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INHIBITION OF *c-myc* EXPRESSION IN HUMAN PROMYELOCYTIC LEUKEMIA AND COLON ADENOCARCINOMA CELLS BY 6-THIOGUANINE

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Abstract—A rapid decrease in expression of the oncogene c-*myc* has been associated with the induction of differentiation of HL-60 human leukemia cells. In this manner, the treatment of a hypoxanthine phosphoribosyltransferase (HPRT)-deficient HL-60 variant (HL-60/var) with 6-thioguanine (TG) was accompanied by lower c-*myc* mRNA levels. This occurred in the absence of 6-thioguanosine 5′-monophosphate (TGMP) synthesis and without alterations in cellular nucleotide pool sizes. Paradoxically, inhibition of c-*myc* expression in the wild type HL-60 (HL-60/wt) cell, which is only weakly induced to differentiate by TG, was 5-fold more sensitive to the thiopurine (IC_{50} = 35 μM). Furthermore, inosine, which blocks the formation of TGMP and enhances the extent of differentiation of HL-60/wt cells, decreased the sensitivity of c-*myc* expression in the HL-60/wt to TG. These actions of TG and inosine on c-*myc* were also observed in the human colon carcinoma cell line COLO 320, further dissociating some of the effects of TG on c-*myc* expression from granylocytic differentiation. The hematopoietic granulocyte-macrophage colony stimulating factor (GM-CSF) elevated c-*myc* expression and antagonized the actions of TG on c-*myc* in the HL-60 cells. GM-CSF more readily antagonized the inhibitory action of TG in the HL-60/var cell line when compared to the HL-60/wt cells, restoring c-*myc* levels to that of the untreated controls. Hence, TG inhibited c-*myc* expression by two distinct mechanisms in cells which express high levels of the oncogene: a TGMP-dependent, differentiation-independent process with an IC_{50} of 35 μM, and a TGMP-independent action with an IC_{50} of 175 μM that was associated with induction of differentiation and was reversed more readily by GM-CSF.

The purine antimetabolite 6-thioguanine (TG†) was developed as a cytotoxic agent and is used in the induction and post-remission treatment of acute myelogenous leukemia [1]. The mechanism of TG cytotoxicity is generally believed to include conversion and accumulation in the cell of the active ribonucleoside monophosphate, 6-thioguanosine 5′-monophosphate (TGMP), which inhibits purine nucleotide synthesis by actions on the enzymes 5-phosphoribosyl 1-pyrophosphate (PRPP) amidotransferase, IMP dehydrogenase, and ATP-GMP phosphotransferase [2]. These combined actions of TG produce a reversible depletion of purine nucleotide pools. In addition, TG is incorporated irreversibly into DNA, which leads to chromatid damage and may ultimately be responsible for cell death [3]. TG also has a separate anti-leukemic activity, the ability to induce cell differentiation under certain conditions. TG is a highly efficacious inducer of erythroid differentiation in murine erythroleukemia cells and granulocytic maturation in human promyelocytic leukemia cells that lack hypoxanthine phosphoribosyltransferase (HPRT) activity, and thus are not susceptible to the cytotoxic actions of TG [4–6].

TG has been reported to have partial, weak, or no activity as an inducer of granulocytic differentiation of wild type HL-60 (HL-60/wt) cells at low concentrations that are also cytotoxic [5–8]. It has been suggested that a TG nucleotide is responsible for the induction of differentiation under these conditions [8]. However, it is also clear from studies of HPRT-deficient variant HL-60 (HL-60/var) cell lines that TG can induce differentiation without conversion to a nucleotide by a mechanism distinct from that producing cytotoxicity [5, 9, 10]. The inconsistent findings of TG induction of differentiation in the wild type HL-60 may reflect variability in expression of this biological pathway due to the concurrent substantial cytotoxic actions of the thiopurine. When the TGMP-mediated cytotoxicity is blocked, either biochemically [11] or by loss of the activating enzyme activity [5, 6], full expression of the differentiation-inducing properties of TG can be observed, albeit at higher concentrations than required to induce partial differentiation in the wild type HL-60.

