

Cell Cycle Events Associated with the Termination of Proliferation by Cytotoxic and Differentiation-inducing Actions of 6-Thioguanine on HL-60 Cells¹

Edward L. Schwartz,² Owen C. Blair, and Alan C. Sartorelli³

Department of Pharmacology and Developmental Therapeutics Program, Comprehensive Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT

Delayed growth arrest was observed in HL-60 acute promyelocytic leukemia cells after exposure to 6-thioguanine (TG). This growth arrest occurred in both wild-type HL-60 cells exposed to 2 μ M TG and an HL-60 clone lacking hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity at a 500-fold higher concentration of drug. Both cell lines continued replication during an initial 4-day period of exposure to TG; however, upon removal of the purine antimetabolite and reincubation in fresh medium in the absence of drug, no further increase in cell number was observed over the next 4 days. Extensive differentiation, as measured by the reduction of nitroblue tetrazolium, occurred in TG-treated, HL-60 HGPRT-negative cells, whereas no significant increase in the number of nitroblue tetrazolium-positive cells was observed in wild-type HL-60 cells exposed to the purinethiol. Thus, termination of proliferation in wild-type cells appeared to be an expression of cytotoxicity, while in the HGPRT-negative clone, cell replication was apparently terminated by conversion of cells to end-stage forms with a mature phenotype. In support of this conclusion, differences occurred in the stage of the cell cycle arrest, determined on Day 6 after exposure to TG. Approximately 85% of parental HL-60 cells treated with TG were present in the S and G₂ + M phases of the cell cycle, with the greatest proportional change from untreated controls being in the G₂-M phase (*i.e.*, a 63% increase over untreated controls). In contrast, HL-60 HGPRT-negative cells treated with TG accumulated in G₁, with 68% of the population located in this phase (*i.e.*, an 80% increase compared to controls), as might be expected for a differentiated population. Dimethyl sulfoxide, which produced differentiation in both parental HL-60 and HL-60 HGPRT-negative cells, was used as a positive control. Both cell lines responded identically to dimethyl sulfoxide, with growth arrest being due at least in part to differentiation, which corresponded to an increase in G₁ cells.

INTRODUCTION

Cell lines, such as the HL-60 human acute promyelocytic leukemia and the Friend murine erythroleukemia, are valuable tools for studying drug-induced reversal of the malignant phe-

notype. These cells undergo functional and morphological maturation in response to the inducer DMSO⁴ (3, 10). The induction of differentiation in these leukemic cells is associated with changes in their cell cycle kinetics (7, 9, 12, 20, 26). In the presence of optimal concentrations of DMSO for induction of differentiation, both cell lines undergo several rounds of division before replication ceases (7, 8, 22). Furthermore, after a defined period of time considered to be required for commitment, the DMSO can be removed without affecting the subsequent irreversible loss of the self-renewing capacity of the cells and their eventual expression of markers of differentiation (7, 13, 25). In the HL-60 cell line, this commitment period is associated with an initial transient increase in the rate of cell multiplication (7) while, in the Friend erythroleukemia, DMSO causes a prolongation of the G₁ phase (9, 12, 26). Furthermore, studies with HL-60 cells have demonstrated that maturation can occur even when cell division is blocked (6, 25).

The antileukemic agent TG is a highly effective inducer of maturation in selected mutant clones of these leukemias (14, 15), although at best it has only weak activity in the wild-type HL-60 and Friend leukemia cell lines (2, 14, 15, 18, 23). In addition, this purine antimetabolite is highly cytotoxic to a variety of cell types (see, *e.g.*, Ref. 19). Typically, the growth arrest associated with the cytotoxic action of the 6-thiopurines and their nucleoside forms is delayed (1, 27, 28) and is not reversed upon removal of the drug once the cells have been exposed for a critical period of time (28). Thus, cells retain the capacity to synthesize DNA and undergo cellular replication during the initial phase of exposure to TG.

