Biochemical Pharmacology, Vol. 38, No. 13, pp. 2069–2075, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

TEMOZOLOMIDE INDUCED DIFFERENTIATION OF K562 LEUKEMIA CELLS IS NOT MEDIATED BY GENE HYPOMETHYLATION

M. ZUCCHETTI, C. V. CATAPANO, S. FILIPPESCHI, E. ERBA and M. D'INCALCI*
Istituto di Recerche Farmacologiche 'Mario Negri', Via Eritrea 62, 20157 Milan, Italy

(*Received* 2 *March* 1988; *accepted* 10 *December* 1988)

Abstract—Temozolomide (8-carbamoyl-3-methylimidazo[5,1d]-1,2,3,5-tetrazin-4-(3H)-one), an experimental antitumor agent which spontaneously decomposes to 5-(3,3-methyl-1-triazeno) imidazole-4-carboxamide, the active metabolite of the antineoplastic drug DTIC, causes erythroid differentiation of K562 leukemia cells. The increase in ε and γ globin gene expression after temozolomide treatment does not appear to be due to drug-induced hypomethylation of the genes. In other genes containing many methylated sequences such as the proto-oncogenes c-myc and C-Ha-ras, temozolomide caused no detectable change in methylation. In contrast, in the same genes 5-aza-2'-deoxycytidine induced hypomethylation. Temozolomide caused DNA alkali-labile sites and an arrest of the cell cycle in G2 phase. Ethazolastone (its 3-ethylimidazo analogue) which does not cause differentiation of K562 produced no significant DNA damage and G2 phase blockade. DNA damage rather than hypomethylation may be responsible for induction of differentiation.

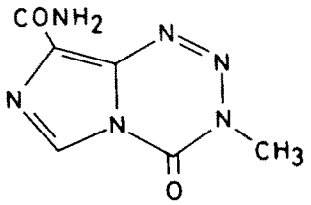

Fig. 1. Chemical structure of temozolomide.

5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) is an antineoplastic agent used for the therapy of several human malignancies [1, 2] and is the drug of choice against melanoma [3]. DTIC undergoes oxidative N-demethylation, forming of 5-(3,3-methyl-1-triazeno) imidazole-4-carboxamide (MTIC) an alkylating agent probably responsible for DTIC's activity [4, 5].

Temozolomide (8-carbamoyl-3-methylimidazo-[5,1-d]-1,2,3,5-tetrazin-4-(3H)-one) (Fig. 1), previously called methazolastone, is a recently synthesized compound which decomposes spontaneously to MTIC [6] without requiring metabolic activation. Temozolomide may overcome the problem of the variable and/or insufficient activation of DTIC and for this reason is under clinical investigation.

In addition it is useful for *in vitro* studies to elucidate the mode of action of methyltriazenes, which is still unclear. These monofunctional alkylators not only have cytotoxic activity, probably due to alkylation of cellular macromolecules, but other interesting effects related to modulation of the expression of some genes have been reported. Methyltriazenes cause increased expression of some tumoral cell-associated antigens [7], and their antitumoral activity might thus be partially due to an increased susceptibility to immunological mechanisms.

Recently Tisdale [8] reported that temozolomide but not ethazolastone (its 3-ethylimidazo analogue), induced differentiation in K562 erythroleukemic cells. He found that after temozolomide treatment the content of 5-methylcytosine fell from 3.5 to 2.2% in the total genome and proposed that differentiation was due to gene hypomethylation [8], as previously described for azacytidine [9, 10]. The data on the total presence of 5-methylcytosine can only be taken as indicative at this stage and more detailed studies on the methylation status of single genes are required to confirm this hypothesis.

In the present study we investigated whether temozolomide induces changes in the methylation status of the ε globin gene, γ globin gene, c-myc oncogene and ras oncogene. The data do not support the hypothesis that temozolomide-induced differentiation of K562 erythroleukemia cells is mediated by gene hypomethylation.

MATERIALS AND METHODS

Drugs. Temozolomide and 8-carbamoyl-3-ethylimidazo[5,1-d]-1,2,3,5-tetrazin-4-(3H)-one (ethazolastone) were kindly provided by Dr C. G. Newton, May & Baker Ltd., Dagenham, Essex (RM107XS). The drugs were dissolved in 0.5% DMSO immediately before use. 5-Aza-2'-deoxycytidine was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute, MD. It was dissolved in RPMI 1640 medium before administration.