Both the cytotoxic and the cytodifferentiative actions of TG are delayed, requiring several days for full expression [9, 12]. However, many of the events accompanying differentiation occur earlier, preceding both the appearance of maturation markers and the commitment of the cells to terminal differentiation [13]. A reduction in the cellular levels of c-*myc* mRNA is an early event in the differentiation of HL-60 cells [14]. It is not known whether TG initiates similar changes in the wild type

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† Abbreviations: DMSO, dimethyl sulfoxide; GM-CSF, granulocyte-macrophage colony stimulating factor; HPRT, hypoxanthine phosphoribosyltransferase; and TG, 6-thioguanine.

HL-60 cells under conditions in which differentiation is not fully expressed, or in non-hematopoietic cells which do not undergo differentiation.

MATERIALS AND METHODS

Cell culture and materials. HL-60 cells [15] were obtained from Dr. Robert Gallo and COLO 320-HSR cells were obtained from ATCC (Rockville, MD). Both were maintained in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum and gentamicin (GIBCO). Cells were passaged weekly, and only HL-60 cells between passage 25 and 50 were used. Variant HL-60 cell line TG/0.5 was obtained from Dr. Robert Gallagher. These cells had been selected by growth in 0.5 μg/mL TG for approximately 2 weeks and were subsequently maintained in the absence of drug [6]. Cells were suspended at 5×10^5/mL and treated for 3 hr with 6-thioguanine (Sigma) and/or purified human recombinant GM-CSF (Genetics Institute).

Measurement of c-myc expression. RNA was extracted from cells in the presence of guanidine isothiocyanate as has been described [16]. Purified RNA (8 μg) was fractionated by electrophoresis on agarose gels, transferred to nitrocellulose, and analyzed by probing with nick-translated cDNA for the human c-*myc* gene (pHSR-1) [17]. RNA was also applied directly to nitrocellulose using a dot-blot apparatus. Blots were stripped and reprobed with a nick-translated cDNA for human actin (LK215). Blots were exposed to X-ray film at −80° with intensifying screens, and autoradiographs quantified by scanning with a Hoeffer densitometer equipped with a HP integrator.

Analysis of thiopurines and cellular nucleotides. HL-60 cells were treated with TG for 3 hr as described above, washed in phosphate-buffered saline, and extracted with 1.2 N cold perchloric acid. The acid-insoluble material was removed by centrifugation, and the supernatant was neutralized with KOH. After removal of insoluble salts, aliquots were assayed by HPLC on a HP1090 system equipped with a Whatman PartiSphere-5 SAX column. A linear gradient of 0.01 to 0.64 M potassium phosphate at a flow rate of 1.6 mL/min was used, as has been described [18]. The eluant was monitored at both 254 and 340 nm using a diode array detector for the analysis of nucleotides and thionucleotides, respectively, and peaks were quantified by comparison to external standards.

RESULTS

Inhibition of cell proliferation and c-myc expression by 6-thioguanine. The cytotoxicity of TG can be readily observed in the wild type HL-60 cells as a concentration-dependent inhibition of cell proliferation with an IC_{50} of 0.7 μM. The thiopurines retained the ability to inhibit cell proliferation under conditions in which the formation of TG nucleotides was blocked, albeit at concentrations two logs higher than when TG nucleotides were synthesized. For example, addition of inosine shifts the IC_{50} for TG in the wild type HL-60 cells from 0.7 to 70 μM, and in the HPRT-deficient variant cell line, TG has an IC_{50} of 110 μM.

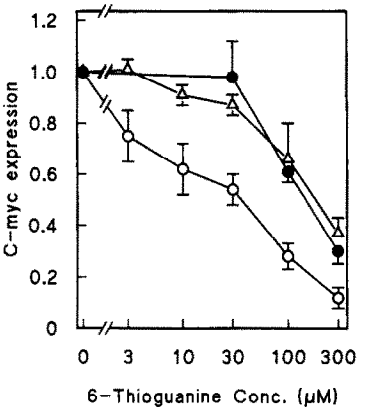

Fig. 1. Effect of TG and inosine on c-*myc* RNA. Wild type (○, ●) or variant HL-60 (△) cells were suspended at 5×10^5/mL with the indicated concentrations of TG, or TG and inosine (1 mM) (●). After 3 hr, the cells were centrifuged, and washed once with phosphate-buffered saline; then the RNA was extracted with guanidine thiocyanate, as described in Materials and Methods. RNA (8 μg) was fractionated by electrophoresis on formaldehyde-containing agarose gels, transferred to nitrocellulose, and probed with a nick-translated human c-*myc* cDNA. After autoradiography at −80°, blots were stripped and reprobed with human actin cDNA to correct for RNA loading. Films were scanned with a densitometer, and the corrected level of c-*myc* was expressed relative to control = 1.0. Data are means ± SE of three experiments.