The present study demonstrates that HL-60 HGPRT-negative cells undergoing granulocytic maturation in response to TG show growth kinetics similar to those cells induced to differentiate by DMSO. Because of the similarity of the cellular growth kinetics that accompanies the differentiation caused by TG in HL-60 HGPRT-negative cells to that associated with the cytotoxicity of TG observed in wild-type HL-60 cells, cell cycle analyses were performed to compare the 2 phenomena. The results show that termination of proliferation resulting from differentiation and from cytotoxicity can be distinguished on the basis of differences in the phase of the cell cycle in which the growth-arrested cells ultimately reside.

MATERIALS AND METHODS

Cell Culture. HL-60 human acute promyelocytic leukemia cells were provided by Dr. Robert C. Gallo of the National Cancer Institute. They were grown in suspension culture in RPMI 1640 medium containing 20% heat-inactivated fetal bovine serum (Grand Island Biological Co.) in a humidified 5% CO₂ atmosphere at 37°. HL-60 HGPRT-negative cells

¹ This research was supported in part by USPHS Grant CA-02817 from the National Cancer Institute.

² Present address: Department of Oncology, Montefiore Medical Center/Albert Einstein College of Medicine, 111 East 210th Street, New York, NY 10467.

³ To whom requests for reprints should be addressed.

⁴ The abbreviations used are: DMSO, dimethyl sulfoxide; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; TG, 6-thioguanine.

Received January 9, 1984; accepted June 12, 1984.

were isolated by mutagenization and selection with TG, as described previously (15). These cells had no measurable HGPRT activity (*i.e.*, less than 0.1% of the enzyme activity of wild-type HL-60 cells), using hypoxanthine, guanine, or TG as substrates.

Drug Treatment. Cells were suspended at a level of 10^5 cells/ml in the presence of RPMI 1640 medium containing TG (Day 0). TG was dissolved in 10 mM NaOH immediately before use and added at final concentrations of $2 \mu\text{M}$ and 1 mM for wild-type and HGPRT-negative cells, respectively. DMSO was used at a concentration of 1.8% for both cell lines. After 4 days of continuous exposure to the inducing agent, the cells were collected by centrifugation and resuspended at a concentration of 10^5 cells/ml in fresh medium in the absence of inducer for 4 additional days. Cell concentrations were measured daily using a Coulter Model ZBI particle counter. Functional maturation of the cells was assessed on Day 7 as described previously (24) by measuring the percentage of cells that were capable of reducing nitroblue tetrazolium.

Flow Cytometry. The cellular DNA content and cell cycle analyses were determined on Day 6. Approximately 10^6 cells, collected by centrifugation, were resuspended in 0.5 ml of phosphate-buffered saline [KCl (0.2 g/liter)- KH_2PO_4 (0.2 g/liter)-NaCl (8 g/liter)- $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (2.2 g/liter)], at which time 1.5 ml of ice-cold 95% ethanol was added dropwise. Fixed cells were refrigerated until stained with 0.01% mithramycin by methodology described by Crissman and Tobey (4). The cellular DNA content was measured using a Becton-Dickinson FACS IV flow cytofluorometer, and cell cycle distributions were estimated by computer analysis of DNA histograms by the method of Dean and Jett (5).

RESULTS AND DISCUSSION

HL-60 cells, which morphologically resemble promyelocytes (11), grow exponentially in suspension culture with a doubling time of approximately 30 hr. The growth characteristics of wild-type HL-60 cells in the presence of 1.8% DMSO and $2 \mu\text{M}$ TG are illustrated in Chart 1. Cell numbers increased at approximately the rate of untreated control cells for the first 4 days of treatment. The minimum levels of DMSO and TG required to suppress further increases in cell number for an additional 4 days, during which cells were incubated in fresh medium in the absence of drug, were chosen; this required a relatively high concentration of each agent. The variant HL-60 HGPRT-negative cell line required a 500-fold higher concentration of TG (1 mM) to produce delayed inhibition of replication. Cells treated for shorter periods of time (*i.e.*, less than 4 days) at these concentrations showed a partial reversal of growth inhibition upon drug removal (data not shown).

