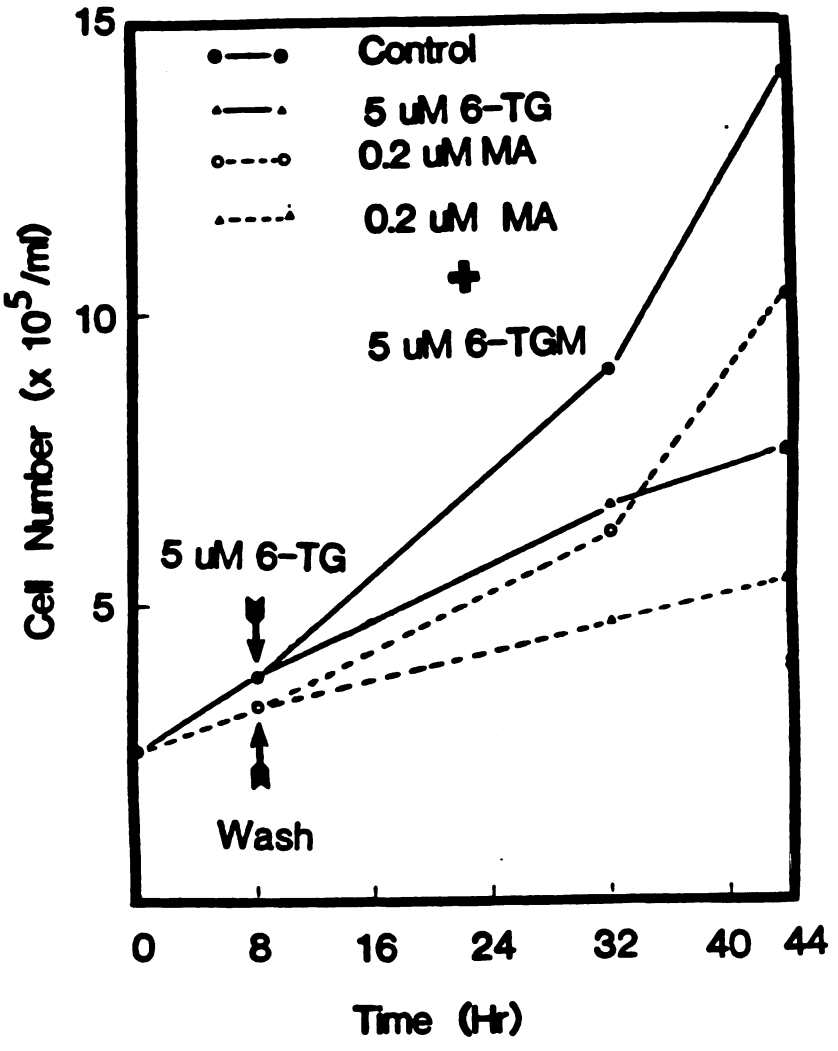

Figure II-2. The sequentially combinational growth-inhibitory effect of MA and 6-TG on S-49 cells. S-49 cells were preincubated with 0.2 μM MA for 8 h, washed, and then incubated with 5 μM 6-TG for an additional 36 h. Cell density was measured at designated times to calculate percentage of control growth. Closed symbols and solid lines indicate no MA preincubation treatment. Each point was assayed in duplicate samples from one experiment.

to demonstrate synergism between these two drugs (Fig. II-2).

It was first thought that this lack of synergism may be due to the inhibition of DNA and RNA synthesis resulting from the effect of MA (39), and therefore no 6-TG could be incorporated into DNA and RNA. To overcome this inhibition effect, exogenous guanine was added back to the medium to stimulate DNA and RNA synthesis. Under these conditions, however, guanine showed little effect on 6-TG toxicity, even at doses that maximally bypass the inhibition of MA. Therefore, guanine reversed the toxicity only of MA, not 6-TG (Fig. II-3).

3-c. No effect of guanine on 6-TG toxicity in S-49 cells

When we added varying concentrations of guanine to the combination of MA and 6-TG to restore the nucleic acid synthesis of S-49 cells, we also added guanine to 6-TG to serve as the control. Since 6-TG is a structural analog of guanine (Fig I-1), one would expect the exogenous guanine to compete with 6-TG for transport, metabolism and incorporation into nucleic acids and it should, therefore, decrease the cell toxicity of 6-TG. However, the results show that guanine concentrations of up to 300 μM failed to have any preventive effect on the growth inhibition action of 6-TG in S-49 cells (Fig. II-3). When 6-TG concentrations below and above the LD_{50} concentration were again coadministered with varying concentrations of guanine, the results further confirmed a general phenomenon of no effect on 6-TG toxicity in S-49 cells. Since 200 μM guanine had neither a preventive effect against 6-TG toxicity nor did it affect S-49 cell growth very much itself, this concentration was selected for use in later competition experiments.

To rule out the possibility of depletion of guanine in the medium

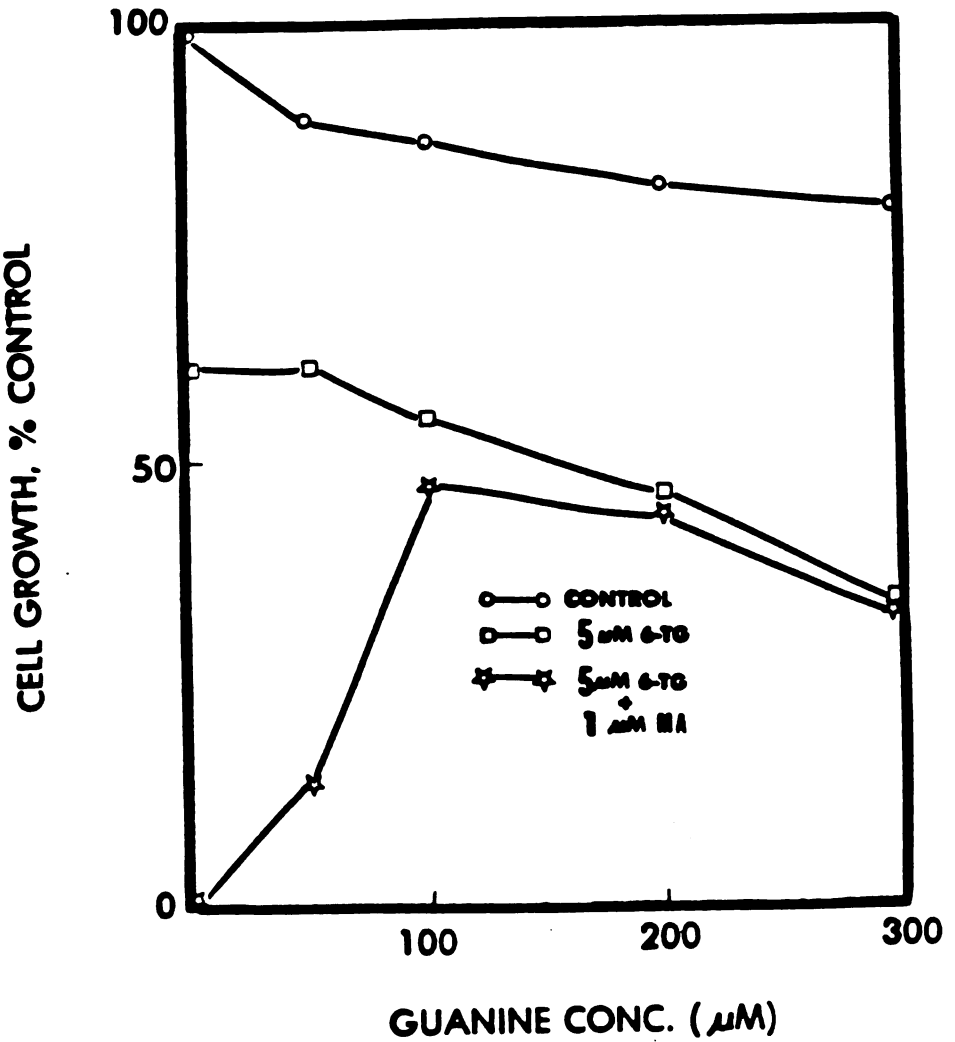

Figure II-3. Guanine rescue of the combinational growth-inhibitory effect of MA and 6-TG on S-49 cells. S-49 cells (density 2 X 10^{-5} cells/ml) were incubated with varying concentrations of guanine, 5 μM 6-TG and 1 μM MA for 24 h. 1 μM MA alone kills all S-49 cells in the medium. Cell density was measured and used to calculated percentage of control growth. Each point was assayed in duplicate samples from one representative experiment. Similar results were obtained in three independent experiments.

during incubation time, guanine concentration was checked by HPLC after 24 h and approximately 30 % of the added guanine still remained in the medium.

3-d. [8-^{14}C]6-TG incorporation into DNA and RNA of S-49 cells

It has been reported that 6-TG cell cytotoxicity is closely related to its incorporation into DNA and causes damage to chromatin of the treated cells (8,9). The results described in part 3-c clearly show guanine does not prevent 6-TG toxicity; therefore the manner in which guanine can affect 6-TG incorporation into DNA was examined to correlate the relationship of incorporation into DNA with toxicity. S-49 cells were incubated with 5 μM 6-TG in the presence/absence of guanine for 4 hours. The results showed significant competition between guanine and 6-TG for incorporation into DNA and RNA (Table II-1). Therefore, incorporation into DNA and RNA may not account for 6-TG toxicity in S-49 cells.

3-e. Guanine effect on 6-TG metabolism in S-49 cells

Activation of 6-TG to its nucleotide metabolite 6-TGMP via HGPRTase is necessary for its toxicity (42). Therefore, the acid soluble fraction from 6-TG treated cells was analyzed by HPLC to reveal 6-TG nucleotide distribution. 6-TGMP appeared as the predominant metabolite of 6-TG and its concentration increased for the first 16 h, then reached a plateau (6 nmoles/10^8 cells) (Fig. II-4). When [8-^{14}C]6-TG was used to label S-49 cells, the radioactivity revealed one metabolite in addition to the mono-, di-, and triphosphate nucleotide. This unknown metabolite was tentatively assigned to 6-deoxythioguanosine triphosphate

TABLE II-1. Competition of guanine with 6-TG for incorporation into DNA and RNA of S-49 cells.

	5 μM 6-TG	5 μM 6-TG + 200 μM Gua
DNA (nM/10^8 cells/30 min)	0.091±0.004	0.022±0.003
RNA (nM/10^8 cells/30 min)	0.106±0.006	0.034±0.006

40 ml S-49 cells were pulsed with 5 μM 6-TG and 0.75 μCi [8-^{14}C] 6-TG (56 mCi/mM) in the presence/absence of 200 μM guanine for 3.5 h. The PCA insoluble cell pellet was hydrolyzed with KOH overnight to separate RNA from DNA. Radioactivity associated with the DNA and RNA was determined by liquid scintillation counting.

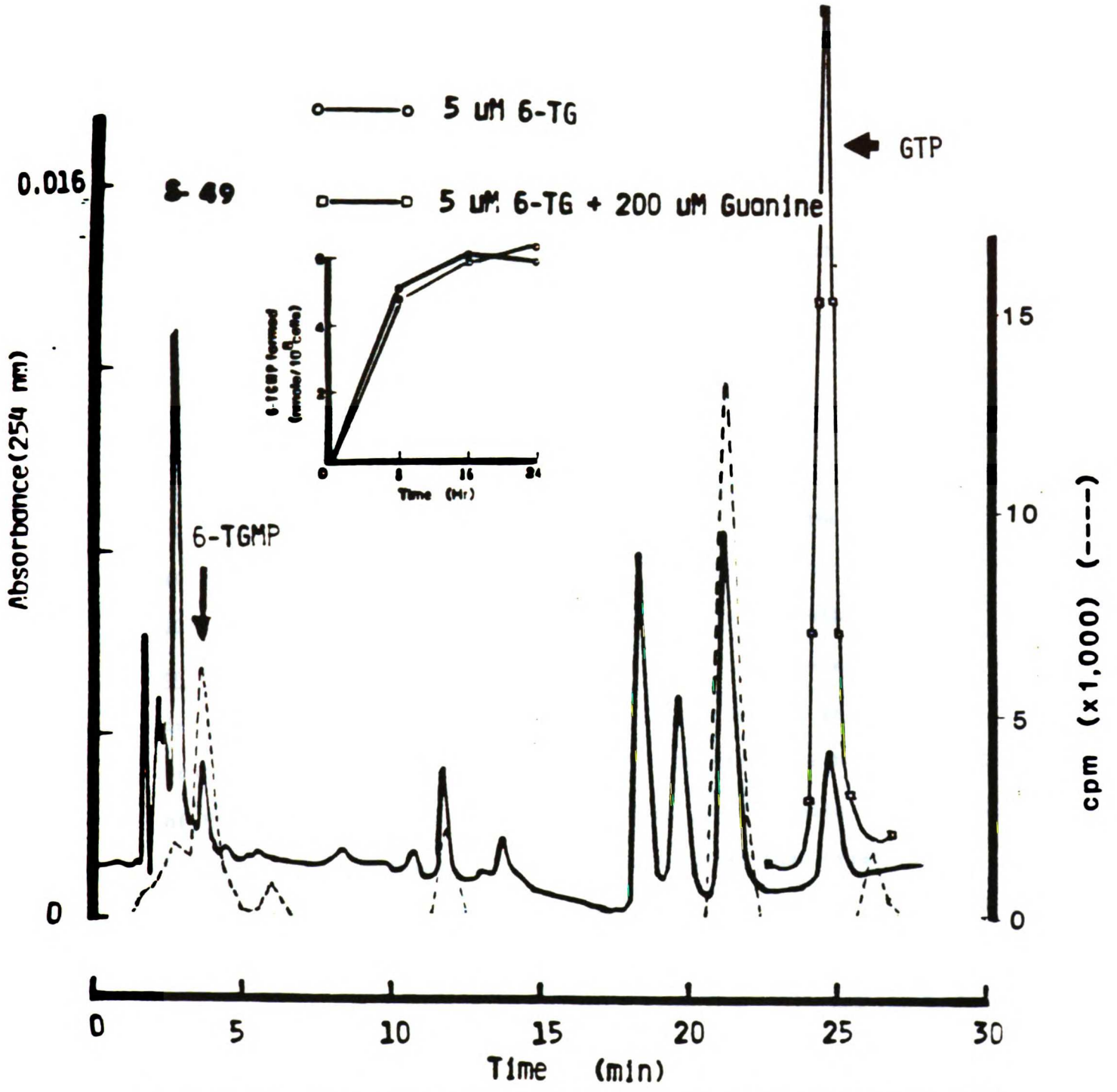

Figure II-4. HPLC chromatogram of acid soluble cell extract from S-49 cells. S-49 cells were incubated with 5 μM 6-TG in the presence or absence of 200 μM guanine. The insert represents the time profile of the accumulation of 6-TGMP. The broken line represents the radioactivity profile after 4-hour incubation of 5 μM 6-TG and 0.75 μCi [8-^{14}C]6-TG.

(6-dTGTP) because of its longer retention time than that of 6-TGMP, but further investigation is needed.

The presence of exogenous guanine does not affect the accumulation of 6-TGMP in S-49 cells; however, it does eliminate the radioactivity associated with 6-dTGTP, possibly through either direct competition with GDP for ribonucleotide reductase or through feedback inhibition of this enzyme by dGTP (43).

4. Discussion

The cytotoxicity of 6-TG in some cultured cell lines has been attributed to the incorporation of 6-TG into DNA as thioguanylate (8-12), rather than incorporation into RNA (13-16) or inhibition of purine de novo synthesis (21-23). To reinforce this idea, Nelson et al. have demonstrated that inhibition of DNA synthesis by 1 mM thymidine can protect H.Ep.2 cells from 6-TG toxicity. On the other hand, inhibition of RNA synthesis by 6-azauridine failed to protect cells against 6-TG toxicity (12). Maybaum and Mandel also reported that treatment of Chinese hamster ovary (CHO) cells with 6-TG produced severe disruption of chromatin structure in G_2 phase following completion of one cell cycle, as visualized by the premature chromosome condensation technique (9). Christie et al. further examined this damage using alkaline elution technique and found that DNA strand breaks are produced, which correlate with the incorporation of 6-TG into DNA and subsequent cytotoxicity (8).