Cell culture. K562 erythroleukemia cells [11] were grown at 37° in suspension culture in RPMI 1640 medium (Gibco Europe, Glasgow, Scotland) supplemented with 10% heat-inactivated (56°, 30 min)

* To whom requests for reprints should be addressed at the Istituto di Ricerche Farmacologiche 'Mario Negri', via Eritrea 62, 20157 Milan, Italy.

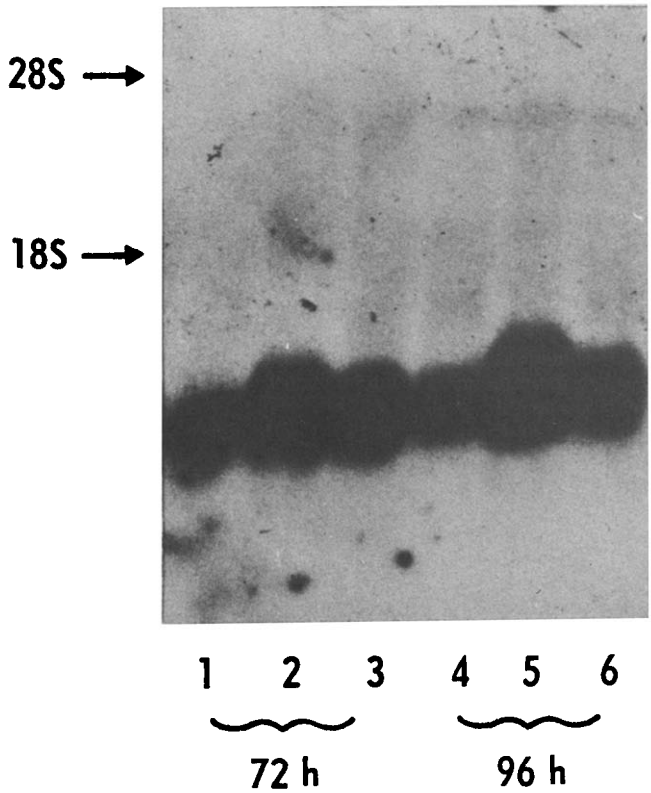

Fig. 2. Northern blot analysis of ε globin mRNA levels from uninduced (lanes 1 and 4), hemin induced (lanes 2 and 5) and temozolomide treated (lanes 3 and 6) K562 cells. The filter was hybridized with the 0.7 Kb Bam HI fragment of the ε globin gene.

fetal bovine serum (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stock cultures were maintained in exponential growth at a density between 0.2×10^6 and 0.8×10^6 cells/ml.

Cells were treated with 100 μM of temozolomide, 100 and 200 μM of ethazolastone or 1 μM of 5-aza-2′-deoxycytidine for 72 hr. At this time the percentage of differentiated cells was evaluated by benzidine staining as previously described [12].

Analysis of methylation pattern. High molecular weight DNA was extracted from treated (as indicated above) and untreated K562 cells by standard techniques. Total DNA (10 μg) was digested overnight as 37° with 10 U/μg of the methylation-sensitive restriction endonuclease MspI, HpaII and HhaI (Bethesda Research Laboratories). The completeness of digestion was assessed by comparing the results obtained with increasing amounts of enzymes. Methylation was studied using HpaII and MspI digesting the 5′-CCGG-3′ sequence (but only MspI can cut when the internal cytosine of this sequence is methylated) and HhaI, digesting unmethylated GCGC sequences [13, 14]. The resulting DNA fragments were separated by DNA electrophoresis through 0.9% agarose gel in a Tris acetate–EDTA buffer (pH 8), denatured with NaOH, then transferred to Gene Screen Plus membrane (Dupont Co. N.E.N. Products, Boston, MA) according to the method of Southern [15]. The membrane was prehybridized for 6 hr at 42° in a solution containing 50% formamide [16]. Salmon sperm DNA (final concentration 100 μg/ml) and denatured DNA probes (4×10^5 dpm/ml) labeled with [α-^{32}P]dCTP (Amersham, U.K.) were added and incubation continued overnight. The membrane was then washed twice in 2 × SSC (0.3 M NaCl–0.03 M sodium citrate) at room temperature for 10 min, twice in 2 × SSC–1% SDS at 65° for 15 min, and finally in 0.1 × SSC at room temperature for 15 min. To reveal the hybridized fragments the membrane was then exposed to Kodak XAR-5 film with Dupont Lightning Plus intensifying screens at −70° for three or four days.