The mechanism by which DMSO produces a delayed loss of the self-renewal capacity of HL-60 cells may be the direct result of terminal differentiation, since commitment to maturation appears to result in the cells being limited to a maximum of 5 subsequent rounds of cell division (22). When assayed on Day 7, only 25% of DMSO-treated cells were nitroblue tetrazolium positive (Table 1), an early functional marker of HL-60 maturation; however, measurement at later times resulted in 65% of cells expressing nitroblue tetrazolium positivity.⁵ It has been reported that the loss of the self-renewing capacity of HL-60 cells does not necessarily result in the expression of all markers of the mature state, particularly after a relatively short period of exposure to DMSO (7). Thus, while the loss of immortality may occur both before cells actually stop dividing and before they express functional characteristics of mature granulocytes, these properties need not necessarily be linked.

⁵ E. L. Schwartz and A. C. Sartorelli, unpublished observations.

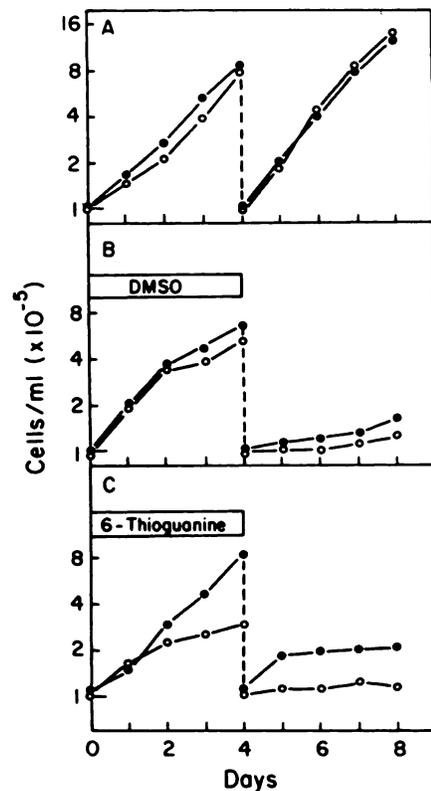


Chart 1. Growth of HL-60 (●) and HL-60 HGPRT-negative (○) cells in suspension culture. Cells were suspended at a level of 10^5 cells/ml in RPMI 1640 medium containing 20% fetal bovine serum without inducing agents (A) or in the presence of DMSO (1.8%; B) or TG ($2 \mu\text{M}$ for HL-60 and 1 mM for HL-60 HGPRT negative; C). On Day 4, the cells were washed and resuspended at a level of 10^5 cells/ml in fresh medium without drug. Values are cell numbers as determined with a Coulter ZBI particle counter and are the means of at least 4 experiments.

Table 1
Induction of cellular differentiation by DMSO and TG

HL-60 or HL-60 HGPRT-negative cells were suspended at a level of 10^6 cells/ml in the presence of DMSO (1.8%) or TG ($2 \mu\text{M}$ for HL-60 and 1 mM for HL-60 HGPRT negative). After 4 days, cells were collected, washed, and resuspended in fresh medium in the absence of inducer at 10^6 cells/ml. After an additional 3 days, functional maturation was assessed by incubating 10^6 cells in 0.1% nitroblue tetrazolium in phosphate-buffered saline and $1 \mu\text{M}$ 12-O-tetradecanoylphorbol-13-acetate for 20 min at 37°. Nitroblue tetrazolium-positive cells were determined by light microscopy after staining with Wright's solution.

Treatment	Nitroblue tetrazolium-positive cells (%)	
	HL-60	HL-60 HGPRT negative
None	3 ± 0.5 ^a	2 ± 0.6
DMSO	24 ± 5	25 ± 7
TG	7 ± 1	48 ± 4

^a Mean ± S.E. of 3 separate experiments.

In contrast to DMSO, biochemical mechanisms have been suggested to account for the delayed effects of the 6-thiopurines on growth arrest (1, 19, 27). These include the suggestion that incorporation of TG into DNA is the critical event, leading to impairment of replication in subsequent rounds of the cell cycle (1, 28) and unilateral chromatid damage (17). This hypothesis, however, cannot explain the delayed growth-arresting effect of TG observed in HL-60 HGPRT-negative cells (Chart 1), since in contrast to wild-type HL-60, these cells do not form TG nucleotides nor incorporate the thiopurine into DNA at the drug concentrations used in this study (15).