Based on this incorporation theory, one might expect that any agent that increases 6-TG incorporation into DNA should theoretically enhance

6-TG toxicity. MA, an IMP dehydrogenase inhibitor which is known to deplete guanine nucleotides in S-49 cells (39-41,43) was chosen for this purpose. Coadminstration of MA and 6-TG was expected to result in more 6-TG nucleotide incorporation into DNA because of the lack of competition with endogenous guanosine nucleotides for nucleic acid synthesis. However, cell growth from either sequential or simultaneous treatment of MA and 6-TG failed to have any synergistic effect. When exogenous guanine was added in the medium along with MA and 6-TG, the growth curve coincided with that of MA and 6-TG. Thus, it is clear that guanine only reverses the MA toxicity by providing guanine nucleotides through a salvage pathway without any effect on 6-TG toxicity.

6-TG is a competitive inhibitor of guanine for HGPRTase with a Ki = 4.5 μM (17). 6-TGMP has also been reported as a competitive inhibitor of GMP for GMP kinase with Ki values in the range of mM concentration in some systems (90). The presence of guanine is expected to compete with 6-TG at different loci. Under the circumstance of guanine presence, the availability of 6-TG to S-49 cells should be decreased, as should the toxicity. However, the results from this study do not reflect this hypothesis. The fact that, while exogenous guanine does not affect the accumulation of 6-TGMP in S-49 cells it still does decrease 6-TG incorporation into DNA and RNA, suggests guanine may compete with 6-TG at other site(s) before the incorporation of 6-TG into nucleic acids occurs. This was noted when [8-^{14}C]6-TG was used to label S-49 cells, and the radioactivity associated with the putative 6-dTGTP peak was eliminated by the exogenous guanine through either direct completion with a larger pool of GDP for ribonucleotide reductase or the feedback inhibition of this enzyme by dGTP formed (43). Other possible sites may

include DNA polymerase. Exogenous guanine also does not prevent S-49 cells from the growth inhibitory action of 6-TG, indicating that incorporation of 6-TG into nucleic acids may not totally account for 6-TG toxicity. To rule out the possibility of depletion of guanine in the medium during the incubation time, guanine concentration was checked after 24 h and reasonably high concentrations still remained in the medium; therefore, other mechanisms such as protein synthesis inhibition (13,15,16) or rRNA maturation inhibition (14,78) may contribute to 6-TG toxicity.

Several other findings are unsupportive of the concept that incorporation of 6-TG into the DNA of neoplastic cells is responsible for antitumor activity. These include: (a) Pretreatment of L1210 cells with 1 μM methotrexate for 3 h, although substantially enhancing the cytotixicity of 6-TG, markedly suppressed the incorporation of 6-TG into DNA over the drug exposure period (99); (b) A mercaptopurine resistant subline of adenocarcinoma 755 incorporated more of 6-mercaptopurine into DNA, in the form of 6-TG, than into that of the parent sensitive cell line (102). It is known that 6-TGMP can inhibit several enzymes involved in purine nucleotide biosynthesis, namely, it serves as a pseudofeedback inhibitor of PRPP amidotransferase (11), an inhibitor of IMP dehydrogenase (19,20), a competitive inhibitor of GMP kinase (90), and a noncompetitive inhibitor of HGPRTase (17). This kind of inhibition of guanosine nucleotide formation at different loci represents a form of sequential blockade which could markedly reduce guanine nucleotide pools (46). However, guanine nucleotide levels are not reduced by 6-TG in S-49 cells, and the addition of exogenous guanine restores the depleted guanine nucleotide pools (500 % increase in the

GTP pool); therefore, guanine starvation cannot account for the toxicity of 6-TG in S-49 cells.

GMP concentration in S-49 cells has been reported as 0.18 nmol/10^8 cells (47). With the high concentration of 6-TGMP accumulated in cells (6 nmol/10^8 cells), the manner in which it would affect the cellular utilization of GMP is not known and further study is needed. Neither is there a clear reason for lack of effect of guanine on 6-TG toxicity, although the incorporation of 6-TG into DNA and RNA was reduced.

5. Summary

1. There is no cytotoxic synergism between MA and 6-TG.
2. Guanine does not affect the cell growth inhibition of 6-TG and the accumulation of 6-TGMP in S-49 cells, but it eliminates the formation of 6-dTGTP.
3. Guanine decreases the incorporation of 6-TG into DNA and RNA in S-49 cells; therefore, the incorporation into DNA and RNA alone cannot account for 6-TG toxicity.

CHAPTER III

MODULATION OF 6-THIOGUANINE INDUCED CELL DIFFERENTIATION BY GUANINE IN HL-60 CELLS

1. Introduction

In recent years the development of human myelomonocytic cell lines has provided useful models for studying the regulation of both cell proliferation and differentiation. This can be very important for studies on the treatment of leukemias. A conceptual approach to treatment is to induce leukemia cells to undergo terminal differentiation and to inhibit their proliferation (63). The growth advantage that myeloid leukemia cells have in vivo over normal cells is not because of a more rapid growth rate, but rather because of an apparent inability to mature to functional, terminally differentiated end cells (Fig. III-1). It is possible that some leukemia cells do not mature either because they have a decreased ability to respond to exogenous differentiation factors, or because the production of specific gene products requisite for differentiation is altered (7). It follows that these leukemias can be viewed as diseases resulting from a blockade at specific stages of cell differentiation. The availability of human promyelocytic leukemia HL-60 cells has made it possible to study the regulation of proliferation and differentiation and the effects on these cells of some known mediators and modulators.

6-TG has been observed to induce HL-60 cells to terminally differentiate (51,53-56). In a recent report, this differentiation-inducing property of 6-TG was proposed to be caused directly by 6-TG itself without any activation to nucleotide form when the cells were exposed to high concentrations of 6-TG (1 mM) (54). Since this high concentration is never attainable in physiological conditions, we feel that there is a need to investigate the low concentration end and characterize the metabolic form responsible for the differentiation-

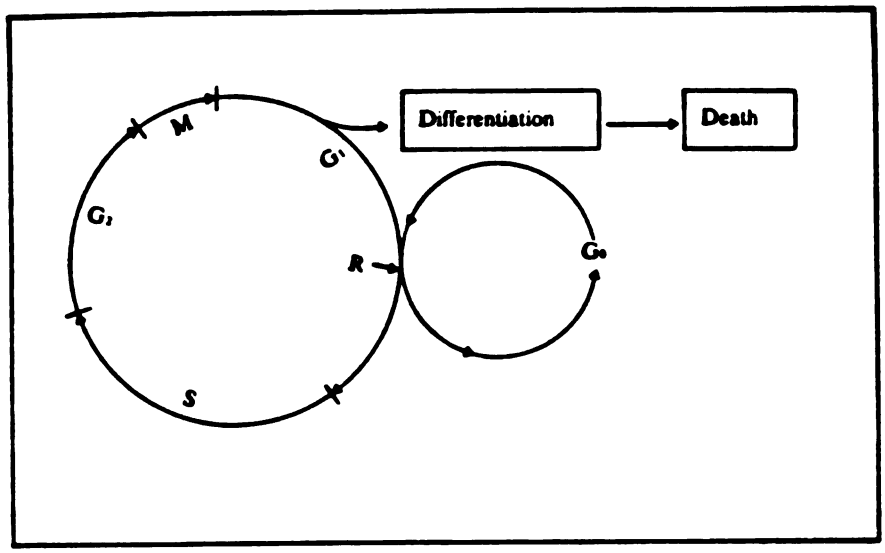

Figure III-1: A cell cycle diagram.

The conventional sequence of cell cycle phases G_1, S, G_2 and M are followed by cell division. Also included is a branch intended to indicate exit in G_1 of differentiating cells from the cycle. Non-differentiating cells exist in the quiescent (G_0) condition if they are unable to pass beyond the restriction point (R), also located in G_1.

inducing effect of 6-TG.

In Chapter II it was observed that guanine does not affect the accumulation of 6-TGMP or cell toxicity of 6-TG in S-49 cells. In this chapter, experiments were designed to examine the effect of guanine on the cellular metabolism of 6-TG with the hope of establishing a relationship between 6-TG metabolite(s) and cell differentiation. Guanine has a different impact on the accumulation of 6-TGMP in HL-60 cells in comparison with S-49 cells, which may result from the two cell lines possessing different capacities for guanine utilization. Coadministration of 6-TG with guanine reduces 6-TGMP by 90% in HL-60 cells, in parallel with suppression of the differentiation process. Therefore, induction of cell differentiation appears to be dependent upon 6-TG nucleotide formation at therapeutic concentrations. Since high concentrations of 6-TGMP can be reached in cells to inhibit several enzymes involved in guanosine nucleotide metabolism thus causing guanine starvation, it is possible that guanine starvation may play an important role in regulating cell differentiation by 6-TG.

2. Materials and Methods

2-a. Chemicals and reagents

All chemicals and reagents were of analytical grade. 6-TG, guanine, tri-n-octylamine, potassium hydroxide, ammonium phosphate, DMSO, NBT reduction kit, and 12-O-tetradecanoylphorbol-13-acetate were purchased from Sigma Chemical Co. (St. Louis, MO). [8-^{14}C]6-TG (56 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA), and further purified through a C-18 reverse phase column (Alltech

Associates, Deerfield, IL) on an HPLC before use. Aquasol® was purchased from New England Nuclear (Boston, MA). Acetonitrile, perchloric acid, and 1,1,2-trichloro-1,2,2-trifluoroethane were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). [Methyl-^{3}H]thymidine and [5-^{3}H]uridine were purchased form Amersham (Arlington Heights, IL). Giemsa stain was purchased from Fisher Scientific Co. (Fair Lawn, NJ).

2-b. Apparatus

HPLC analysis was performed on a liquid chromatograph (Waters Associates, Milford, MA) equipped with a Rheodyne model 7105 injector, a model 660 solvent programmer, two model M6000-A pumps, and a Perkin-Elmer model LC-15 UV detector, 254 nm (Norwalk, CT). Cell counts were determined on a model Z_F coulter counter (Coulter Electronics, Hialeah, FL). Radioactive analyses were performed on a Beckman LS 7800 liquid scintillation counter (Beckman Institute, Palo Alto, CA). The NBT dye incubated cells were fixed onto a slide by cytospin (Shandon Southern Instruments, Inc., Sewickley, PA). The cells were maintained in a 37°C model 6300 incubator (National Appliance Co., Portland, OR).

2-c. Cell culture

Human promyelocytic HL-60 cells were established in this lab with seed cell cultures provided to us by Dr. John Stobo (Howard Hughes Foundation, University of California, San Francisco). They were maintained in RPMI 1640 medium containing 2 g/ℓ $NaHCO_3$ and supplemented with 10% fetal calf serum, 100 units penicillin, and 100 ng streptomycin at 37°C in a 10% humidified incubator. Studies were performed with

cells in the logarithmic phase. The growth inhibition experiments were performed in Costar (24-well) tissue culture plate (Costar Inc., Cambridge, MA). Two ml of cells in complete medium (2 X 10^5 cells/ml) were added to the combination of agents to be tested. After 48 h incubation, cell density was measured. Percentage of growth was defined as described in Chapter II.2-c. Treatment was considered lethal when the final cell density was less than the initial density after 48 h incubation.

2-d. Inducement and assessment of HL-60 cell differentiation

HL-60 cells were induced to differentiation by their inoculation into fresh medium at a density of 2 X 10^5 cells/ml together with a variety of inducing compounds. Final concentrations of these inducing agents were: 10 μM 6-TG, 360 mM DMSO, 10 μM 6-MMPR, 200 μM guanine, and 1.0 μM MA. Cell suspensions were incubated in volumes of 20 ml for up to 5 days. At various intervals, 2 ml of cell suspension was removed from the flask. 0.5 ml of this was used for cell density determination, and another 1.5 ml was used for differentiation assessment.

Functional differentiation of HL-60 cells in culture was quantitated by the reduction of nitroblue tetrazolium (NBT) dye modified from a previously described method (48,49,62,97). Undifferentiated HL-60 cells generate little O_2^- in the resting state. As the cells mature, the rate of O_2^- production increases (97). NBT reduction assay allows qualitative examination of each cell that generates O_2^-. Close correlation is found between the percentage of cells induced to morphological maturation and the percentage of cells capable of reducing NBT (51,97). Newberger *et al.* suggests that NBT reduction is one of the

earliest differentiation markers expressed in mature HL-60 cells (97). Thus, NBT reduction is a sensitive and easily quantitated method to assess HL-60 cell differentiation.

The cells, washed twice with RPMI 1640 medium and resuspended at a concentration of 1 X 10^6 cells/ml, were incubated at 37°C for 30 min in RPMI 1640 medium with 100 ng/ml 12-O-tetradecanoylphorbol-13-acetate with 0.1% NBT. After incubation, cells were placed onto slides by cytospin, air dried, and further stained with Wright stain and Giemsa stain. The percentage of cells that contained blue-black formazan deposits, the product of NBT dye reduction, was determined by direct microscopic inspection of at least 200 cells per slide.

2-e. Staining procedure

Wright stain: The thoroughly dried slide was placed in a Coplin jar filled with Wright stain solution for exactly 30 seconds. The slide was removed from stain solution and place in a Coplin jar filled with deionized water for exactly 50 seconds. It was briefly rinsed in running deionized water and air dried.

Giemsa stain: Two parts of Giemsa stain concentrate were mixed with 50 parts of 1/150 M sodium phosphate buffered solution, pH 7.0 to make working Giemsa solution (this working solution was always made up fresh before each use). The slide was placed in Giemsa working solution for 25 min. It was then removed from the staining solution, dipped briefly in 1/150 M sodium phosphate buffered solution, drained quickly and dried.

2-f. Chromatographic conditions

Purification of [8-^{14}C]6-TG. Column: Alltech C-18 (25 cm X 46 mm) 10 µ reverse phase column; solvent: 10 mM $NH_4H_2PO_4$, 1% acetonitrile, pH 3.6; flow rate: 2 ml/min; retention time: 7.8 min.

Analysis of cellular nucleotide pools. Column: Whatman Partisil 10 SAX anion exchange (Whatman Laboratories, Clifton, NJ); solvent A: 5 mM K Citrate, 0.2 M KCl pH 3.6; solvent B: 0.8 M $NH_4H_2PO_4$, 0.4 M KCl, pH 3.6; flow rate program: isocratic elution with solvent A at 2 ml/min for 5 min followed by a linear gradient, maintaining total flow rate at 2 ml/min, reaching 100% solvent B in 20 min; retention time: 6-TGMP = 4.3 min; GTP = 24 min.

2-g. Preparation of HPLC samples

The acid soluble fraction (200 µl) from 6-TG treated cells was first neutralized with 440 µl of 1,1,2-trichloro-1,2,2-trifluoroethane/tri-n-octylamine mixture (78/22). The aqueous layer was removed after microcentrifugation and ready to be analyzed as described in part 2-f.

2-h. Reversal of DNA and RNA synthesis inhibition by guanine

The ability of exogenous guanine to reverse the DNA and RNA synthesis inhibition caused by 6-TG was measured by incorporation of [methyl-^{3}H]thymidine and [5-^{3}H]uridine into DNA and RNA. HL-60 cells at a density of 2 X 10^5 cells/ml were incubated with 10 µM 6-TG in the presence/absence of 200 µM guanine at 37°C. At the appropriate times, duplicate samples of 1 ml cell suspension was removed and either 0.5 µCi of [methyl-^{3}H]thymidine or [5-^{3}H]uridine was added to the cell

suspension 45 min prior to harvest. The cell suspension was filtered through Whatman GF/B glass filter paper presoaked with ice-cold 0.4 N PCA. The acid insoluble precipitate was washed with 5 ml 0.4 N PCA twice and dissolved in 10 ml Aquasol®, and radioactivity was determined by liquid scintillation counting.

3. Results

3-a. Toxicity of 6-TG on HL-60 cells

The growth inhibitory effect of 6-TG on HL-60 cells was determined by incubating the cells with varying concentrations of 6-TG for 48 h. The results are shown in Fig. III-2. The 50% growth inhibition value is estimated to be 10 μM. Unless it is specifically mentioned otherwise, this concentration is used in later experiments. Higher doses of 6-TG failed to completely suppress the growth of HL-60 cells at 48 h, possibly because of its differentiating effects that protected the HL-60 cells from further 6-TG induced damage.