A reduction in expression of the c-*myc* oncogene occurs upon treatment of leukemia cells with agents which induce cell differentiation; similarly, the direct inhibition of c-*myc* expression with antisense constructs leads to an inhibition of cell proliferation and the induction of differentiation [14, 19–24]. Hence it was of interest to examine the effect of TG on c-*myc* expression in the HL-60 cell lines. Cells were treated with TG for a brief period of time, since other inducers of differentiation had been shown to produce an early decline in c-*myc* expression [25, 26]. TG treatment of variant HL-60 cells for 3 hr produced a 50% reduction in c-*myc* RNA levels at a concentration of 175 μM (Fig. 1), approximately equal to the EC_{50} for induction of differentiation after 7 days, and somewhat higher than the concentration that inhibited cell proliferation by 50% in these cells. In contrast, the HL-60/wt cells were 5-fold more sensitive (IC_{50} = 35 μM) to the reduction of c-*myc* by TG than were the HL-60/var cells. However, since the EC_{50} for the inhibition of proliferation and clonogenicity in these cells is approcimately 1 μm [8, 27], inhibition of c-*myc* was not closely correlated with this action of TG. In all experiments quantitation of the level of c-*myc* RNA was corrected by reprobing blots with actin, whose expression did not change with any of the treatments. Quantitation of c-*myc* expression from autoradiographs of dot blots (using 0.5 to 4 μg RNA) gave results identical to those obtained from Northern (RNA) blots.