After treatment with TG, both L1210 (16, 28) and Chinese

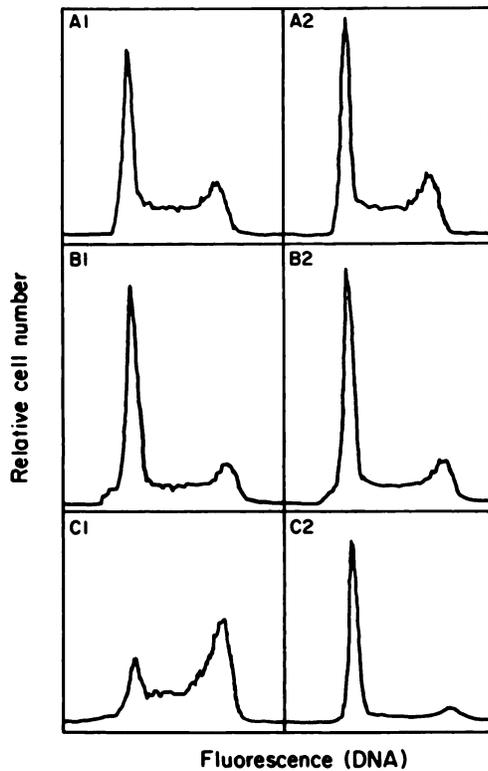


Chart 2. HL-60 cells (Column 1) and HL-60 HGPRT-negative cells (Column 2) were untreated (Row A) or treated as described in Chart 1 with DMSO (Row B) or TG (Row C). On Day 6, cells were collected and washed, fixed, and stained with mithramycin, as described in "Materials and Methods." DNA histograms were generated by measuring cellular fluorescence using a Becton-Dickinson FACS IV cytofluorometer, and representative results for each treatment are presented.

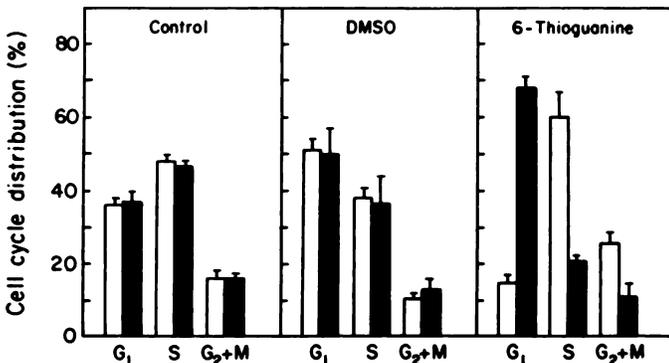


Chart 3. Cell cycle distributions estimated by computer analyses of DNA histograms. HL-60 cells (□) and HL-60 HGPRT-negative cells (■) were treated with DMSO (1.8%) or TG (2 μ M for HL-60 and 1 mM for HL-60 HGPRT negative) for 4 days and resuspended in fresh medium for 2 additional days. The proportions of cells in G₁, S, and G₂-M phases of the cell cycle were determined as described in Chart 2. Columns, results of 3 separate experiments; bars, S.E.

hamster ovary (17) cells accumulate in late-S and G₂-M phases of the cell cycle. Similar findings were observed with parental HL-60 cells after exposure to 2 μ M TG (Chart 2). The relative increases in the S and G₂-M phases of the cell cycle in TG-treated HL-60 cells were 25 and 63%, respectively, when compared to untreated controls (Chart 3). Thus, the characteristics of the growth arrest were similar to those reported in response to the cytotoxic action of the purine antimetabolite in other cell lines. In contrast, treatment of HL-60 HGPRT-negative cells with

1 mM TG produced a 55 and 30% reduction in S- and G₂-M-phase cells, respectively, compared to untreated controls (Chart 3). The pronounced difference in the response of the 2 cell lines to TG can be further illustrated by comparing the percentage of the total population of growth-arrested cells in the G₁ phase of the cell cycle (*i.e.*, 15% for the wild-type HL-60 and 67% for the HL-60 HGPRT-negative cells, compared to approximately 36% for the untreated controls of both cell lines).