3-b. Guanine effect on 6-TG induced cell differentiation in HL-60 cells

6-TG has been demonstrated to induce cell differentiation in cell lines such as Friend murine erythroleukemia (50), and human promyelocytic leukemia HL-60 cells (7,25,51-56). The active form to initiate differentiative process was reported to be 6-TG itself in HL-60 cells when a high concentration (1 mM) of 6-TG is used (53,54). The results from Chapter II revealed that guanine does not affect the growth inhibitory property of 6-TG. The same observation was also noted in hL-60 cells (Fig. III-3). Since guanine does not appear to induce cell

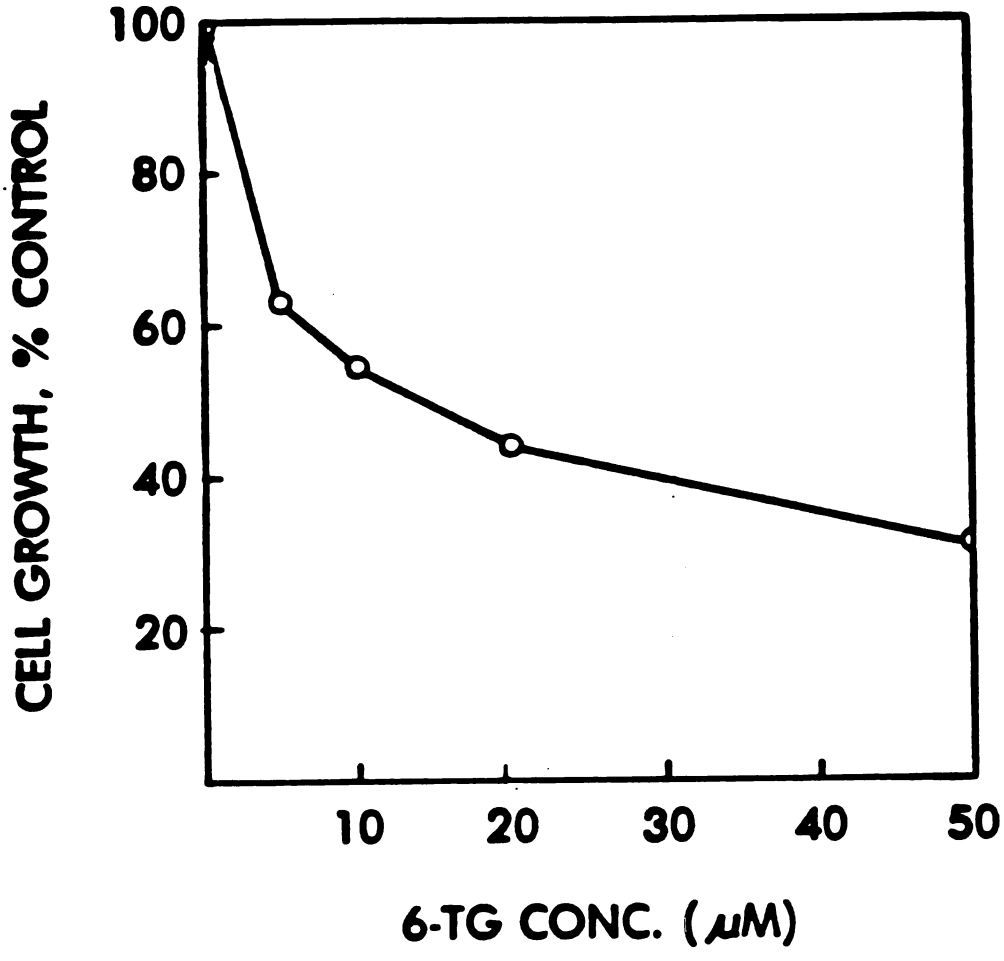

Figure III-2. Toxicity of 6-TG on HL-60 cells. The growth-inhibitory effect of 6-TG on HL-60 cells was determined by incubating the cells (density 2 X 10^5 cells/ml) with varying concentrations of 6-TG for 48 h. Cell density was measured and used to calculate percentage of control growth. The 50% growth inhibition concentration is estimated to be 10 μM. Results represent the mean of two separate experiments.

differentiation by itself (15), the manner in which guanine affects the differentiation-inducing action of 6-TG was examined. HL-60 cells were incubated with 10 μM 6-TG and 200 μM guanine, and DMSO was chosen as a positive control inducing agent. The result of cell counts and percentage of differentiation of cells is shown on Fig. III-4. Guanine apparently did not affect the cell growth at first (see Fig. III-4A, 64-hour point). However, when the cell population was examined by NBT reduction test, it appeared that 28% of 6-TG treated cells developed terminal differentiation, and guanine apparently was able to suppress this process (i.e., more than 65% decrease over treated cells). Measurement at later points showed more and more cells (69% after 5 days) expressing NBT positivity. On the other hand, the percentage of differentiation cell curves remained flat for both control and guanine added cells, indicating that guanine was able to suppress 6-TG differentiation-inducing action.

3-c. Guanine effect on 6-TG metabolism in HL-60 cells

The acid soluble fraction from 6-TG treated cells was analyzed by HPLC to reveal 6-TG nucleotide distribution and to determine the manner in which guanine can affect it. In cells incubated with 10 μM 6-TG, 6-TGMP appeared as the predominant metabolite. (Fig. III-5). The presence of 200 μM guanine shows a different effect on the accumulation of TGMP in HL-60 cells when it is compared with S-49 cells. The 6-TGMP pool was reduced about 90% in the presence of exogenous guanine; therefore, guanine is a potent competitor of 6-TG for transport and/or HGPRTase.

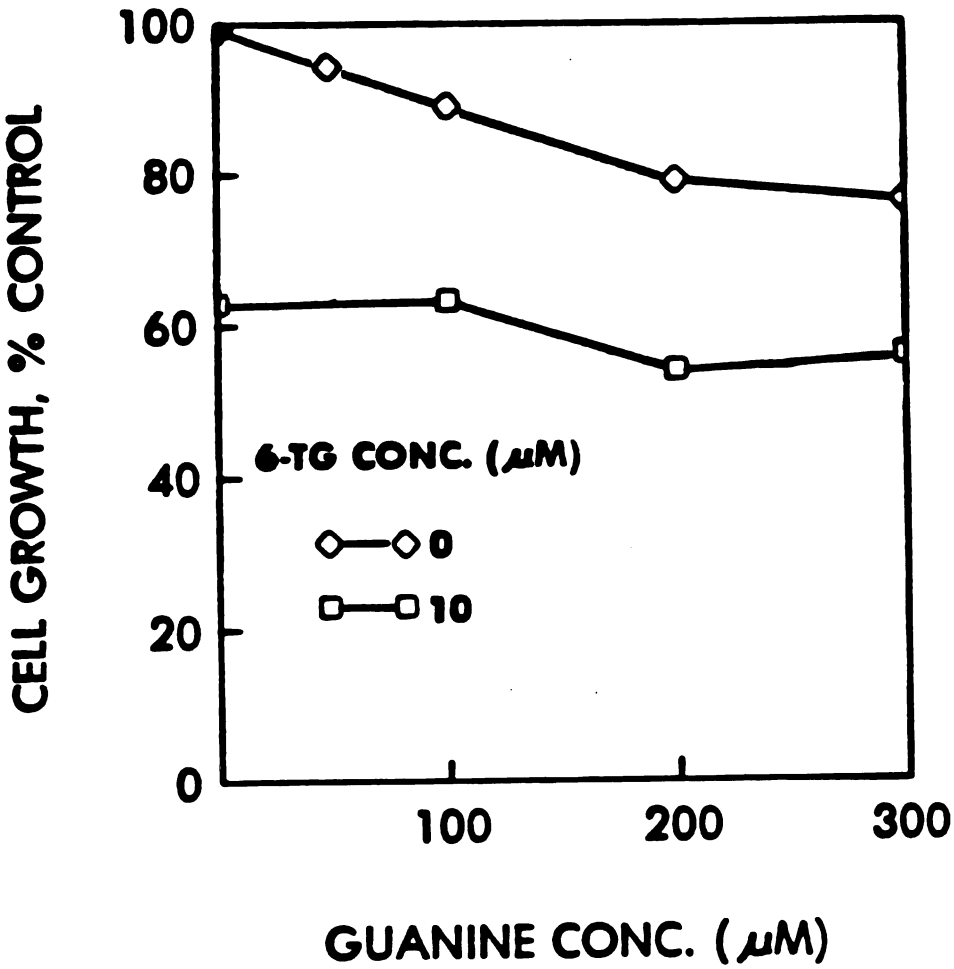

Figure III-3. Guanine effect on the growth-inhibitory action of 6-TG in HL-60 cells. HL-60 cells (density 2 X 10^5 cells/ml) were incubated with 10 μM 6-TG and varying concentrations of guanine for 48 h. Cell density was measured and used to calculated percentage of control growth. Results represent the mean of two separate experiments.

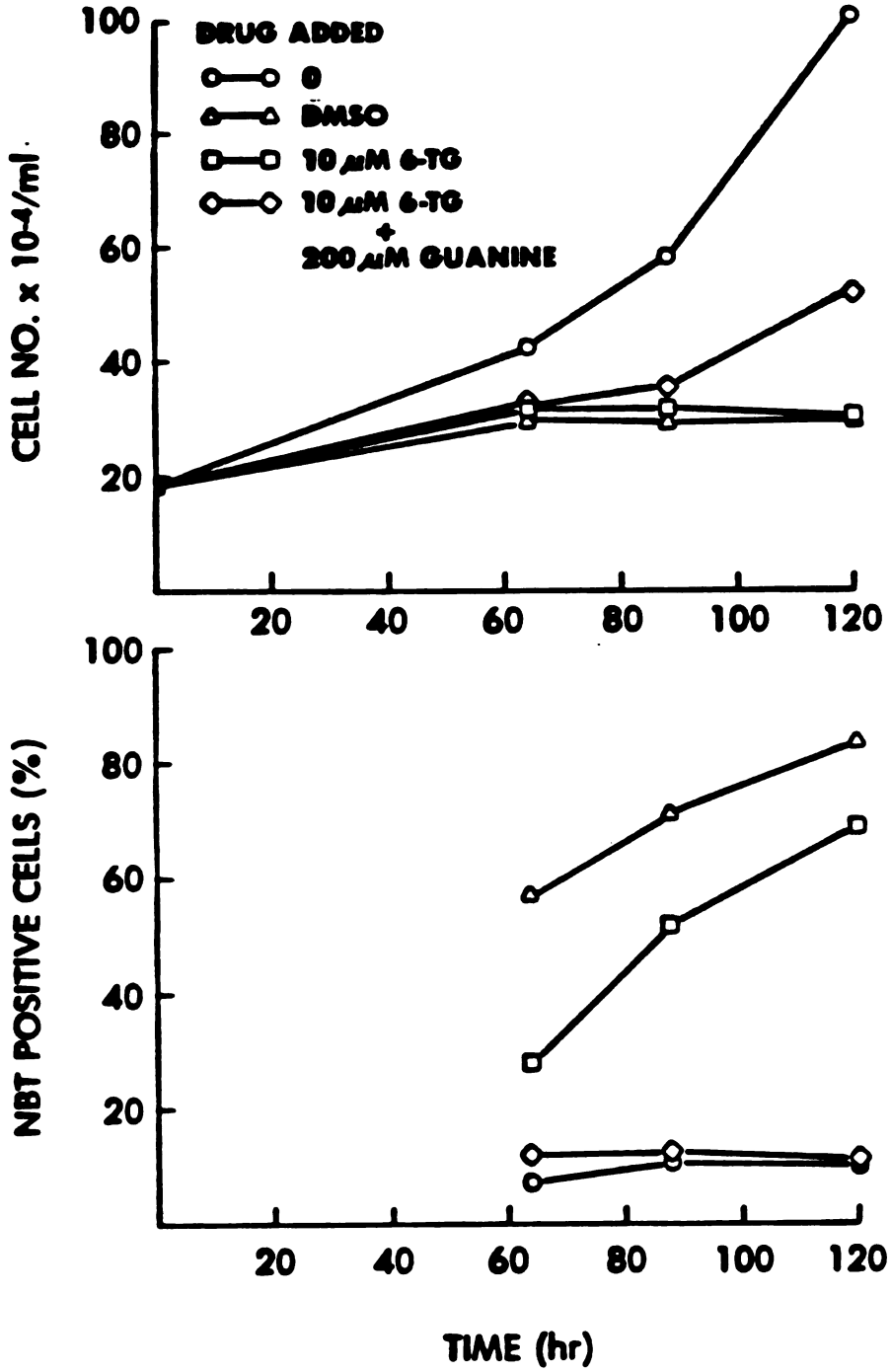

Figure III-4. Guanine effect on 6-TG induced cell differentiation in HL-60 cells. These cells (density 2 X 10^5) were incubated with 10 μM 6-TG and 200 μM guanine. DMSO was chosen as a positive control inducing agent. (Cell suspension was removed for cell density and differentiation measurement at appropriate times.) The terminally functional differentiation was quantitated by NBT test. Each point was assayed in duplicate samples from one representative experiment. Similar results were obtained in three independent experiments.

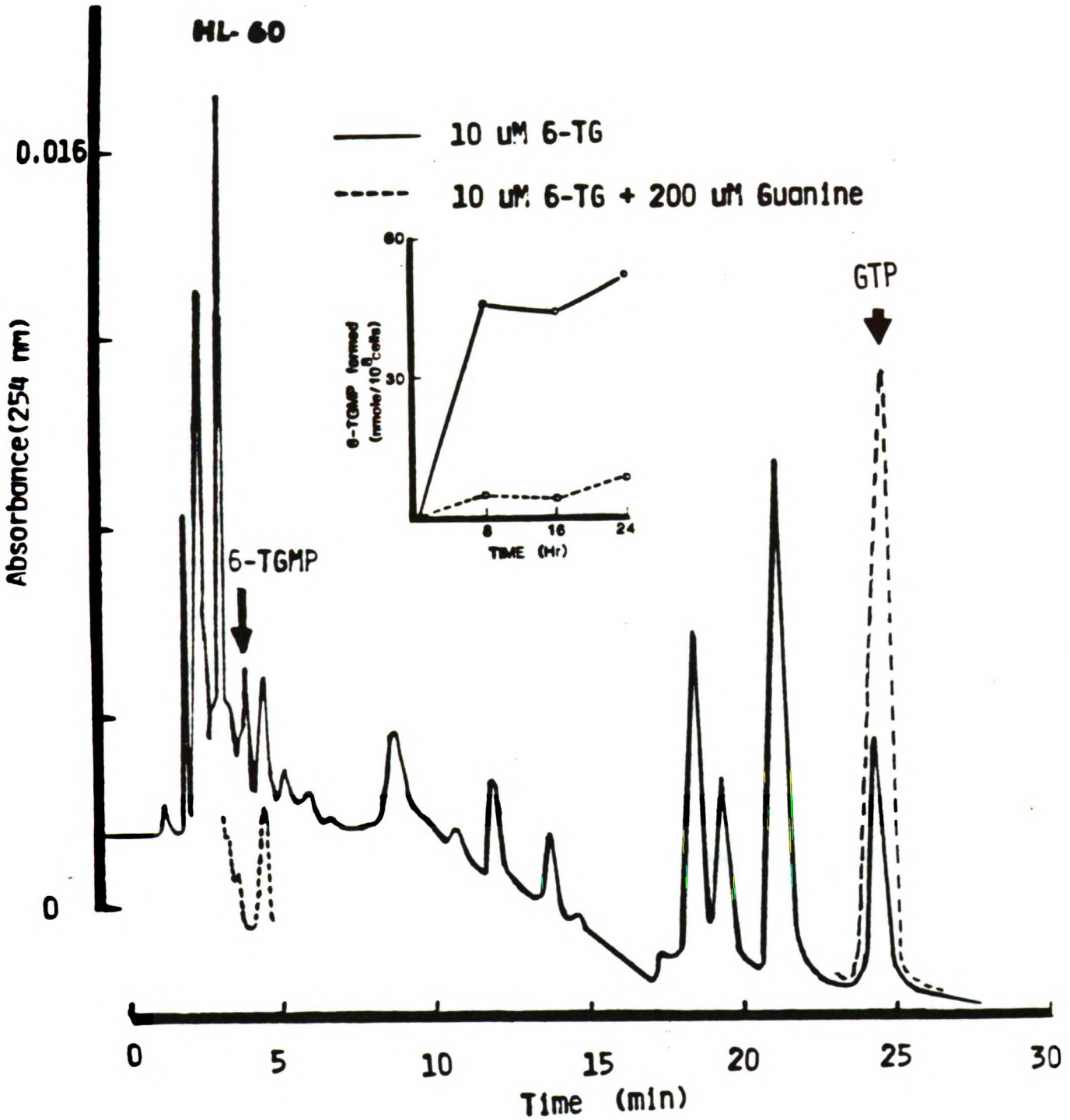

Figure III-5. HPLC chromatogram of acid-soluble cell extract from HL-60 cells. These cells were incubated with 10 μM 6-TG in the presence or absence of 200 μM guanine.