TG also inhibited the expression of c-*myc* in the

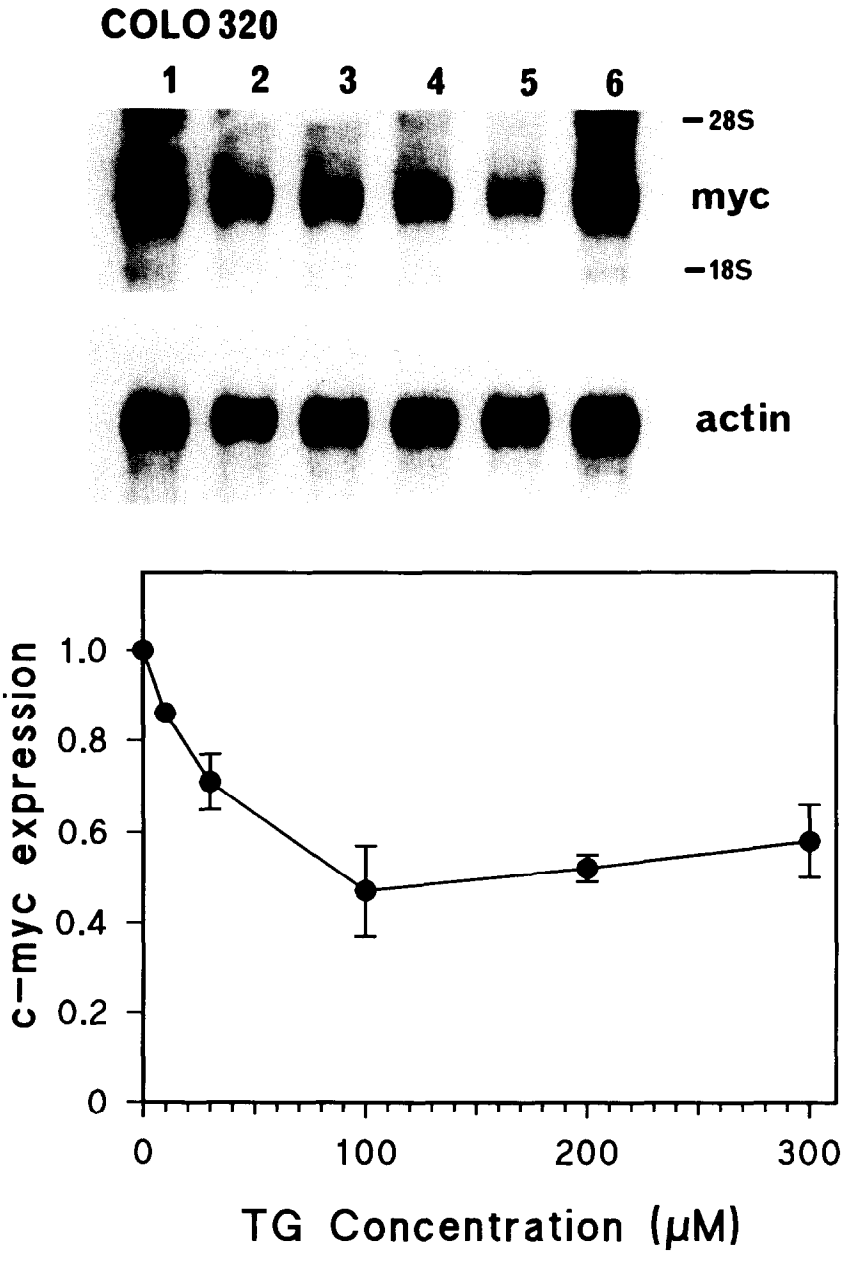

Fig. 2. Effect of TG and inosine on c-*myc* expression in COLO 320 cells. Human colon adenocarcinoma cells were treated with TG or inosine for 3 hr, and RNA was extracted and analyzed as described in the legend of Fig. 1. Treatments were (upper panel): lane 1, control; lane 2, 30 μM TG; lane 3, 100 μM TG; lane 4, 200 μM TG; lane 5, 300 μM TG; and lane 6, 100 μM TG + 1 mM inosine. Lower panel: densitometer quantitation of autoradiograms from two experiments.

COLO 320 human colon adenocarcinoma cells, which have a high constitutive level of c-*myc* mRNA (Fig. 2). There was an approximately 50% reduction in c-*myc* mRNA at 100 μM TG, a concentration intermediate to those observed in the two HL-60 cell lines. In contrast to the HL-60 cells, however, there was no further reduction in c-*myc* mRNA levels at higher TG concentrations up to 300 μM.

Role of TG nucleotides in c-myc *inhibition.* The HL-60/var cells have approximately 1% of the HPRT activity when compared to the HL-60/wt cells, as judged by [^{3}H]hypoxanthine uptake and incorporation (data not shown). Synthesis of the TG nucleotide TGMP under various conditions was assessed by HPLC and compared with the accompanying effect on c-*myc* expression (Table 1). No TGMP was detected in the HL-60/var cells at any of the TG concentrations tested, under conditions in which c-*myc* was inhibited. TGMP levels in HL-60/wt cells increased 4.5-fold when the TG concentration was raised from 3 to 30 μM (Table 1), conditions under which c-*myc* inhibition increased

Table 1. TGMP levels in wild type and variant HL-60 cells

Treatment	(nmol TGMP/10^7 cells) HL-60/wt	HL-60/var
TG (3 μM)	1.17 ± 0.08	0.00*
TG (30 μM)	5.18 ± 0.83	0.00*
TG (300 μM)	2.81 ± 0.03	0.00*
TG (300 μM) + GM-CSF	2.08 ± 0.41	0.00*
TG (300 μM) + inosine	0.40 ± 0.15	ND†

HL-60 cells were incubated for 3 hr with the indicated concentrations of TG, and in some instances with inosine (1 mM) or GM-CSF (2.5 nM), and in some instances with insoine (1 mM) or GM-CSF (2.5 nM). Cells were washed once with PBS, extracted with perchloric acid, neutralized, and analyzed by HPLC as described in Materials and Methods. Values are means ± SE of three to five experiments.

* Less than 0.01 nmol/10^7 cells.

† ND, not determined.