RNA analysis. We used a recently reported procedure to isolate total RNA from whole cells [17]. Briefly: 1 ml of suspended K562 cells (5×10^5) were pelleted by centrifugation, washed and resuspended in 10 μl of cold PBS containing bentonite. Three volumes of formaldehyde-SDS buffer [17] preheated to 95° were added to the cell suspension and heated at 65° for 10–15 min. The samples were centrifuged for 2 min, and the resulting supernatant containing the RNA species was loaded on formaldehyde-SDS 1% agarose gel. Electrophoresis, conventional blotting and hybridization techniques were then used to indicate the RNA species [18].

Alkaline Elution Assay. DNA Alkaline-Labile Sites (ALS) were determined by alkaline elution techniques previously described in detail [19]. Cells were labeled for 24 hr using a medium supplemented with 0.05 μCi/ml ^{3}H-thymidine (specific activity

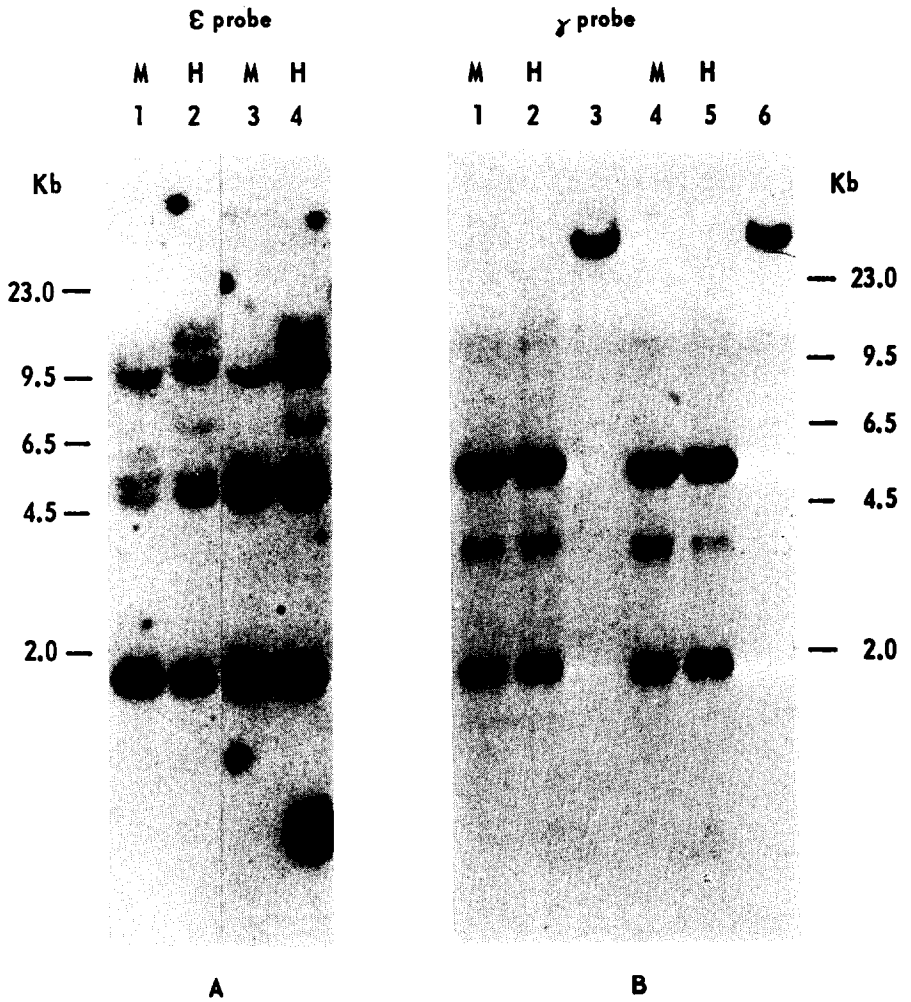

Fig. 3. Pattern of DNA methylation of ε (part A) and γ (part B) globin genes. DNA from untreated (lanes 1, 2) or temozolomide treated (lanes 3 and 4 part A; lanes 4 and 5 part B) K562 cells were digested with MspI (M) or HpaII (H) and hybridized with the 0.7 Kb Bam HI fragment of the ε globin gene (part A) and with the 3.3 Kb HindIII fragment of the Aγ globin gene (part B). Lanes 3 and 6 (part B) correspond to undigested DNA from untreated and from temozolomide treated K562 cells.