The accumulation of growth-arrested HL-60 HGPRT-negative cells in the G₁ phase is comparable to the effects of DMSO on both HL-60 and HL-60 HGPRT-negative cell lines (Charts 2 and 3). Furthermore, the findings are in agreement with those obtained from experiments using Friend erythroleukemia cells, where arrest occurs in G₁ after DMSO treatment (8). Thus, like DMSO-treated HL-60 and Friend leukemia cells, TG-treated HL-60 HGPRT-negative cells arrest in G₁ when they undergo terminal differentiation. Arrest of cells in G₁ occurs in not only differentiated acute leukemia cells, but also many normal mammalian cell types in advanced stages of maturation (see, *e.g.*, Ref. 21). That this occurs in human leukemia cells in response to TG further underscores the likelihood that this agent has novel biochemical actions not described previously by studies of its cytotoxicity and suggests that its therapeutic usefulness in the treatment of acute nonlymphocytic leukemia may result from growth arrest produced by both distinct cytotoxic and differentiation-inducing actions.

ACKNOWLEDGMENTS

We wish to express our gratitude to Anita L. Stepanowski for her excellent technical assistance.

REFERENCES

- Barranco, S. C., and Humphrey, R. M. The effects of β -2'-deoxythioguanosine on survival and progression in mammalian cells. *Cancer Res.*, 31: 583-586, 1971.
- Collins, S. J., Bodner, A., Ting, R., and Gallo, R. C. Induction of morphological and functional differentiation of human promyelocytic leukemia cells (HL-60) by compounds which induce differentiation of murine leukemia cells. *Int. J. Cancer*, 25: 213-218, 1980.
- Collins, S. J., Ruscetti, F. W., Gallagher, R. E., and Gallo, R. C. Terminal differentiation of human promyelocytic leukemia cells induced by dimethylsulfoxide and other polar compounds. *Proc. Natl. Acad. Sci. USA*, 75: 2458-2462, 1978.
- Crissman, H. A., and Tobey, R. A. Cell cycle analysis in 20 minutes. *Science (Wash. DC)*, 194: 1297, 1974.
- Dean, P. N., and Jett, J. H. Mathematical analysis of DNA distributions derived from flow microfluorometry. *J. Cell Biol.*, 60: 523-527, 1974.
- Ferrero, D., Tarella, C., Gallo, E., Ruscetti, F. W., and Breitman, T. R. Terminal differentiation of the human promyelocytic leukemia cell line, HL-60, in the absence of cell proliferation. *Cancer Res.*, 42: 4421-4426, 1982.
- Fibach, E., Treves, A., Peled, T., and Rachmilewitz, E. A. Changes in cell kinetics associated with differentiation of a human promyelocytic cell line (HL-60). *Cell Tissue Kinet.*, 15: 423-429, 1982.
- Friedman, E. A., and Schildkraut, C. L. Terminal differentiation in cultured Friend erythroleukemia cells. *Cell*, 12: 901-913, 1977.
- Friedman, E. A., and Schildkraut, C. L. Lengthening of the G₁ phase is not strictly correlated with differentiation in Friend erythroleukemia cells. *Proc. Natl. Acad. Sci. USA*, 75: 3813-3817, 1978.
- Friend, C., Scher, W., Holland, J. G., and Sato, T. Hemoglobin synthesis in murine virus-induced leukemic cells *in vitro*. *Proc. Natl. Acad. Sci. USA*, 68: 378-382, 1971.
- Gallagher, R., Collins, S., Trujillo, J., McCredie, K., Ahearn, M., Tsai, S., Metzgar, R., Aulakh, G., Ting, R., Ruscetti, F., and Gallo, R. Characterization of the continuous differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. *Blood*, 54: 713-733, 1979.
- Geller, R., Levenson, R., and Housman, D. Significance of the cell cycle in commitment of murine erythroleukemia cells to erythroid differentiation. *J. Cell. Physiol.*, 95: 213-222, 1978.