3-d. Guanine utilization by HL-60 cells in the presence of MA

Because guanine has a differential effect on 6-TG metabolism in S-49 and HL-60 cells, it also differently affects the growth inhibitory and differentiation-inducing properties of 6-TG on these two cells lines. Therefore, the utilization of guanine in HL-60 cells was investigated and compared with the utilization of guanine in S-49 cells (44). Guanine nucleotide pools in HL-60 cells were reduced more than 70% in the presence of 1 μM MA (59). This concentration, therefore, was used to block de novo guanine nucleotides biosynthesis (39-41,59) and varying concentrations of guanine were added to the medium to overcome this inhibitory effect. It was found that while 20 μM guanine could fully reverse MA induced guanine starvation in HL-60 cells (Fig. III-6), it took 300 μM guanine to achieve the same effect in S-49 cells (44). This finding reflects the different guanine utilization capacities that these two cell lines have, and this discrepancy can account for the different effects of guanine on 6-TG metabolism in these two cell lines.

3-e. Reversal of 6-TG caused DNA and RNA inhibition by guanine

Investigations on the metabolic effects of 6-TG in ascites tumor cells indicated the possibility of several metabolic blockades in purine de novo biosynthesis (60,61). These blockades could deplete precursors for nucleic acid synthesis, thus reducing DNA and RNA synthesis (24). The ability of guanine to reverse nucleic acid synthesis inhibition caused by 6-TG was measured by incorporation of radiolabeled thymidine and uridine into DNA and RNA. The incorporation of [methyl-^{3}H]thymidine and [5-^{3}H]uridine into PCA insoluble materials was linear over the 45-min period studied, and was therefore considered an acceptable estimate

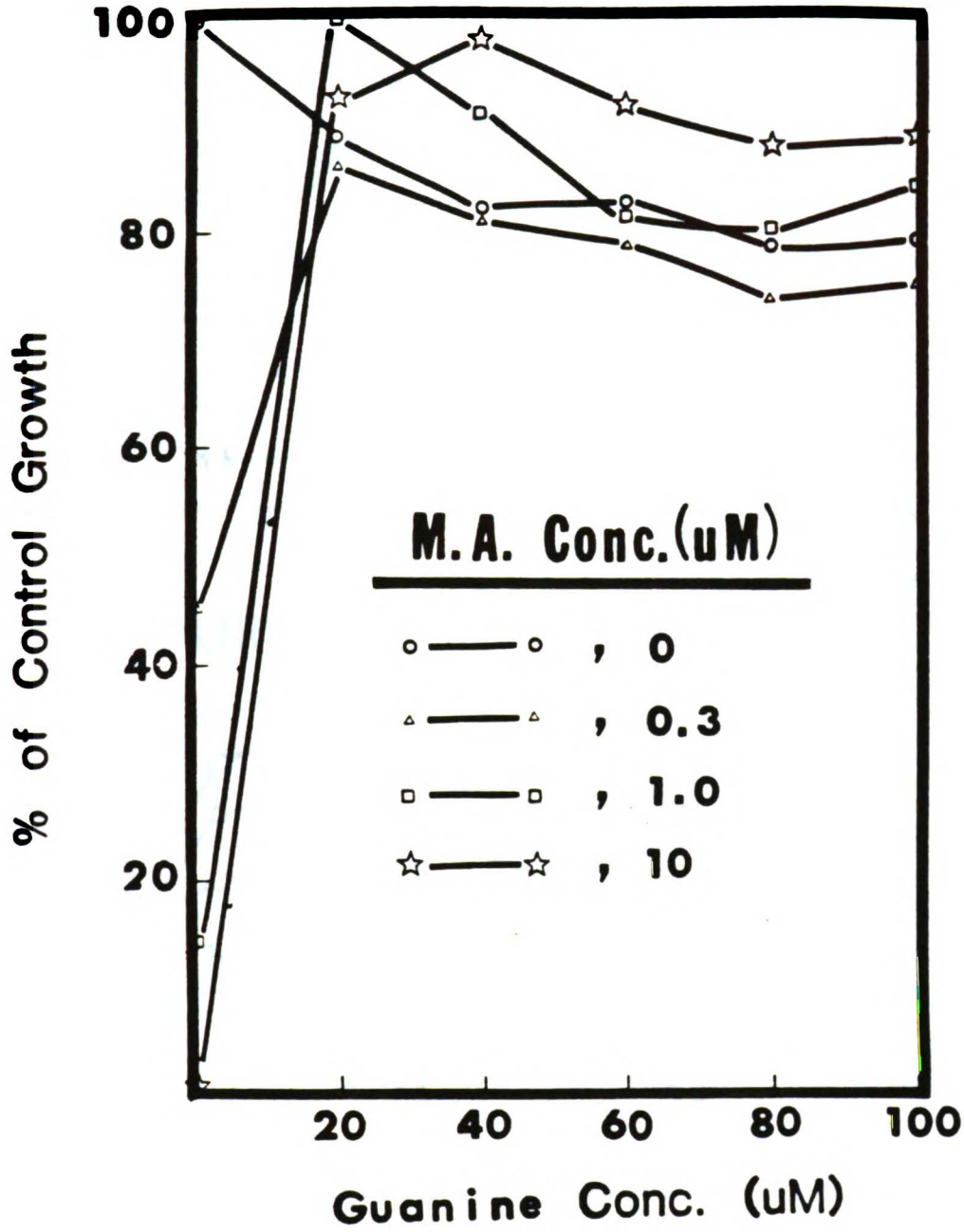

Figure III-6. Guanine rescue of the toxicity of mycophenolic acid (MA) on HL-60 cell growth. These cells (density 2 X 10^5 cells/ml) were incubated with varying concentrations of guanine and MA for 48 h. Each point was assayed in duplicate samples from one representative experiment. Similar results were obtained in three independent experiments.

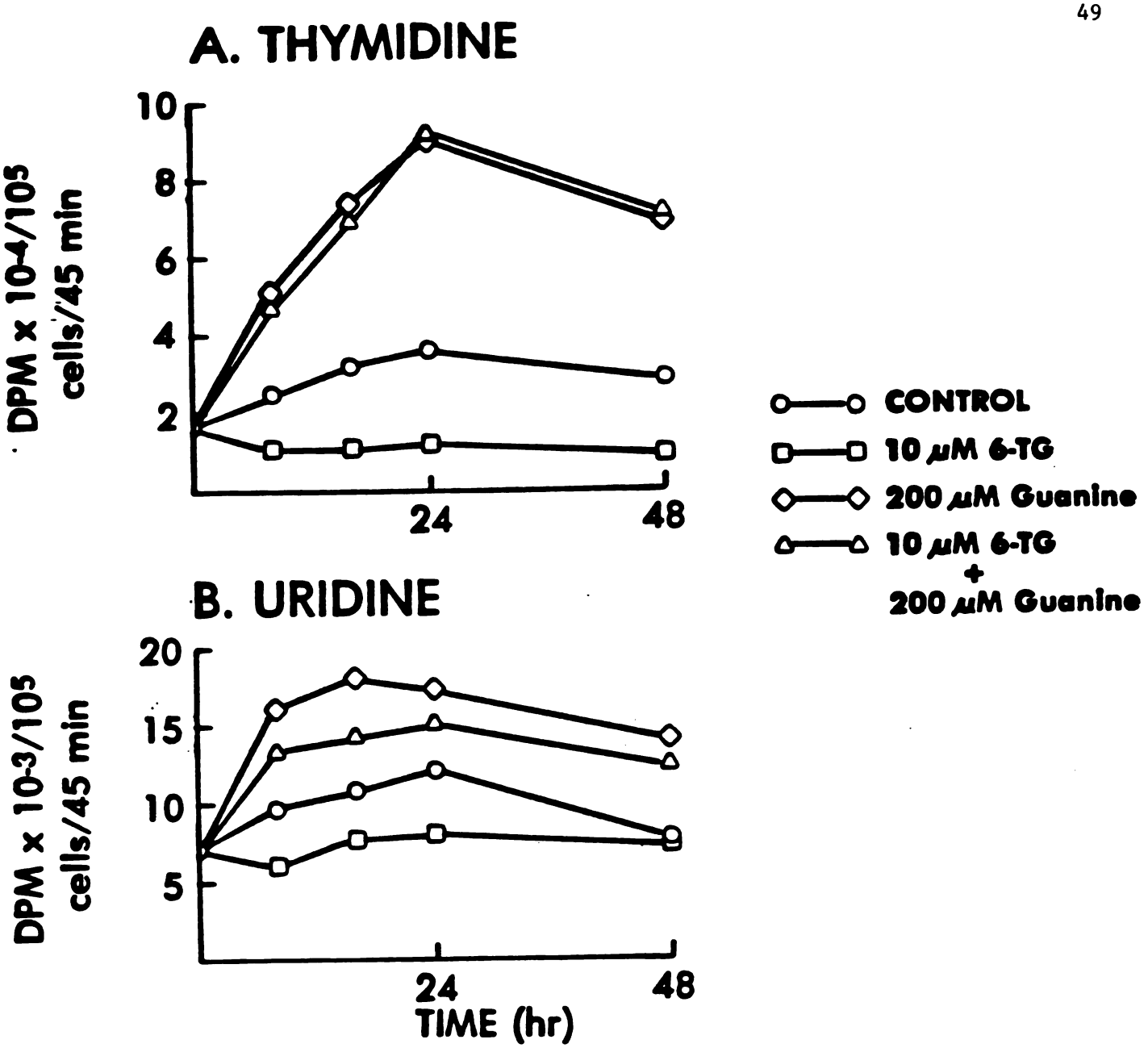

Figure III-7. Reversal of 6-TG caused DNA and RNA inhibition by guanine. HL-60 cells were incubated with 10 μM 6-TG in the presence or absence of 200 μM guanine. At appropriate times, 0.5 μCi radiolabeled thymidine or uridine was added to 1 ml cell suspension, 45 min prior to harvest. The radioactivity associated with PCA insoluble precipitate was determined by liquid scintillation counting. Each point was assayed in duplicate samples from one representative experiment. Similar results were obtained in two independent experiments.

of the relative rate of DNA and RNA synthesis rate. HL-60 cells were incubated with 10 μm 6-TG in the presence or absence of 200 μM guanine. Guanine *per se* (200 μM) increased the level of tracer incorporation, presumably by changing pyrimidine nucleotide metabolism. Both thymidine and uridine incorporation decreased upon treatment of 6-TG and this effect developed as early as 8 hr (Fig. III-7). This nucleic acid inhibition has more significant impact on the DNA synthesis side than RNA synthesis side, and this reduction could be reversed by the addition of exogenous guanine.

4. Discussion

6-TG has been observed to induce cell differentiation in human promyelocytic leukemia HL-60 cells (7,25,51-56). In a recent report, Schwartz and coworkers had succeeded in cloning an $HGPRT^-$ mutant. When this mutant was incubated with 1 mM 6-TG, extensive differentiation, as measured by the reduction of NBT, occurs. Flow cytometry analysis showed that 68% of these 6-TG treated cells accumulated in the G_1 phase (i.e., an 80% increase compared to controls), as might be expected for a differentiated population (see Fig. III-1). Since this mutant lacks the capability of converting 6-TG to its corresponding nucleotides, Schwartz *et al.* concluded that 6-TG is the active form that initiates the cell differentiation process in HL-60 cells (25,53,54). However, the mechanism of its action still remains unclear.

We have observed that guanine could suppress the differentation-inducing property of 6-TG in HL-60 cells when they were exposed to low concentrations (10 μM in this study, vs. 1 mM in a previous study

conducted by Schwartz et al. (25)) of 6-TG (11% in control cells vs. 69% in 6-TG treated cells expressing NBT positivity) (Fig. III-4). Analysis of the acid-soluble cell fraction revealed a marked decrease of the intracellular accumulation of 6-TGMP in the presence of exogenous guanine, indicating that suppression of differentiation in HL-60 cells is being achieved by the interference of guanine with 6-TG activation to its nucleotide form. Guanine can be seen as protecting HL-60 cells against the differentiation-inducing action of 6-TG by preventing the anabolism of this drug to its nucleotide forms by competing with it at several points. These include (a) the transport carrier: The uptake of 6-TG is mediated by a Hyp-Gua carrier, K_m,K_i values for hypoxanthine and 6-TG from Novikoff rat hepatoma cells being reported as 412 and 200 μM, respectively (65); (b) HGPRTase: K_m values for guanine and 6-TG with HGPRTase from adenocarcinoma 755 are 2.7 and 10 μM (17); and (c) PRPP: Activation of guanine to GMP leads to a decreased availability of PRPP for the synthesis of 6-TGMP by HGPRTase.

There is diverse experimental evidence to suggest that purine nucleotide metabolism plays an important role in the control of cell differentiation. Studies on Friend erythroleukemia cells in culture has shown that cell differentiation can be initiated by purine bases or purine analogs that inhibit purine nucleotide biosynthesis (50,64,67). Some investigators have therefore suggested that there is a link between purine biosynthesis and the event(s) required to trigger cell differentiation (64). Additionally, other investigators have shown that when HL-60 cells were exposed to different differentiation-inducing compounds, consistent alterations in purine metabolism were observed to occur within 24 H (59). Perturbation of guanosine nucleotide synthesis

and decrease of intracellular guanylate pool sizes were associated with the induced differentiation in response to diverse inducing agents (49,59). On the other hand, no consistent alterations in adenosine nucleotide pools were found to occur with induced differentiation. Moreover, these changes, which occurred early during induced differentiation, could be attributed in part to a discrete inhibition of guanine nucleotide biosynthesis (59). 6-TGMP is known to inhibit several enzymes involved in guanine nucleotide biosynthesis, namely, it serves as a pseudofeedback inhibitor of PRPP amidotransferase (11), and inhibitor of IMP dehydrogenase (19,20), and a noncompetitive inhibitor of HGPRTase (17). This kind of inhibition of guanosine nucleotide formation would markedly reduce the guanosine nucleotide pool; thus, guanine starvation may play an important role in the regulation of terminal differentiation. Wotring and Roti noticed that 6-TG could cause both a G_2 and an S cell progression block in L1210 cells (24). The arrest of cells in the G_2 phase was not reversible, and had been suggested to play a major role in the cytotoxic effect of 6-TG (24,75); particularly *in vivo*, where the effective exposure time of target cells may be limited. On the other hand, some cells were also retained in S phase during 6-TG treatment. This S phase block was readily reversible upon drug removal, and had been suggested to be a lack of precursors for DNA synthesis, such as would result from inhibition of purine *de novo* synthesis (24). The latter effect was therefore considered to be secondary to the mechanism of cell toxicity. Radiolabeled thymidine and uridine incorporation into HL-60 cells were used to assess this inhibitory effect upon DNA and RNA synthesis. The observation indicated that DNA and RNA synthesis decreased upon 6-TG treatment; however, this

inhibition of nucleic acid synthesis could be overcome by addition of exogenous guanine. Therefore, we suggest that the accumulation of 6-TGMP may result in guanine starvation which then initiates cell differentiation in HL-60 cells. With the presence of exogenous guanine, the 6-TGMP level is reduced (~90%) and the GTP level is increased (~140%) (Fig. III-5); therefore, the cell differentiation process is suppressed. In one experiment, when 10 μM 6-methylmercaptopurine riboside (6-MMPR) (LD_{50}), an early purine de novo synthesis inhibitor, was incubated with HL-60 cells for 72 h, no change was observed with the percentage of NBT positive cells between control and 6-TG treated cells (15% vs. 14%). It is thus suggested that inhibition of early de novo purine synthesis may not contribute to HL-60 cell differentiation.