20 Ci/mmol; Amersham, U.K.) and 10^{-6} M unlabeled thymidine. Postlabeling 18–24 hr chasing in medium without ^{3}H-thymidine was done before drug treatment. Some standard controls were irradiated with 450 rads. After 1 hr temozolomide or ethazolastone treatment, the cells ($5–10 \times 10^5$) were washed with cold PBS and layered on polycarbonate filters, 0.8 μm pore size and 25 mm diameter (Nucleopore Corp, Pleasanton, CA). Cells were then lysed with a solution containing 2% SDS, 0.02 M Na_2 EDTA, 0.1 M glycine, pH 10.0 (lysis solution), which was allowed to flow through the filter by gravity. The outlet of the filter holders was connected to the pumping system and 2 ml proteinase K, 0.5 mg/ml (EM Laboratories, Darmstadt, West Germany) dissolved in the lysis solution were added to a reservoir over the polycarbonate filters and cycled through the filters for approximately 1 hr at a rate of 0.35 ml/min.

DNA was eluted from the filters by pumping 0.02 M EDTA solution adjusted to pH 12.6 with tetrapropylammonium hydroxide (Fluka, West Germany) containing 0.1% SDS through the filters at approximately 2 ml/hr. Three-hour fractions were collected, and fractions and filters were processed as described previously [19].

Flow cytometry. K562 cells were treated with temozolomide (100 μM), ethazolastone (100 and 200 μM) and 5-aza-2′-deoxycytidine (1 μM). The effects on cell cycle phase distribution were evaluated after 24, 48 and 72 hr recovery time. K562 cells were centrifuged and directly stained with 2 ml of propidium iodide (Calciochem Behring) in 0.1% sodium citrate and 25 μl RNAse 0.5 μg/ml in water (Calciochem Behring) at room temperature for 30 min.

RESULTS

The number of benzidine positive K562 cells increased from 5 to 30% and 27% after 72 hr from temozolomide or 5-aza-2′-deoxycytidine treatment