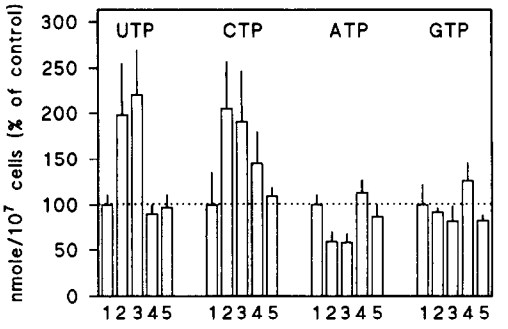

Fig. 3. Cellular nucleotide triphosphate levels: effect of 6-thioguanine and GM-CSF. Acid-soluble extracts from HL-60/wt (treatments 1–4) or HL-60/var cells (treatment 5) were analyzed by HPLC as described in Materials and Methods. Treatments were for 3 hr, as follows: 1, control; 2, 300 μM TG; 3, TG + GM-CSF; 4, 300 μM TG + 1 mM inosine; 5, 300 μM TG (HL-60/var cells). The control concentrations (treatment 1; nmol/10^7 cells) of each nucleotide were: UTP, 4.59 ± 0.49; CTP, 2.32 ± 0.81; ATP, 20.6 ± 2.3; and GTP, 4.76 ± 1.07. Data are means ± SE of three experiments.

from approximately 22 to 45% (Fig. 1). As the TG concentration was increased to 300 μM, inhibition of c-*myc* doubled again to 90%, but the TGMP levels decreased instead of showing a further increase. Hence, the reduction in c-*myc* was not correlated with formation of TGMP at TG concentrations above 30 μM.

To further evaluate the roles of TG versus TG nucleotides in the inhibition of c-*myc*, inosine (1 mM) was used to modulate the formation of TG nucleotides in the HL-60/wt and COLO 320 cells. Treatment with inosine alone caused a 30% reduction in c-*myc* expression in the HL-60/wt cells (data not shown). Inosine completely abrogated the inhibitory effect of TG at concentrations up to 30 μM in the HL-60 cells (Fig. 1), and at all TG concentrations tested in the COLO 320 cells (100 μM shown in Fig. 2). Higher concentrations of TG produced substantial decreases in c-*myc* expression in the presence of inosine in the HL-60 cells, and the inhibition curves of TG with and without inosine were roughly parallel at TG concentrations above 30 μM (Fig. 1). Although inosine nearly completely blocked the formation of TG nucleotides in the HL-60/wt cells at a concentration of 300 μM TG (Table 1), it did not block the reduction in c-*myc*.

Nucleotide pool size. Previous studies have shown that TG causes alterations in ribonucleotide pool sizes as a consequence of the inhibition of enzymes involved in purine synthesis [28, 29]. These changes have been reported to play a role in the regulation of cell differentiation in the HL-60 cells [30, 31]. Nucleotide pools were examined in the wild type and variant HL-60 cells to determine their relationship to c-*myc* expression. As expected, a 3-hr treatment with TG caused a 75–100% elevation in cellular UTP and CTP levels, and a 50% reduction in ATP levels (Fig. 3). In contrast to previous reports using other cell lines [29], TG produced only a minimal depletion of cellular GTP levels. None of these changes in nucleotide pool sizes were observed in HL-60/wt cells treated with TG and inosine, or in HL-60/var cells treated with TG (Fig. 3), conditions in which c-*myc* levels were reduced by at least 60%.

GM-CSF stimulation of c-myc *expression.* The hematopoietic growth factor GM-CSF stimulates the proliferation of HL-60 cells, and cells undergoing differentiation are particularly sensitive to this action [27]. GM-CSF treatment (2.5 nM for 3 hr) stimulated the expression of c-*myc* to a similar degree (75–95%) in both the wild type and variant HL-60 cell lines (Figs. 4 and 5). A similar degree of stimulation of c-*myc* by GM-CSF was observed in HL-60/wt cells treated with 300 μM TG. In contrast, GM-CSF had a larger than expected effect on c-*myc* expression in the TG-treated HL-60/var cells, producing a greater than 3-fold stimulation and restoring the level of c-*myc* in the TG-treated HL-60/var cells to that found in the untreated control cells (Fig. 5).

To test if the HL-60/var cells undergoing differentiation were particularly sensitive to the actions of GM-CSF on c-*myc*, the above experiments were repeated using DMSO as an inducer. Under these conditions, the two HL-60 cells lines had very similar responses to GM-CSF (Figs. 4 and 5). The actions of GM-CSF were also not due to effects on TGMP levels (Table 1) nor to alterations in ribonucleotide pool sizes (Fig. 3), dissociating the reversal of c-*myc* inhibition from the extent of nucleotide formation.