Guanine apparently differs in its effect upon the accumulation of 6-TGMP in S-49 and in HL-60 cells. The presence of 200 μM guanine was able to reduce 6-TGMP level about 90% in HL-60 cells after one cell cycle (Fig. III-4). This indicates that guanine is a potent competitor of 6-TG for transport and/or HGPRTase. On the other hand, the same concentration of guanine has little effect on the 6-TGMP level in S-49 cells. One of the explanations for the discrepancy between these two cell lines may be saturation of guanine transport in S-49 cells. To test this hypothesis, mycophenolic acid, an IMP dehydrogenase inhibitor, was used to specifically induce guanine starvation to differentiate the guanine utilization capacity in these two cell lines. The optimum guanine concentrations for reversal of the effect of MA are 300 and <20 μM for S-49 and HL-60 cells, respectively (44). All the evidence strongly suggests guanine transport may be the rate-limiting step for its utilization in S-49 cells.

5. Summary

1. Metabolism of 6-TG to its nucleotides is necessary to initiating cell differentiation in HL-60 cells at therapeutic concentrations.
2. The accumulation of 6-TGMP may result in guanine starvation, which may augment cell differentiation in HL-60 cells.
3. Guanine reduced the accumulation of 6-TGMP in HL-60 cells, which may suppress the differentiation process.

CHAPTER IV

6-THIOGUANINE MONOPHOSPHATE EFFECT ON ENZYMES METABOLIZING GMP

1. Introduction

Metabolism of 6-TG to the 5'-nucleotide level is thought to be necessary to exert its antitumor action. Neoplastic cells can carry out this conversion very actively, and Moore and LePage showed that 6-TGMP accumulated to ~1 X 10^{-4} M in Ehrlich ascites cells isolated from animals treated with 6-TG (68). A number of enzymes involved in purine biosynthesis are inhibited by this high concentration of 6-TGMP (Fig. IV-1), i.e., PRPP amidotransferase (21,22), IMP dehydrogenase (19,20,46), GMP kinase (18), and HGPRTase (17). These demonstrated inhibitions are proposed to diminish the rate of biosynthesis of guanine nucleotides, which would result in a decreased availability of guanine nucleotides for both coenzyme function and nucleic acid synthesis (46). In view of the accumulation of TGMP as the major intracellular product, it is necessary to test its effects on any of the many enzymes that interact with GMP, each of which could provide a biochemical target of 6-TG toxicity.

Miech et al. reported that it was not possible to demonstrate the occurrence of more than trace amounts of either the di- or triphosphates of 6-thioguanosine after i.p. injection of 2.5 mg/kg 6-TG into mice bearing 6-day implants of sarcoma 180 cells (46). Similar findings of relatively high concentrations of 6-TGMP and negligible amounts of 6-TGDP and 6-TGTP were observed here in S-49 and HL-60 cells (Chapters II and III). These observations suggest that 6-TGMP is very slowly converted to its higher nucleotides and/or degraded to 6-thioguanosine. The reactivity of 6-TGMP with 5'-nucleotidase, acid phosphatase, and alkaline phosphatase was tested and it appeared to be a noncompetitive inhibitor of GMP for those three enzymes. 6-TGMP is a

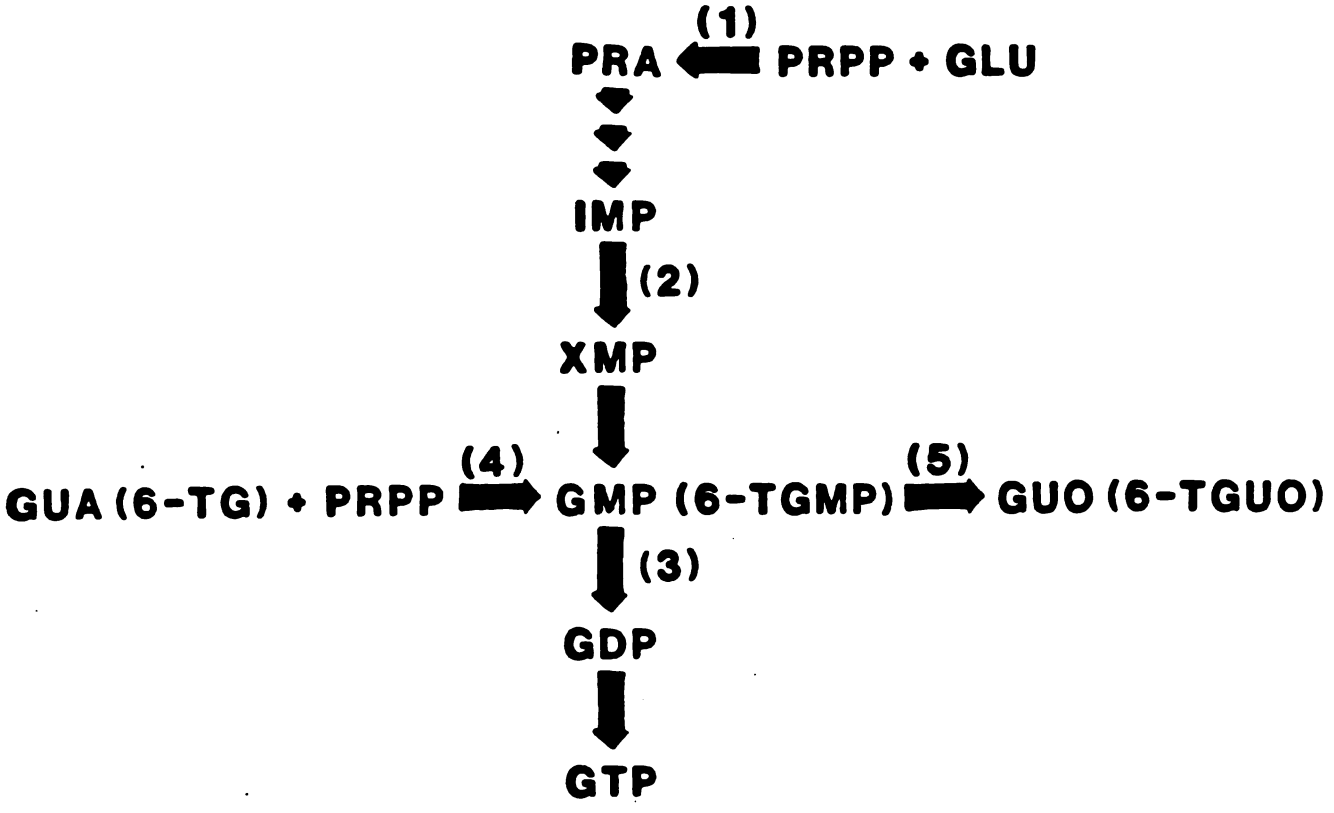

Figure IV-1. Sites of inhibition of 6-TGMP. (1) PRPP amidotransferase, k^1 = 170~200 μM (23); (2) IMP dehydrogenase; (3) ATP/GMP phosphotransferase, k_i = 2.1 mM (90); (4) HGPRTase; (5) 5'-nucleotidase, acid phosphatase, or alkaline phosphatase, k_i = 112.5 μM, 30 μM, and 390 μM, respectively (Table IV-1). Abbreviations: PRA, 5-phosphoribosyl-1-amine; Gua, guanine; Guo, guanosine; 6-TG, 6-thioguanine; 6-TGuo, 6-thioguanosine; PRPP, phosphoribosyl pyrophosphate; Glu, glutamine.

poor substrate for all of these enzymes and for GMP kinase from HL-60 cells with a very low V_m value and k_m = 384 mM, which could account for the high 6-TGMP concentrations observed in the cells.

2. Materials and Methods

2-a. Chemicals and reagents

All chemicals and reagents were of analytical grade. 6-TG, PRPP, tri-n-octylamine, ammonium phosphate, acid phosphatase type II from potato (EC 3.1.3.2), alkaline phosphatase type III from E. coli (EC 3.1.3.1), guanosine 5'-nonophosphate sodium salt, β-NADH (a reduced form of disodium salt) grade II from yeast, ATP, 6-thioguanosine, m-resol, lithium hydroxide, pyrophosphoyl chloride, and 5'-nucleotidase type IV from Crotalus atrox venom (EC 3.1.3.5), were all purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, perchloric acid, and 1,1,2-trichloro-1,2,2-trifluoroethane were purchased from J.T. Baker Chemical Co. (Philipsburg, NJ). 6-TGMP was generously provided by Dr. R.L. Miller from Wellcome Research Laboratories (Research Triangle Park, NC).

2-b. Apparatus

HPLC analysis was performed on a liquid chromatograph (Waters Associates, Milford, MA), equipped with a Rheodyne model 7105 injector, a model 660 solvent programmer, and two model M 6000A pumps. A Perkin-Elmer (Norwalk, CT) model LC-15 (254 nm) or a Waters model 440 (280 nm) was used as a detector. Oxidation of NADH was measured at 340 nm spectrophotometrically on an LKB model 4050 spectrophotometer (LKB

Instruments, Inc., Gaithersburg, MD), equipped with a model 4070 controller and an Apple IIe PC (Apple, Inc., Cupertino, CA) to calculate the resultant data.

2-c. Cell culture

S-49 and HL-60 cells were used for cell extract preparation. Maintenance of these two cell lines is described in Chapters II and III.

2-d. Preparation of crude cell extract

Mid-log phase of either S-49 or HL-60 cells were harvested and washed twice with phosphate buffer saline, pH 7.4, resuspended in one volume of 25 mM Tris, pH 7.4, 20 mM KCl, 6 mM $MgCl_2$, and 1 mM dithiothreitol. The cell suspension was homogenized in an ice-bath using a Brinkman polytron (Westbury, NY) for three 15-s periods at a setting of 10. The homogenate was centrifuged at 17,000 g for 1 h to remove the cell debris, and the resulting supernatant (crude cell extract) was used either for synthesizing 6-TGMP or enzyme assay.

2-e. Synthesis of 6-TGMP

Enzymatic synthesis. The synthesis employed here was modified from a previously described method (18,68). One volume of crude S-49 cell extract was added to 2.5 volumes of reaction mixture composed of 100 mM Tris, pH 8.07, 20 mM $MgCl_2$, 7 mM 6-TG, and 7 mM sodium 5-phospho-α-D-ribosyl-pyrophosphate. The reaction mixture was allowed to stand for 16 h at room temperature before adding 0.6 ml concentrated PCA to stop the reaction. The reaction mixture was centrifuged at 17,000 g for 15 min to remove the precipitated protein. The resulting supernatant was

neutralized with KOH to pH 7.0. The neutralized reaction mixture was chromatographed on a Whatman Pantsil 10 SAX analytical anion-exchange column to separate the nucleotide formed from the unreacting 6-TG and PRPP according to the method described in 2-g. The eluted 6-TGMP solution was lyophylized to dryness for subsequent use.

Chemical synthesis. The synthesis employed here was modified from a previously described method (67). One-tenth gram (0.33 mmol) thioguanosine was suspended in 3 ml m-cresol. Two hundred fifty microliters (1.8 mmol) pyrophosphoryl chloride was added to the suspension under ice cooling. The mixture was stirred at 0-10°C for 3 hr to give a homogenous dark yellow solution. The solution was extracted with 2.5 ml cold de-ionized water three times. Combined aqueous layers were extracted with 5 ml ether 5 times to remove m-cresol. The remaining aqueous solution was neutralized to pH 7.5 with 1 N LiOH and the white precipitate of Li_3PO_4 produced was removed by centrifugation. The identification of products was accomplished with HPLC.

2-f. Enzyme assay

Acid phosphatase. The assay method employed here was a modification of a previously described method (71). The enzyme activity was assayed in 100 mM potassium citrate buffer pH 4.8, with GMP as the substrate. Various volumes of 500 μM 6-TGMP were added to the reaction mixture to make a final concentration of 25, 50, or 100 μM. The reaction was started by adding 10 μl acid phosphatase (1 U/200 μl) to 200 μl reaction mixture. The reaction mixture was incubated at 37°C for 15 min before 8.8 μl concentrated PCA was added to stop the reaction. The mixture was then centrifuged to remove the precipitated enzyme. The

resulting supernatant was neutralized with 440 µl 1,1,2-trichloro-1,2,2-trifluoroethane/tri-n-octylamine (78/22). The aqueous layer was chromatographed by HPLC, and the formation of guanosine and 6-thioguanosine was measured at 280 nm to determine acid phosphatase activity.

Alkaline phosphatase. The assay method employed here was a modification of a previously described method (73-75). The enzyume activity was assayed in 0.1 M glycine, pH 10.4, 1.0 mM $MgCl_2$, 1.0 mM $ZnSO_4$, with GMP as the substrate. Various amounts of 1 mM 6-TGMP were added to the reaction mixture to inhibit the enzyme activity. The reaction was started by adding 10 µl alkaline phosphatase (1 U/ml) to 190 µl reaction mixture. The reaction mixture was incubated at 37°C for 15 min before 8.8 µl concentrated PCA was added to stop the reaction. Measurement of the formation of guanosine and 6-thioguanosine was the same as the above description for acid phosphatase.

5'-nucleotidase. The assay method employed here was a modification of a previously described method (87,88). The enzyme activity was assayed in 50 mM Tris buffer, pH 7.5, with GMP as the substrate. Various amounts of 1 mM 6-TGMP were added to the reaction mixture to inhibit the enzyme activity. The reaction was started by adding 10 µl 5'-nucleotidase (EC 3.1.3.5 from Crotalus atrox venom, 1 U/ml) to 190 µl reaction mixture. It was then incubated at 37°C for 15 min before 8.8 ml concentrated PCA was added to stop the reaction. Measurement of the formation of guanosine and 6-thioguanosine is the same as described for acid phosphatase.

GMP kinase. HPLC method: The assay method employed here was a modification of a previously described method (18,69,70). The enzyme activity was assayed in 100 mM Tris-HCl buffer, pH 8.0, 200 mM KCl, 20

mM $MgCl_2$, 2.5 mM ATP, with GMP as the substrate. Various volumes of 1 mM 6-TGMP were added to the reaction mixture to inhibit the enzyme activity. The reaction was started by adding 10 µl crude HL-60 cell extract (17,000 g supernatant from HL-60 cell homogenate) to 490 µl reaction mixture. It was then incubated at 37°C for 7.5 min before 22 µl concentrated PCA was added to stop the reaction. Formation of GTP was used to calculate the enzyme activity. Measurement of the formation of GTP is described below, in section 2-g.

Spectrophotometric method: The composition of the assay mixture is listed in Table IV-1. GMP kinase activity was determined by coupling the formation of the products ADP and GDP to the pyruvate kinase and lactase dehydrogenase mediated oxidation of NADH, as described in Oeschger (69):

$$(d)GMP + (d)ATP \longrightarrow (d)GDP + (d)ADP$$

$$2 \text{ phosphoenolpyruvate} + (d)ADP + (d)GDP \xrightarrow{\text{pyruvate kinase}} 2 \text{ pyruvate} + (d)ATP + + (d)GTP$$

$$2 \text{ pyruvate} + 2 \text{ NADH} + 2H^+ \xrightarrow{\text{lactate dehydrogenase}} 2 \text{ lactate} + 2 \text{ } NAD^+$$

Various volumes of 1 mM 6-TGMP were added to the reaction mixture to inhibit the enzyme activity. The reaction was started by adding 20 µl crude HL-60 cell extract to 980 µl reaction mixture. The oxidation of NADH was followed spectrophotometrically at 340 nm at 20°C, and the decrease in absorbance was used to calculate the enzyme activity.

2-g. Chromatographic conditions

Purification of 6-TGMP and measurement of guanosine nucleotide pools. Column: Whatman Partsil 10 SAX anion exchange (Whatman

TABLE IV-1. Spectrophotometric GMP kinase assay mixture.

Component	Volume (ml)	Final Concentration (mM)
H_2O	0.39	--
Tris•HCl, 1 M (pH 8 at 50 mM)	0.10	100
KCl, 2 M	0.12	240
$MgCl_2$, 0.1 M	0.20	20
ATP, 0.1 M (pH 7)	0.05	5
Phosphoenolpyruvate, 50 mM	0.02	1
NADH, 12.5 mM	0.04	0.5
Lactic dehydrogenase[a]	0.03	--
Guanylate kinase[b]	0.02	--
GMP, 5 mM	--	--
6-TGMP, 1 mM	--	--
Vf	1.00	

[a]Lactic dehydrogenase is Type I from Sigma which contains pyruvate kinase. Pyruvate kinase is essential for the assay. The enzyme is prepared by dilution 1:5 with 0.05 M Tris•HCl (pH 8 at 0.05 mM).