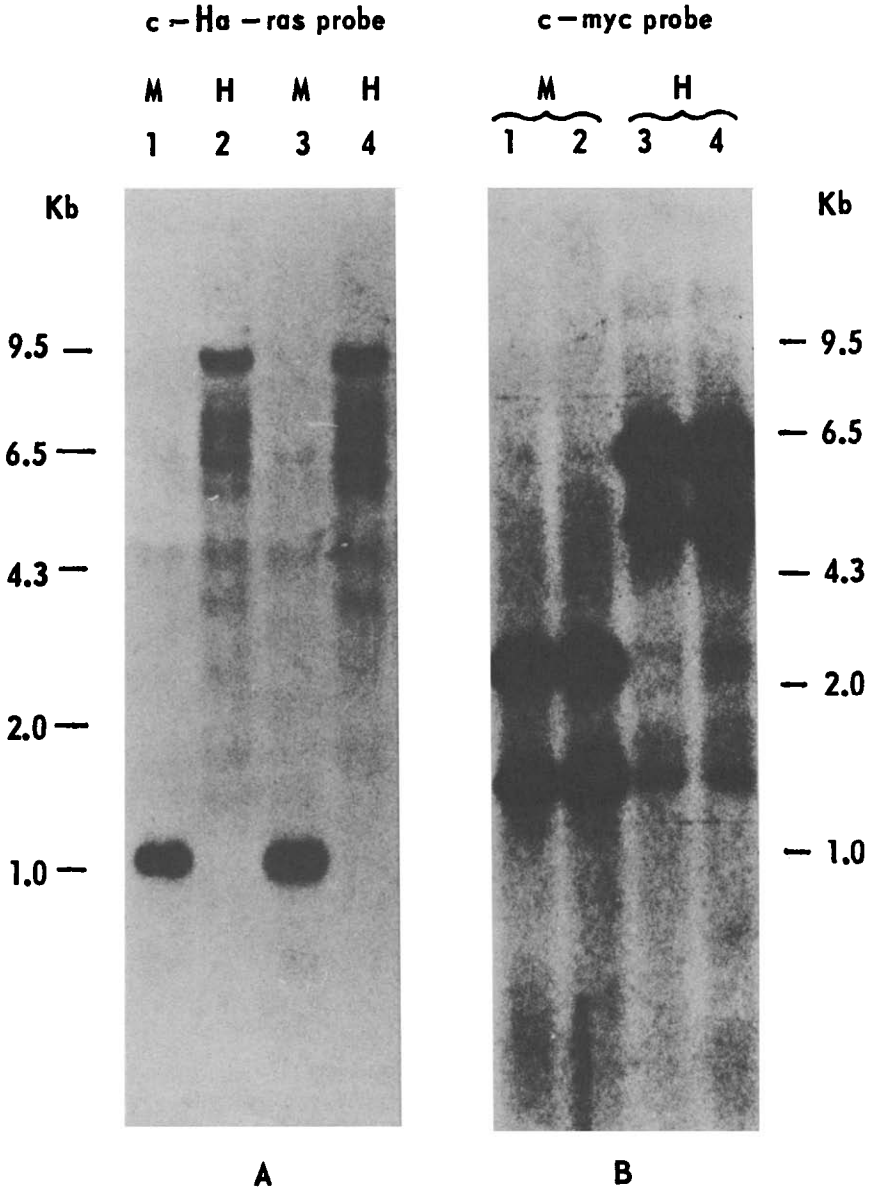

Fig. 4. DNA methylation pattern of c-Ha-ras and c-myc proto-oncogenes. DNAs from untreated (lanes 1 and 2 part A; lanes 1 and 3 part B) and temozolomide treated (lanes 3 and 4 part A; lanes 2 and 4 part B) K562 cells were digested with MspI (M) and HpaII (H) and hybridized with the 6.5 Kb Bam HI fragment of the c-Ha-ras (part A) and with the 1.4 Kb ClaI-EcoRI fragment (third exon) of c-myc oncogenes (part B).

respectively. No increase of benzidine positive cells was found after ethazolastone. Expression of the ε globin gene was increased after temozolomide exposure, in line with the induction of differentiation (Fig. 2). Hybridization of mRNA with the 0.7 Kb Bam HI 5′ fragment of the ε globin gene detected a considerably higher mRNA level in the K562 cells treated with temozolomide than the untreated cells. To determine whether this rise in globin mRNA was related to the presumed inhibition of gene methylation we analysed the methylation state around the ε and γ globin genes. Figure 3 reports the Southern blot analysis of DNA obtained from untreated K562 (lanes 1 and 2 part A and B) and from temozolomide treated K562 cells (lanes 3 and 4 part A and 4,5 part B). DNA was digested with MspI, HpaII and HhaI (not shown) restriction endonucleases, processed as described in Methods, and hybridized with the ε probe previously described (Fig. 3, part A) and with the 3.3 Kb HindIII fragment of the Aγ globin gene (Fig. 3, part B). In uninduced K562 cells ε and γ globin genes were largely hypomethylated and their state of methylation was not altered by temozolomide. We then extended our studies to two other genes known to contain many methylated sites, the ras and myc proto-oncogenes [20–22]. Even in these