DISCUSSION

The purine analog 6-thioguanine has been studied extensively and its biochemical actions are well defined. TG has been shown to inhibit *de novo* purine synthesis, induce a sequential block of guanine nucleotide formation, and be incorporated into RNA and DNA leading to an impairment of nucleic acid function [2, 3, 28, 29]. These actions all require the conversion of TG to the nucleotide TGMP, which readily occurs via the purine salvage pathway in HL-60 cells. Hence, blockade of this critical metabolic

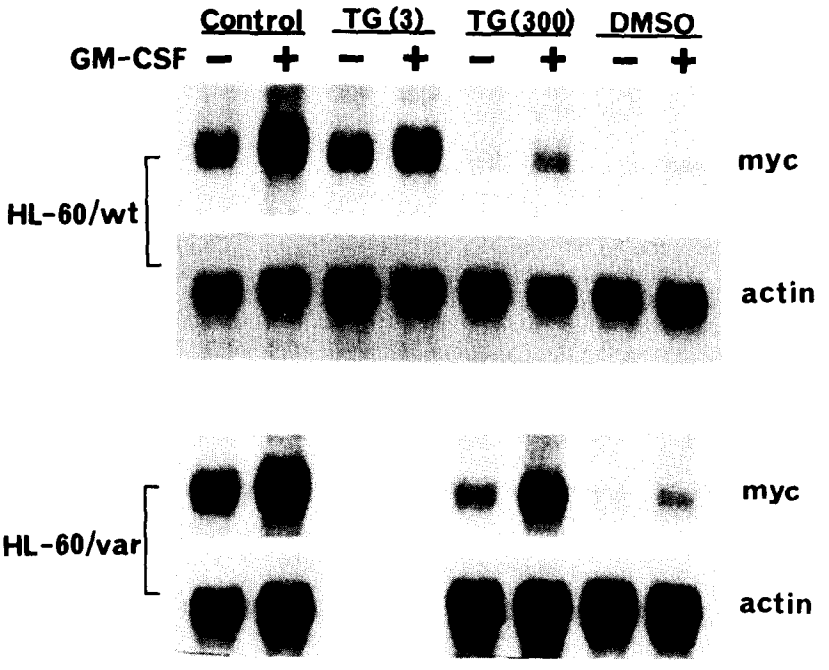

Fig. 4. Effect of GM-CSF on c-*myc* expression. HL-60/wt and HL-60/var cells were treated with TG (3 or 300 μM) or DMSO (1.25%) in the presence or absence of GM-CSF (2.5 nM), as indicated. After 3 hr, RNA was extracted and fractionated as described in Materials and Methods. Blots were probed sequentially with c-*myc* and actin cDNA.

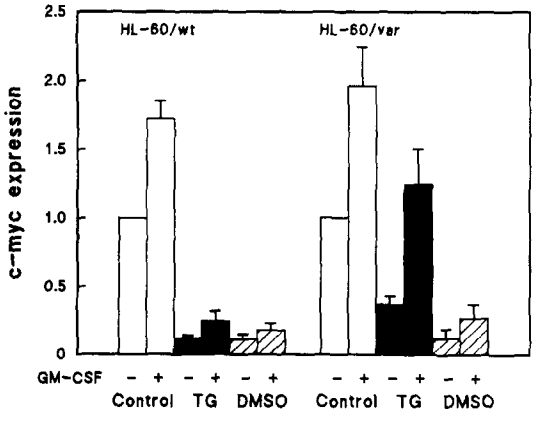

Fig. 5. Quantitation of the effect of GM-CSF on c-*myc* RNA levels. Autoradiographs of blots of HL-60 RNA were prepared as described in the legend of Fig. 4, and were scanned with a densitometer. RNA from both HL-60/wt and HL-60/var cells were used after control (open bars), TG (300 μM, solid bars), and DMSO (hatched bars) treatment for 3 hr. GM-CSF was added as indicated. Data are means ± SE of at least three experiments.