[b]Crude HL-60 cell extract (17,000 g supernatant from HL-60 cell homogenate).

Laboratories, Clifton, NJ); solvent A: 5 mM potassium citrate, 0.2 M KCl; solvent B: 0.8 M $NH_4H_2PO_4$, 0.4 M KCl, pH 3.6; flow rate program: isocratic elution with solvent A at 2 ml/min for 5 min, followed by a linear gradient, maintaining total flow rate at 2 ml/min, reaching 100% solvent B in 20 min; retention time: 6-TGMP = 4.3 min; GMP = 3.5 min; GDP = 15.3 min; GTP = 23.1 min.

Measurement of the formation of guanosine and 6-thioguanosine. Column: Alltech C-18 (25 cm X 46 mm) 10 μ reverse phase column; solvent: 10 mM $NH_4H_2PO_4$, 2% acetonitrile pH 3.6; flow rate: 2 ml/min. Retention time: guanosine = 9 min; 6-TGuo = 11.5 min.

3. Results

3-a. Synthesis of 6-TGMP

Since there is no authentic 6-TGMP available commercially, chemical synthesis of this compound was first used to produce this nucleotide. The procedure was followed exactly according to that of Irie (67). Examination of the resultant aqueous solution on HPLC revealed several unknown products in the chromatogram; this method was therefore discarded.

When the enzymatic synthesis method was used, crude cell extract from S-49 cells was incubated with PRPP for 16 h. Examination of the resultant solution on HPLC showed that two products were formed, i.e., 6-TGMP and GMP, with a yield of 40% for 6-TGMP. GMP formation might have resulted from impurity in the 6-TG used. 6-TGMP thus obtained was homogeneous; this was further confirmed by acid phosphatase digestion that yielded 6-thioguanosine with identical HPLC retention as the

authentic compound. This material was also idential to the major itracellular nucleotide metabolite, previously identified as 6-TGMP.

3-b. 6-TGMP as a noncompetitive inhibitor of GMP for acid phosphatase, alkaline phosphatase, and 5'-nucleotidase

Acid phosphatase, alkaline phosphatase, and 5'-nucleotidase are all able to hydrolyze 5'-monophosphate nucleotides to their corresponding nucleosides. This is important for the regulation of intracellular nucleotide concentration. Intracellular 6-TGMP concentration was noted to be much higher than GMP concentration when the cells are exposed to 6-TG. One of the possible mechanisms for this is the slower degradation rate of 6-TGMP to 6-thioguanosine. Detailed kinetic analysis of acid phosphatase (type II from potato), alkaline phosphatase (type III from E. coli) and 5'-nucleotidase (type IV from Crotalus atrox venom) was therefore performed with GMP as substrate and 6-TGMP as the potential inhibitor. The data, analyzed by Lineweaver-Burke plots (72) and presented in Fig. IV-2, IV-3, and IV-4, demonstrated that 6-TGMP is a noncompetitive inhibitor of GMP, and the k_i values are estimated to be 30 μM, 390 μM, and 112.5 μM, respectively.

It has been proposed that 6-TGMP can form a disulfide bond with enzymes (46,90,93). This was tested by adding either 1 mM glutathione (GSH) or dithiothreitol (DTT) to the 5'-nucleotidase reaction mixture to restore the free sulfhydryl groups on the enzyme. However, both agents failed to reverse this inhibition. Preincubation of 6-TGMP with 5'-nucleotidase from 0 to 10 min also does not produce progressive inhibition as seen in IMP dehydrogenase (46). Therefore, formation of disulfide bonds between 6-TGMP and this enzyme is unlikely.

When the final reaction mixture was analyzed on HPLC, formation of

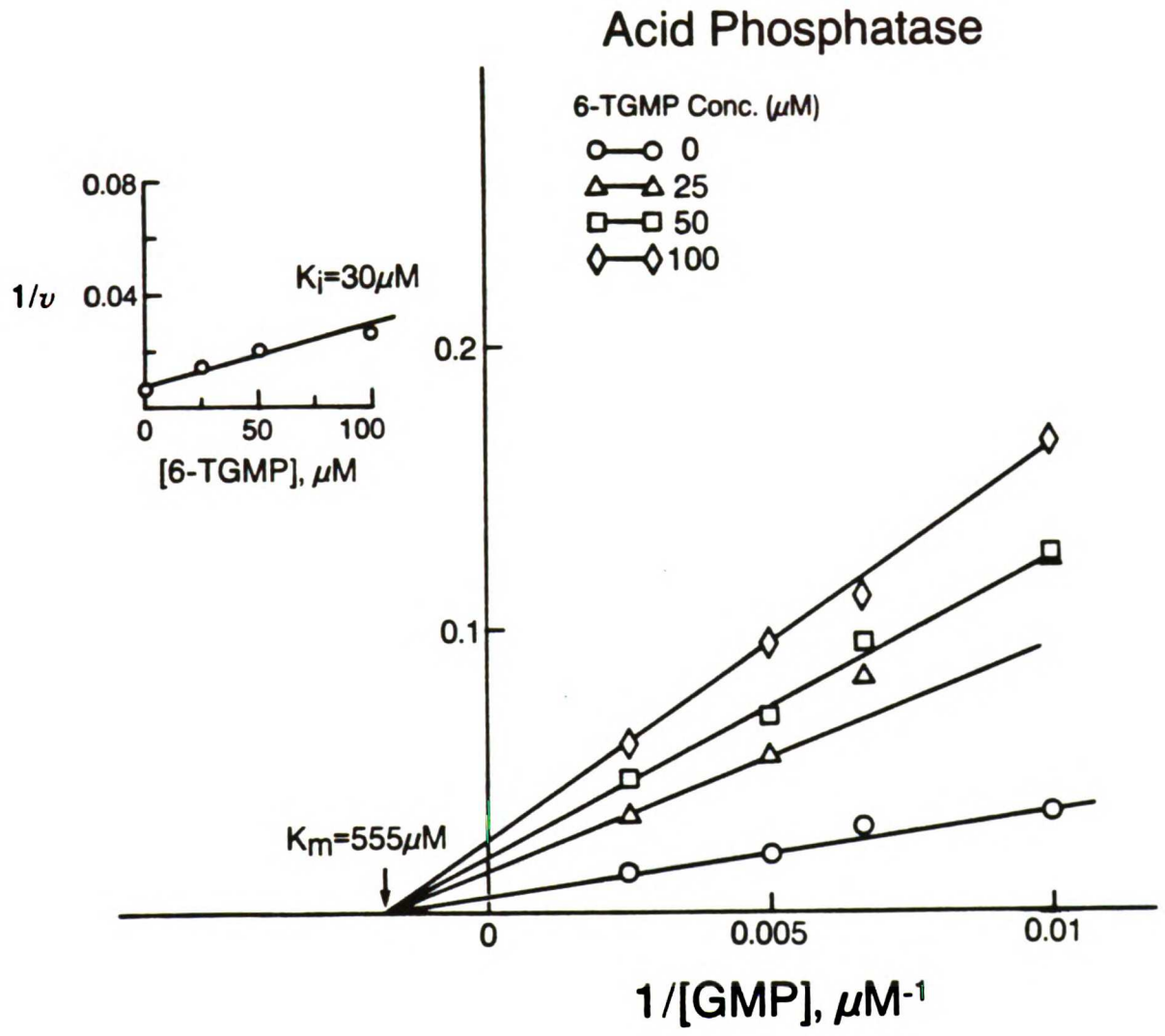

Figure IV-2. Double reciprocal plot of the initial rates of guanosine formation from GMP hydrolyzed by acid phosphatase. 6-TGMP was present as the inhibitor at different fixed concentrations.

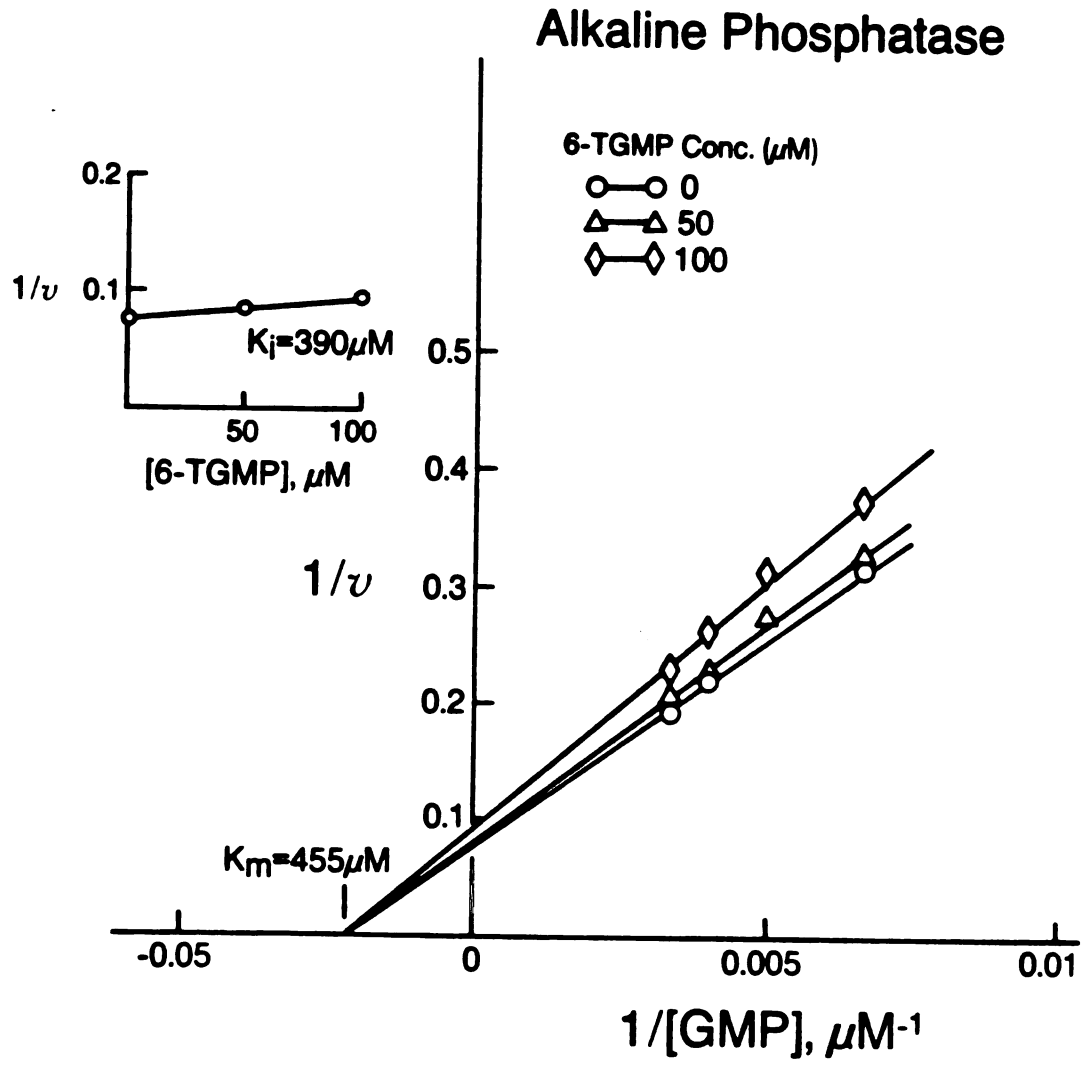

Figure IV-3. Double reciprocal plot of the initial rates of guanosine formation from GMP hydrolyzed by alkaline phosphatase. 6-TGMP was present as the inhibitor at different fixed concentrations.

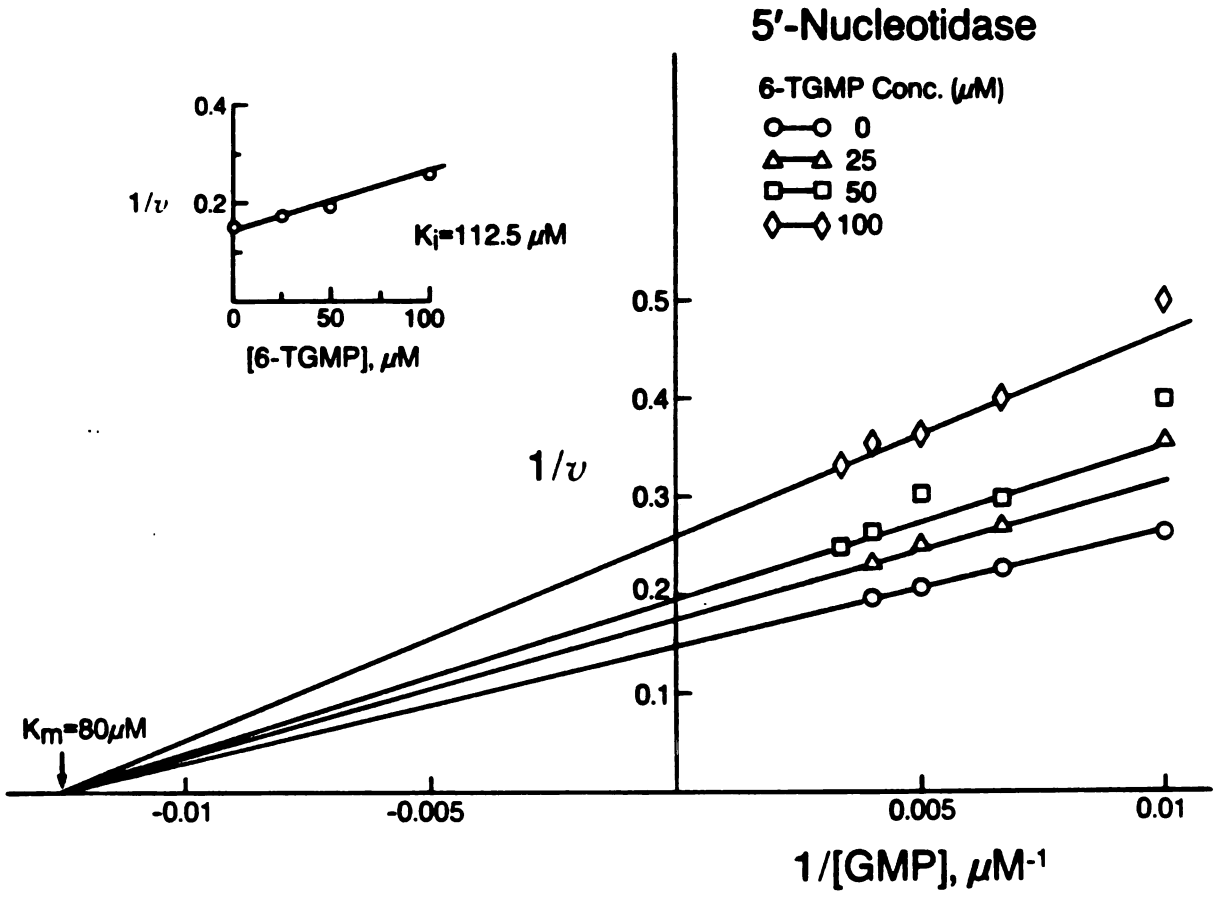

Figure IV-4. Double reciprocal plot of the initial rates of guanosine formation from GMP hydrolyzed by 5'-nucleotidase. 6-TGMP was present as the inhibitor at different fixed concentrations.

6-thioguanosine appeared much less than that of guanosine under the same situations, indicating that 6-TGMP is a poorer substrate than GMP for these three enzymes.

It has been reported that HL-60 cells lack alkaline phosphatase (7). The location of 5'-nucleotidase of HL-60 cells was therefore investigated. GMP was incubated with 17,000 g membraneous fraction and supernatant, and only the membraneous fraction was capable of digesting 6-TGMP.