13. Gusella, J., Geller, R., Clarke, B., Weeks, V., and Housman, D. Commitment to erythroid differentiation by Friend erythroleukemia cells: a stochastic analysis. *Cell*, 9: 221-229, 1976.
14. Gusella, J. F., and Housman, D. Induction of erythroid differentiation *in vitro* by purines and purine analogs. *Cell*, 8: 263-269, 1976.
15. Ishiguro, K., Schwartz, E. L., and Sartorelli, A. C. Characterization of the metabolic forms of 6-thioguanine responsible for cytotoxicity and induction of differentiation of HL-60 acute promyelocytic leukemia cells. *J. Cell. Physiol.*, in press, 1984.
16. Lee, S. H., and Sartorelli, A. C. The effects of inhibitors of DNA biosynthesis on the cytotoxicity of 6-thioguanine. *Cancer Biochem. Biophys.*, 5: 189-194, 1981.
17. Maybaum, J., and Mandel, H. G. Unilateral chromatid damage: a new basis for 6-thioguanine cytotoxicity. *Cancer Res.*, 43: 3852-3856, 1983.
18. Papac, R. J., Brown, A. E., Schwartz, E. L., and Sartorelli, A. C. Differentiation of human promyelocytic leukemia cells *in vitro* by 6-thioguanine. *Cancer Lett.*, 10: 33-38, 1980.
19. Paterson, A. R. P., and Tidd, D. M. 6-Thiopurines. In: A. C. Sartorelli and D. G. Johns (eds.), *Antineoplastic and Immunosuppressive Agents*, Vol. 2, pp. 384-403. Berlin: Springer-Verlag, 1975.
20. Pragnell, I. B., Amdt-Jovin, D. J., Jovin, T. M., Fagg, B., and Ostertag, W. Commitment to differentiation in Friend cells and initiation of globin mRNA synthesis occurs during the G₁ phase of the cell cycle. *Exp. Cell Res.*, 125: 459-470, 1980.
21. Prescott, D. M. Cell cycle and control of cellular reproduction. *Adv. Genet.*, 18: 99-177, 1976.
22. Schwartz, E. L., Brown, B. J., Nierenburg, M., Marsh, J. C., and Sartorelli, A. C. Evaluation of some anthracycline antibiotics in an *in vivo* model for studying drug-induced human leukemia cell differentiation. *Cancer Res.*, 43: 2725-2730, 1983.
23. Schwartz, E. L., Ishiguro, K., and Sartorelli, A. C. Induction of leukemia cell differentiation by chemotherapeutic agents. *Adv. Enzyme Regul.*, 21: 3-20, 1983.
24. Schwartz, E. L., and Sartorelli, A. C. Structure-activity relationships for the induction of differentiation of HL-60 human acute promyelocytic leukemia cells by anthracyclines. *Cancer Res.*, 42: 2651-2655, 1982.
25. Tarella, C., Ferrero, D., Gallo, E., Pagliardi, G. L., and Ruscetti, F. W. Induction of differentiation of HL-60 cells by dimethylsulfoxide: evidence for a stochastic model not linked to the cell division cycle. *Cancer Res.*, 42: 445-449, 1982.
26. Terada, M., Fried, J., Nudel, U., Rifkind, R. A., and Marks, P. A. Transient inhibition of initiation of S-phase associated with dimethylsulfoxide induction of erythroleukemia cells to erythroid differentiation. *Proc. Natl. Acad. Sci. USA*, 74: 248-252, 1977.
27. Tidd, D. M., and Paterson, A. R. P. A biochemical mechanism for the delayed cytotoxic reaction of 6-mercaptopurine. *Cancer Res.*, 34: 738-746, 1974.
28. Wotring, L. L., and Roti Roti, J. L. Thioguanine-induced S and G₂ blocks and their significance to the mechanism of cytotoxicity. *Cancer Res.*, 40: 1458-1462, 1980.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Cell Cycle Events Associated with the Termination of Proliferation by Cytotoxic and Differentiation-inducing Actions of 6-Thioguanine on HL-60 Cells

Edward L. Schwartz, Owen C. Blair and Alan C. Sartorelli

Cancer Res 1984;44:3907-3910.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/44/9/3907>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link <http://cancerres.aacrjournals.org/content/44/9/3907>. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.