activation step would be expected to eliminate the growth inhibitory actions of TG. However, while such a blockade does reduce the potency of TG as a cytotoxic agent, it still retains considerable growth inhibitory properties. For example, in one study 10 μM TG inhibited HL-60 cloning efficiency by 80% despite the presence of a concentration of guanine that reduced TGMP biosynthesis by 85%, leading these investigators to conclude that a portion of the toxicity of TG is unrelated to its incorporation into RNA and DNA [8]. In our and other studies, TG retained the ability to inhibit HL-60 proliferation when the formation of TG nucleotide was blocked. In the present study, we observed that TG caused a rapid reduction in the mRNA levels of the oncogene c-*myc* in HL-60 and COLO 320 cells. A reduction of c-*myc* was first detected at 3–10 μM TG, and 30–100 μM TG produced a 50% reduction in both cell lines. This reduction was blocked completely in both cell lines by inosine, which prevents the synthesis of TGMP, indicating that the effect of TG under these conditions was dependent upon the synthesis of a nucleotide. The two cell lines differed, however, in their response to higher TG concentrations. While no further reduction in c-*myc* was observed in the COLO 320 cells, there was a nearly complete loss of c-*myc* RNA at higher TG concentrations in the HL-60 cells. The inhibitory effect of the higher concentrations of TG in the HL-60 cells was blocked only slightly by inosine, suggesting that at these TG concentrations, formation of TG nucleotide was not required. The studies with the variant HL-60 cells, in which TGMP synthesis cannot be detected, clearly demonstrate that formation of the nucleotide is not required for the inhibition of c-*myc* by TG.

From these observations, we conclude that both TG and TGMP can produce an inhibition of c-*myc* expression, with the nucleotide apparently more potent than the unmodified thiopurine. In support of this hypothesis is the finding that there was little inhibition of c-*myc* when the HL-60/var cells were treated with 30 μM TG, since no TGMP was formed in these cells. Why are higher concentrations of TG (without further metabolism) effective in the HL-60 but not the COLO 320 cells? Presumably this relates to the induction of differentiation that these concentrations of TG produce in the HL-60/var cells, as well as in the HL-60/wt in the presence of inosine (EC_{50} of 200–250 μM for induction of differentiation by TG) [5, 6, 11]. It is of interest that comparable changes in c-*myc* expression occur in the HL-60/wt cells as well although these cells do not undergo extensive differentiation in response to TG; it is likely that the cytotoxic actions of TG prevent full expression of maturation in this cell line. The activity of TG in the COLO 320 cells demonstrates that either TG induces differentiation-like changes in this cell line as well, or that TG has a novel effect on c-*myc* that occurs independent of the induction of differentiation. That the effect of TG on c-*myc* in COLO 320 cells was also not likely due to nonspecific cytotoxicity is supported by the observation that short-term exposure to cytotoxic concentrations of alkylating agents causes an increase in c-*myc* levels in these cells [32].

The growth factor GM-CSF stimulates the proliferation of normal and leukemic hematopoietic cells including HL-60 [27], and causes a rapid increase in c-*myc* mRNA in HL-60 cells. The relative increase in c-*myc* mRNA in HL-60/wt cells with GM-CSF treatment was the same in the presence or absence of TG; in contrast, GM-CSF more complrely antagonized the actions of TG on c-*myc* in the HL-60/var cells, under conditions in which TGMP is not formed. This further supports the hypothesis that TG and TGMP act by different mechanisms in inhibiting c-*myc* expression, with the action of TG related to cell differentiation and more sensitive to antagonism by GM-CSF. GM-CSF also more effectively blocks the growth inhibitory actions of TG in the HL-60/var cells when compared to the wild type HL-60 [27], completely consistent with this hypothesis.

The importance of a high level of c-*myc* expression in maintaining the phenotype of the HL-60 cell has been described [14, 18, 23–26]. There is substantial evidence suggesting that decreasing the high level of c-*myc* expression of HL-60 cells reduces proliferation and initiates or contributes to their terminal maturation [22–24], although c-*myc* expression has been shown not to be linked to cell cycle traverse *per se* nor to be a marker of a specific cell lineage, stage of maturation, or a secondary effect of growth stimulation [14, 20, 33, 34]. The contribution of the high level of c-*myc* expression to the phenotype of the COLO 320 cells has not been investigated as thoroughly. It remains to be determined what the consequences of the TGMP-mediated reduction of c-*myc* are in these and other cells.

It has been reported recently that the presence of high levels of c-*myc* protein in leukemic blast cells from patients with acute myelocytic leukemia is associated with a poor response to chemotherapy [35]; this suggests that inhibition of c-*myc* expression may be a potential therapeutic approach for those malignancies which overexpress c-*myc*. Our demonstration that TG inhibits c-*myc* expression in both human solid tumor and leukemia cell lines suggests that studies of the inhibition of c-*myc* expression *in vitro* may aid in the design and identification of agents with clinical efficacy.

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