3-c. Effect of 6-TGMP on GMP kinase activity

Studies of the inhibition of GMP kinase by 6-TGMP were carried out with the partially purified enzyme from a number of sources (18,89,90). These studies indicated that 6-TGMP acts as a competitive inhibitor of GMP with a k_i value in the range of 50 μM to 2.8 mM. Initial velocities were measured to establish the kinetic parameters of GMP kinase from HL-60 cells with either GMP or 6-TGMP as the substrate. The k_m and apparent V_{max} thus obtained were 22.5 μM and 0.0255 nM/min/μl crude cell extract for GMP (Fig. IV-5); and 384 μM and 0.00275 nM/min/μl crude cell extract for 6-TGMP (from a single experiment, data not shown). It should be noted that nucleotide distribution was different with these two substrates when the final reaction mixture was analyzed on HPLC. GTP was the predominant product (>90%) with little GDP found when GMP was used as the substrate. On the other hand, 6-TGDP appeared as the main product (~70%) with some 6-TGTP formation when 6-TGMP was used.

Varying concentrations of GMP (ranging from 25 to 150 μM, approximately equivalent to 1-6 X k_m) were added to two fixed concentrations of 6-TGMP (50 and 100 μM) to determine the inhibitory

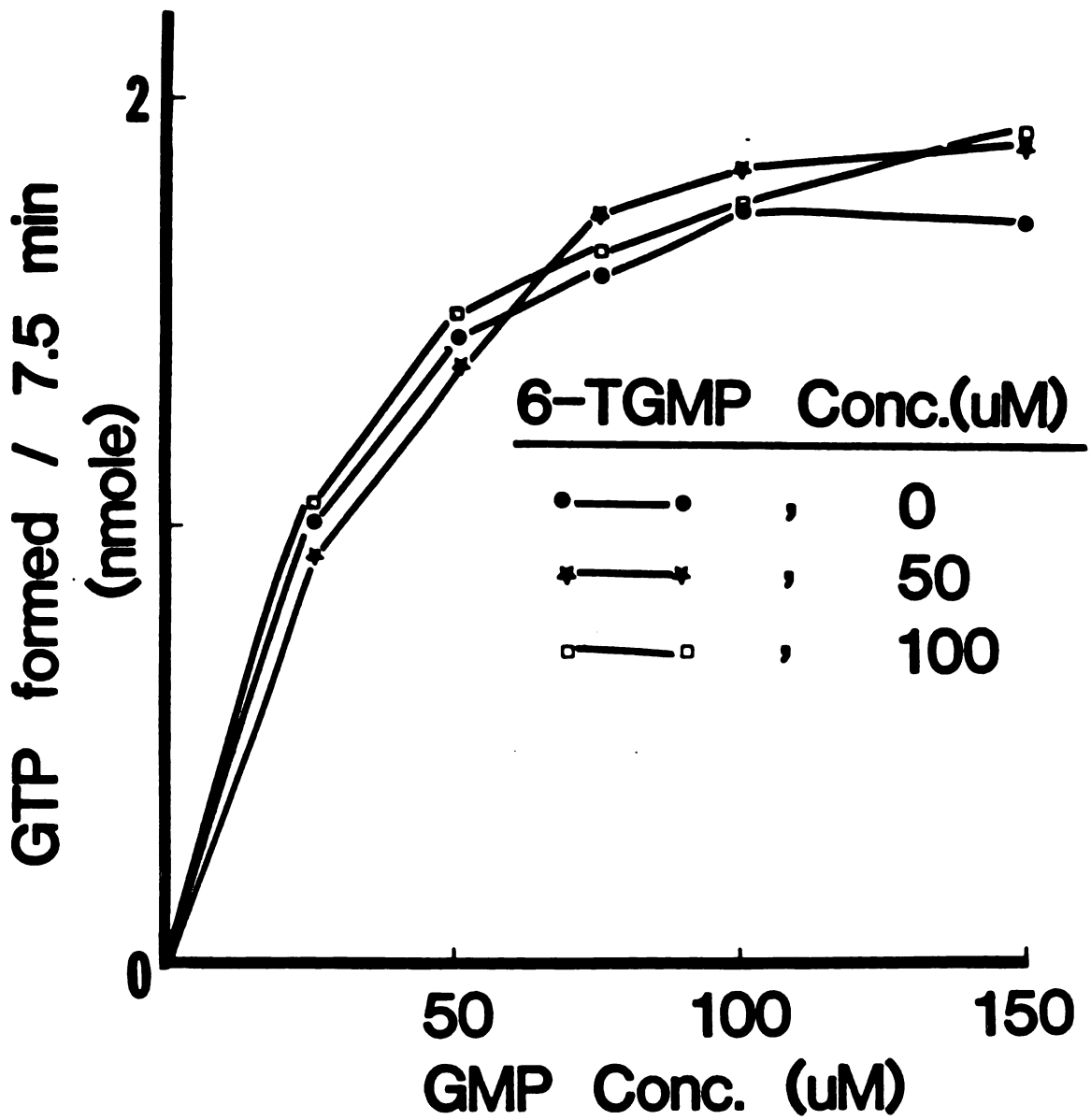

Figure IV-5. 6-TGMP effect on HL-60 GMP kinase activity. Initial rates of GTP formation from GMP catalyzed by HL-60 crude cell extract. 6-TGMP was present as the inhibitor at different fixed concentrations.

effect of 6-TGMP on HL-60 GMP kinase activity. The results, presented in Fig. IV-5, illustrate that 6-TGMP failed to have any inhibitory effect on HL-60 GMP kinase at these two concentrations.

4. Discussion

Metabolism of 6-TG to its nucleotide metabolite, 6-TGMP, via HGPRTase, is thought necessary to express its cell toxicity. It has been known for a number of years that 6-TGMP accumulates to surprisingly high concentrations (as high as 0.1 -0.2 mM) in susceptible tumor cells (46,68,91). This concentration appears to be considerably greater than the normal level of GMP based on the reported value for S-49 cells (0.18 nmol/10^8 cells) (47), and may be explained by its slow conversion to its higher nucleotide(s) and/or degradation to 6-thioguanosine. This hypothesis was challenged by testing the reactivity of 6-TGMP with 5'-nucleotidase, acid phosphatase, and alkaline phosphatase, all of which are known to hydrolyze 5'-monophosphate nucleotides very efficiently. The results showed that 6-TGMP acts as a noncompetitive inhibitor of GMP for all three enzymes (Table IV-2). This kind of inhibition implies that 6-TGMP can combine with an enzyme molecule to produce an inhibitor-enzyme complex regardless of whether a substrate molecule (i.e., GMP) is bound or not (92). Hence, 6-TGMP must bind at a different site from the substrate. This type of inhibition might well occur on other proteins that bind with GMP and thus cause metabolic effects that are responsible for certain of the cytotoxic manifestations of this drug.

It has been reported that 6-TGMP under certain conditions inactivates IMP dehydrogenase and GMP reductase of Aerobacter aerogens,

and evidence suggests that these effects could be associated with formation of disulfide bonds between 6-TGMP and a sulfhydryl group located near the substrate site of each enzyme (46,93). However, the following facts suggest that a covalent bond was not formed between 6-TGMP and 5'-nucleotidase: (a) the inhibition by 6-TGMP was not progressive with time; (b) the inhibition could not be reversed by the addition of 1 mM GSH or DTT.

Conversion of 6-TGMP to 6-TGDP through GMP kinase was also tested, and it appeared that 6-TGMP was a poor substrate (k_m=384 μM) of this enzyme with a low V_{max} value (10% of the velocity attainable if GMP was used). Miller et al. reported a much higher k_m value (2.1 mM) with a lower V_{max} (2.6% of that of GMP) on human erythrocyte GMP kinase (90). This reported k_m is one order of magnitude higher than our value, and this discrepancy may be due to (a) species difference; (b) the concentrations of 6-TGMP used here (25 to 150 μM) were considerably lower than the k_m concentrations; (c) different assay methods were used; HPLC analysis was adopted instead of spectrophotometric analysis. In a previous study with GMP kinase, the enzyme activity was spectrophotometrically determined by coupling the formation of the products ADP and 6-TGDP to the pyruvate kinase and lactase dehydrogenase mediated oxidation of NADH at 373 nm (90). This indirect measurement of enzyme activity may cause errors when interfering enzymes such as ATPase and NAD oxidase are present. On the other hand, direct measurement of 6-TGDP and 6-TGTP formation was used to determine enzyme activity in the present work. Therefore, it should reflect the true enzyme activity regardless of the presence of the interfering enzymes.

The inhibitory activity of 6-TGMP on HL-60 GMP kinase was

studied. Contrary to a previously published study (18), 6-TGMP was found to be a relatively weak inhibitor of GMP kinase. 6-TGMP failed to show any inhibitory effect on this enzyme at 50 and 100 μM concentrations, which is equivalent to 1 and 2 X k_i concentrations, as previously reported. This apparent discrepancy between our observation and the previous report was found to be due to a spectrophotometric artifact that was associated with the high absorbance of 6-TGMP at the wavelength previously used (340 nm) for the velocity measurement (90).

When the final reaction mixture was analyzed on HPLC, it revealed that the nucleotide products distribution was different. GTP was the predominant product (>90%) with little GDP formed when GMP is used as the substrate. On the other hand, 6-TGDP appeared as the main product (~70%) with some formation of 6-TGTP when 6-TGMP was used. Although nucleoside diphosphate kinase is extremely active and is able to convert any amount of GDP synthesized by GMP kinase to GTP, 6-TGDP also appears to be a poor substrate with a low V_{max} value for this nucleoside diphosphate kinase.

TABLE IV-2. Comparison of kinetic parameters of enzymes metabolizing GMP. The Michaelis constants were determined with GMP as the substrate and 6-TGMP as the potential inhibitor.

Enzyme	Source	K_m (M)	K_i (M)	Mode of Inhibition
5'-Nucleotidase	Crotallus atrox venom	8.0×10^{-5}	1.125×10^{-4}	NC[†]
Acid Phosphatase	potato	5.55×10^{-4}	3×10^{-5}	NC
Alkaline Phosphatase	E. coli	4.55×10^{-4}	3.9×10^{-4}	NC
GMP Kinase	HL-60	2.25×10^{-5}		
GMP Kinase[*]	human erythrocytes	1.7×10^{-5}	2.8×10^{-3}	C[*]

[*]Reference (90).

[†]NC = Noncompetitive.

C = Competitive.

5. Summary

1. 6-TGMP behaves as a noncompetitive inhibitor of GMP for 5'-nucleotidase, acid phosphatase, and alkaline phosphatase.
2. 6-TGMP is a poor substrate of GMP kinase with a very low V_{max} value and a k_m value of 384 μM.

CHAPTER V.

EFFECT OF 6-TG ON RNA METABOLISM IN S-49 CELLS

1. Introduction

It has been demonstrated that 6-TG is incorporated into both the DNA and RNA of susceptible tissues. While recent research has concentrated on the incorporation of 6-TG into DNA and has correlated the cytotoxicity of 6-TG to its incorporation into DNA, much less effort has been expended to determine the importance of incorporation of 6-TG into RNA to its cytotoxic properties.

Kwan et al. showed that 6-TG is readily incorporated into all of the major species of RNA and no selective concentration of this drug in a particular RNA fraction could be found (13). This finding has stimulated studies designed to determine whether this agent has an effect on rRNA maturation (14,78).

rRNA formation in eukaryotes involves a unique maturation process that begins with the synthesis of a 45 S RNA precursor. The 45 S RNA becomes methylated and is transformed sequentially into various intermediate precursors (38S, 32S, and 20S RNA) and, finally, into the mature 28S and 18S rRNA (Fig. V-1) (79). Weiss and Pitot, using ^{3}H-uridine and ^{3}H-methionine to label rRNA, have demonstrated that while synthesis of 45S RNA was not inhibited by 6-thioguanosine, further maturation of 28S and 18S rRNA was severely inhibited in Novikoff hepatoma cells (14). Armstrong et al. reported similar findings in C3-L5178Y cells when ^{3}H-cytidine was used to label cellular RNA (78). 6-TG suppressed the labeling of the 28S and 18S rRNA but increased the labeling of nuclear 32 to 45S rRNA.

Preliminary results in S-49 cells show that 10 μM 6-TG can abolish the synthesis of 28S and 18S rRNA after 16 h exposure. However, due to a failure to radiolabel the 45S rRNA in these experiments to detectable

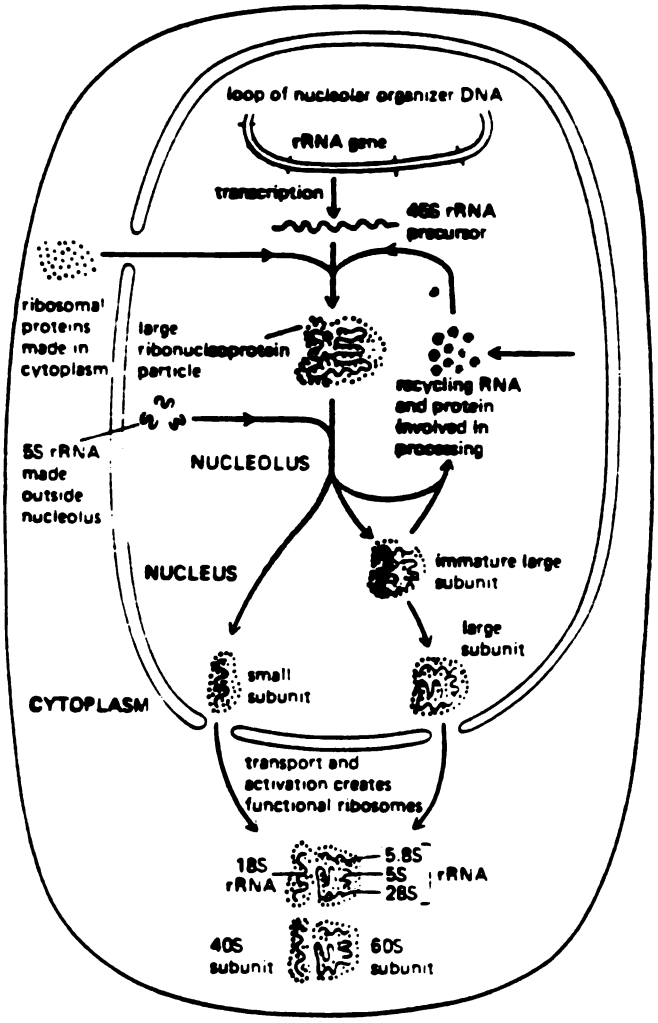

Figure V-1. Schematic view of ribosomal RNA synthesis (79). The large 45S rRNA transcript is packaged in a large ribonucleoprotein particle containing many ribosomal proteins imported from the cell cytoplasm. While this particle remains in the nucleolus, selected pieces are discarded as it is processed into immature large and small ribosomal subunits. These two subunits are thought to attain their final functional form only as they are individually transported through the nuclear pores into the cell cytoplasm.

levels, it was not possible to determine the effect of 6-TG on RNA metabolism.

2. Materials and Methods

2-a. Chemicals and reagents

All chemicals and reagents were of analytical grade. 6-TG, guanine, Tris base, Tris-HCL, sodium chloride, 8-hydroxyquinoline, iodoacetate, EDTA, and agarose (type II, medium EED), were purchased from Sigma Chemical Co. (St. Louis, MO). [5-^{3}H-]Uridine was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). Vanalyl ribonucleoside complex (VRC) was purchased form Bethesda Research Laboratories (Gaithersburg, MD). DNaseI was purchased from Cooper Biochemical (Long Valley, NJ). Proteinase K was purchased from Boehringer Mannhaim (Indianapolis, IN). Sodium lauryl sulfate (SDS) and urea were purchased from International Biotechnologies, Inc. (New Haven, CT). Phenol and chloroform were purchased from J.T. Baker Chemical Co. (Philipsburg, NJ). 200 proof ethanol was purchased from U.S. Industrial Chemicals Co. (Tuacola, IL). Beckman Ready-Solv® was purchased from Beckman Chemical Co. (Palo Alto, CA).

2-b. Apparatus

All centrifugation was performed on a Beckman model J-6M centrifuge (Beckman Institute, Palo Alto, CA). Gel electrophoresis was performed using a Pharmacia model ECPS 3000/150 power supply (Pharmacia, Inc., Piscataway, NJ) and a vertical gel slab apparatus (Aladin Enterprises, San Francisco, CA). RNA content was quantitated on a Beckman DU-8B

spectrophotometer (Beckman Institute, Palo Alto, CA). Radioactive analyses were performed on a Beckman LS-3801 liquid scintillation counter (Beckman Institute, Palo Alto, CA).

2-c. Composition of buffers and solutions

Lysis buffer A: 10 mM Tris, pH 7.0, 10 mM NaCl, 0.75% Triton-X, 1% ethanol.

Lysis buffer B: 10% SDS, 50 mM EDTA, 100 mM Tris, pH 7.0.

Lysis buffer C: 5 parts lysis buffer A plus 1 part lysis buffer B.

DNase buffer: 50 mM Tris, pH 7.5, 1 mM EDTA, 50 mM $MgCl_2$.

DNase buffer/VRC: 40 parts DNase buffer plus 1 part VRC.

Phenol, 0.1% 8-hydroxyquinoline: Extracted with equal an volume of 1.0 M Tris, pH 8.0; then with equal volume of 0.1 M Tris, pH 8.0 until pH is above 7.6. This solution was stored under 0.1 M Tris at 4°C.

Chloroform solution: 24 parts chloroform plus 1 part isoamyl alcohol (V/V).

Electrophoresis running buffer: 0.04 M Tris, pH 7.4, 0.036 M NaH_2PO_4, 1mM EDTA.

Electrophoresis gel buffer: 0.04 M Tris, pH 7.4, 0.036 M NaH_2PO_4, 1mM EDTA, 6 M urea, 0.016 M iodoacetate; the solution was filtered through millipor-type GS 0.22 μM filter after preparation.

Loading solution: 30% glycerol, 0.24% bromophenol blue, 6 M urea in electrophoresis running buffer.

2-d. [5-^{3}H]Uridine incorporation into RNA

Two hundred milliliters of S-49 cells, at a density of 5-6 X 10^5 cells/ml, were incubated at 37°C with 0, 5, or 10 μM 6-TG and 50 uCi [5-

^{3}H]uridine in the presence or absence of 200 μM guanine for 16 h. After incubation the cells were harvested by centrifugation, then resuspended in phosphate buffered saline (pH 7.4) in a 50 ml centrifuge tube and pelleted again. The cell pellets were then ready for RNA isolation.

2-e. Subcellular fractionation of cytoplasmic and nuclear RNA (76,77)

Eight milliliters of lysis buffer A and 0.4 ml VRC were added to the cell pellet, vortexed vigorously, placed in an ice bath for 10 min, and then vortexed again. The nuclei were spun down by centrifugation (3000 rpm) for 10 min at 0°C and the supernatant was removed. Another 5 ml lysis buffer A was added to the nuclear pellet, vortexed, and centrifuged to repellet the nuclei; the supernatant was isolated and combined with the previously obtained supernatant. This supernatant containing cytoplasmic RNA was placed in an ice bath.

Ten milliliters of lysis buffer C was added to the nuclear RNA pellet and vortexed vogorously. 0.5 ml 1 mg/ml stock proteinase K was added and vortexed well or aspirated several times with a 19-gauge needle, and then incubated at 37°C, with occasional vortexing, for 30 min.

After incubation half volumes of phenol were added to the nuclear and cytoplasmic RNA, capped tightly, and vortexed; half volumes of chloroform were then added and revortexed. This was spun at 3000 rpm for 10 min at room temperature to separate and discard the lower organic layer while leaving the interface with the upper aqueous layer. This extraction was repeated once more; extraction was then performed twice more with an equal volume of chloroform, aspirating the protein interface on the last extraction. One-twentieth volumes of 5 M NaCl and

2-2.5 volumes of absolute ethanol were added to each nuclear or cytoplasmic RNA fraction; this was vortexed well and the RNA was precipitated overnight at -20°C.

2-f. DNase treatment of nuclear RNA samples

The nuclear nucleic acid was spun down from ethanol at 4000 rpm for 15 min at 0°C and the supernatant was discarded. Two milliliters DNase/VRC buffer and 0.5 ml of 200 µg/ml DNase were added to the nucleic acid pellet, vortexed, and incubated at 37°C for 30 min. After incubation, 2.5 ml sterile water was added and the pellet was extracted twice with 5 ml phenol/chloroform (50/50) and three times with 5 ml chloroform. The RNA was precipitated as described in 2-e., with 5 M NaCl and ethanol.

The cytoplasmic RNA was spun down from ethanol at 4000 rmp for 15 min at 0°C and the RNA pellet was resuspended in 5 ml sterile water. This was extracted with phenol/chloroform, chloroform, and the RNA was precipitated as described above.

2-g. RNA content quantitation

The RNA pellet was spun down and the supernatant discarded. The pellet was dissolved in 5 ml sterile water and 0.1 ml of this was removed for quantitation. A 0.10 ml aliquot was added to 0.9 ml sterile water and absorbance was measured at 260/280 nm on a Beckman DU-8B spectrophotometer. RNA content was calculated as follows:

$$\text{Amount } (\mu g) = 40\ \mu g \times \text{O.D. } (260).$$

If the 260/280 absorbance ratio was below 1.80, the sample was considered impure and was re-extracted with phenol/chloroform,

chloroform, and then precipitated with ethanol as described above.

2-h. Electrophoresis of RNA through agarose gel containing urea

20 µl loading solution was added to 20 µg RNA and loaded on vertical 1.5% agarose slab gels containing 6 M urea and 0.016 M iodoacetate. The RNA was fractionated at 40 mV for 16 h with a Tris-phosphate running buffer.

The gel was stained with 0.15 µg/ml ethidium bromide staining solution for 15 min, then destained with deionized water for 30 min. RNA bands were visualized under UV light after staining and photography was taken at this time. Incorporation of [5-^{3}H]uridine into the different size classes of RNA was determined by slicing the RNA bands out of the gel into 2mm slices and incubating the slices in 1 M NaOH overnight to hydrolyze the RNA. The gel slices were neutralized with HCl and the radioactivity associated with each slice was determined by liquid scintillation counting.

3. Results

3-a. 6-TG Effect on [5-^{3}H]uridine incorporation into RNA fractionated by gel electrophoresis

Following a 16-h exposure to either 5 or 10 µM 6-TG in the presence or absence of 200 µM guanine, cytoplasmic and nuclear RNA from S-49 cells were isolated and gel electrophoresis was used to examine the RNA produced. [5-^{3}H]Uridine was added concurrently to label the newly synthesized RNA. Electrophoresis of the RNA on 1.5% agarose/6 M urea gels allowed for the separation of the precursor rRNAs (45S, 38S, and

32S), the mature rRNAs (28S and 18S), and the transfer and small molecular weight RNAs (4S to 10S) (Fig. V-2).

Incorporation of [5^{3}H]uridine into different classes of rRNA was determined by slicing the RNA bands and measuring the radioactivity associated with each band (Table V-1). It revealed no incorporation of [5-^{3}H]uridine into 45S or 32S precursor rRNAs, which may result from breakdown of the RNA samples. 6-TG apparently inhibited the labeling of 28S rRNA into both nucleus and cytoplasm; however, the inhibitory effect was more profound in nucleolus (more than 50% compared with 27%). Labeling of cytoplasmic 20S and 18S rRNA was not reduced very much by 6-TG, but that of nuclear (20S and 18S) RNA was reduced very significantly by 6-TG. Exogenous guanine itself does not have much effect on labeling of any classes of cytoplasmic or nuclear RNAs (except that it somehow increases the labeling of nuclear 20S rRNA and decreases that of 18S rRNA). It does possess some preventive effect against 6-TG on labeling of cytoplasmic 28S rRNA but not on 20S or 18S rRNA.

4. Discussion

We demonstrated, in Chapter II, that 6-TG is incorporated into RNA of S-49 cells. The relative extent of incorporation of 6-TG into various species of RNA of sarcoma 180 ascites cells was examined to determine whether 6-TG is selectively concentrated in one species of RNA, and no selective concentration of this drug in a particular RNA fraction was found (13). 6-TG was reported to inhibit maturation of 28S and 18S rRNA in Novikoff hepatoma cells and C3-L5178 Y cells (14,78). All these findings have stimulated our interest in studying the manner

Table V-1. 6-TG Effect on [5-^{3}H]uridine incorporation into cytoplasmic and nuclear RNA of S-49 cells.

	Nuclear RNA (% control)			Cytoplasmic RNA (% control)			
Drug Added	28S	20S	18S	28S	20S	18S	4S-10S
5 μM 6-TG	49	96	104	73	86	89	89
10 μM 6-TG	14	15	16	73	91	95	138
200 μM Guanine	86	131	60	112	96	108	91
50 μM 6-TG + 200 μM Guanine	26	58	80	106	78	110	181
100 μM 6-TG + 200 μM Guanine	10	23	24	89	88	104	115

S-49 cells were incubated with either 0, 5, or 10 μM 6-TG and 50 μCi [5-^{3}H]uridine in the presence or absence of 200 μM guanine for 16 h. Cytoplasmic and nuclear RNA were fractionated according to the methods described in **Materials and Methods.** Radioactivity associated with each class of RNA is expressed as percentage of [5-^{3}H]uridine incorporation into control cells (no drug treatment).

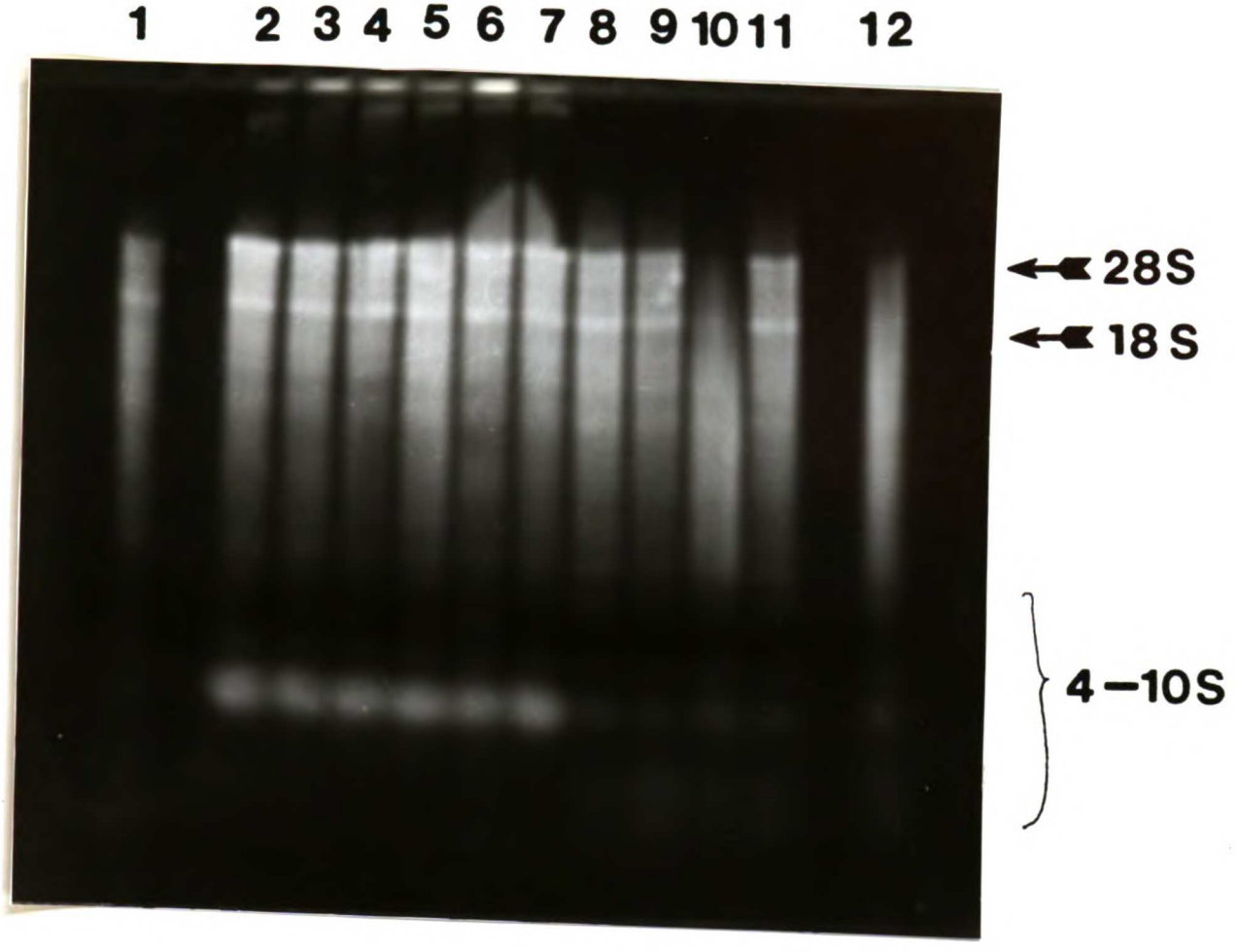

Figure V-2. Agarose/urea gel electrophoresis of S-49 cytoplasmic and nuclear RNA. S-49 cells were incubated with 5 (10) μM 6-TG and [5-^{3}H]uridine in the presence or absence of 200 μM guanine. The cytoplasmic and nuclear RNA were isolated and 0.5 $O.D._{260}$ unit was electrophoresed, as described in **Materials and Methods.**

Lanes from left to right: 1. 100 μM 6-TG (N); 2. Control (C); 3. 5 μM 6-TG (C); 4. 5μM 6-TG + 200 μM guanine (C); 5. 200 μM guanine (C); 6. 100 μM 6-TG; 7. 10 μM 6-TG + 200 μM guanine (C); 8. Control (N); 9. 5 μM 6-TG (N); 10. 5 μM 6-TG + 200 μM guanine; 11. 200 μM guanine (N); 12. 10 μM 6-TG + 200 μM guanine (N). C: cytoplasmic RNA; N: nuclear RNA.

in which 6-TG and guanine interaction can affect this rRNA processing.

The subcellular fractionation of total cellular RNA was adapted from a method developed by Penman et al. (77). Although VRC was present to inhibit RNase during all stages of RNA extraction and purification (81,82), possibly due to 6-TG containing RNA being more susceptible to RNase, 45S and 32S precursor rRNA (more liable than other RNA) were broken down and, therefore, could not be detected after fractionation by electrophoresis. Other methods using the guanidium/cesium chloride centrifugation may be a better approach to isolate the RNAs (83).

Inhibition of synthesis of 28S rRNA was observed when S-49 cells were exposed to 6-TG. The same observations were noticed in other cell lines (14,78), and incorporation of 6-TG into RNA has been proposed to explain the inhibition of the maturation process (15). Other purine and pyrimidine nucleoside and base analogues such as 5-fluorocytidine, 5-fluorouridine (85), toyocamycin (14,85), and aminonucleoside of puromycin (86), which are able to be incorporate into RNA, have been shown to stop or markedly alter the pattern of rRNA processing. The fact that structurally different analogues can produce the same inhibition suggests that the rRNA processing inhibition is not a site-specific alteration in the rRNA precursor, but that it is more likely that alterations in secondary structure of the rRNA precursors resulting from analog incorporation may actually change their configuration so that rRNA precursor-processing enzymes cannot function properly (14,84,86).

28S rRNA formation is accompanied by 18S formation (Fig. V-1); therefore, any reduction in labeling of 28S rRNA should also be seen in labeling of 18S rRNA. Examination of the data in Table V-1 does not

show this relationship and, again, it is likely due to the degradation of RNA preparation. The reduction in labeling of nuclear 28S and 18S rRNA is much more significant than that of cytoplasmic 28S and 18S rRNA. This may be due to the instability of 6-TG-containing RNA and, therefore, its very rapid degradation in the nucleus.

Although incorporation of labeled uridine into RNA is a common means of measuring the rate of RNA synthesis, monitoring RNA synthesis with labeled uridine in the presence of 6-TG should be interpreted with caution. Weiss and Pitot demonstrated that 6-TGuo significantly reduced the incorporation of labeled uridine into the acid-soluble uridine pool in Novikoff cells, which would result in reduction of labeling of RNA (14). Reduction of incorporation into RNA may thus reflect the effect of 6-TG on both RNA metabolism and uridine precursor pool. This problem can be avoided by the use of labeled methionine as a precursor to correct the variations in the precursor pool. Nevertheless, whether this inhibition of labeling of rRNA represents a primary effect due to the direct incorporation into RNA or a secondary effect due to DNA synthesis inhibition is unclear at this stage, and further study to resolve this issue is warranted. However, because it seemed unlikely that such studies would provide more insight into the major objectives of this thesis, no further experiments were conducted.

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