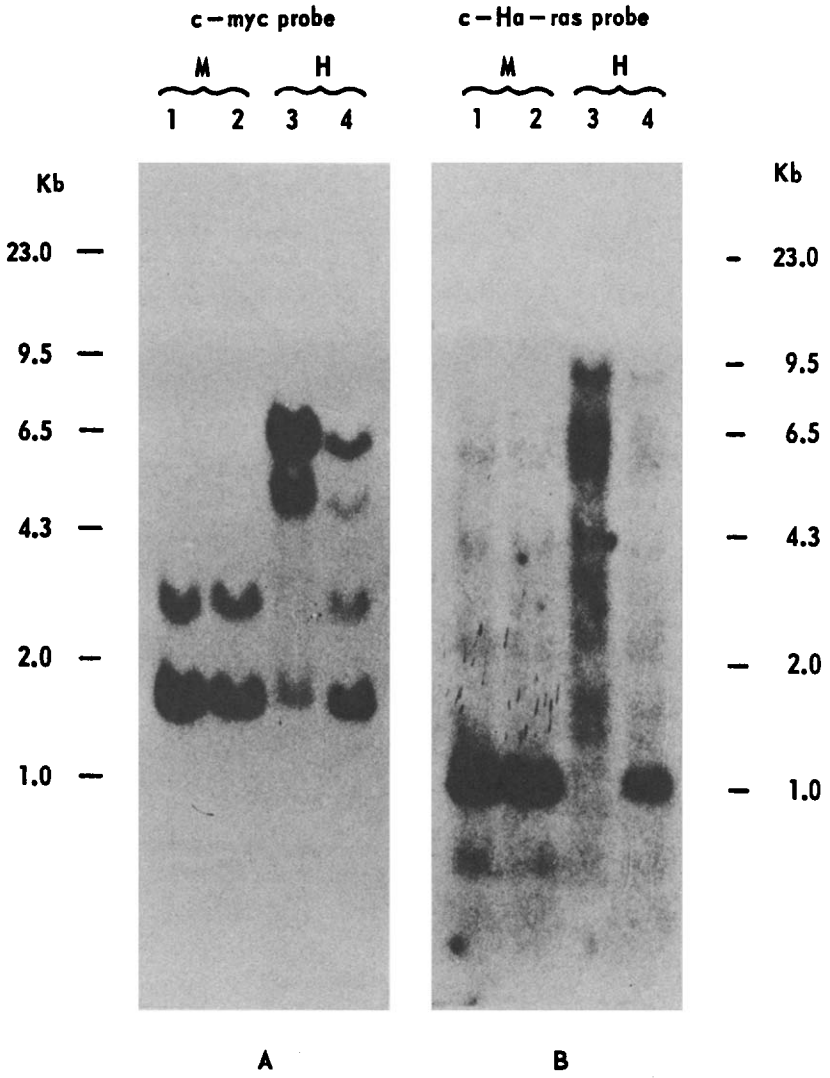

Fig. 5. DNA methylation pattern of c-myc (part A) and c-Ha-ras (part B) proto-oncogenes. DNAs from untreated (lanes 1 and 3) and 5-aza-2′-deoxycytidine treated (lanes 2 and 4) K562 cells were digested with MspI (M) and HpaII (H) and hybridized using the probes described in Fig. 4.

cases, there were no differences in the pattern of DNA methylation around the two oncogenes between temozolomide treated and untreated K562 cells (Fig. 4). Neither was any difference found in the status of methylation of the GCGC sequences as assessed by HhaI (data not shown). In contrast to temozolomide 5-aza-2′-deoxycytidine caused evident hypomethylation around the third exon of the c-myc proto-oncogene (Fig. 5, part A) and to a lesser extent in the c-HA-ras proto-oncogene (part B). In fact, low molecular weight bands were present from 5-aza-2′-deoxycytidine treated K562 cells DNA (lane 4) but not in untreated cell DNA (lane 3) digested with HpaII.

The alkaline elution method showed temozolomide caused dose-related DNA breakage in K562 cells (Fig. 6). The elution profiles were not linear, but convex upward, indicating that the drug induces DNA alkali-labile sites. Ethazolastone caused no detectable DNA damage at the concentration of 100 μM and very low DNA breaks at 200 μM. In K562 cells temozolomide induced an arrest in G2-M phase of the cell cycle; the maximum effect was found 72 hr after treatment (Fig. 7). Ethazolastone did not cause a significant cell cycle perturbation whereas 5-aza-2′deoxycytidine caused a G2-M phase blockade too.

DISCUSSION

In a previous report Tisdale [8] showed that the anticancer agent temozolomide, but not its inactive ethyl analogue ethazolastone, induced differentiation of the human erythroleukemia K562 cell line. He found that temozolomide was able to induce hypomethylation of total genomic DNA and thus proposed that the increased expression of genes associated with differentiation (e.g. globin genes)

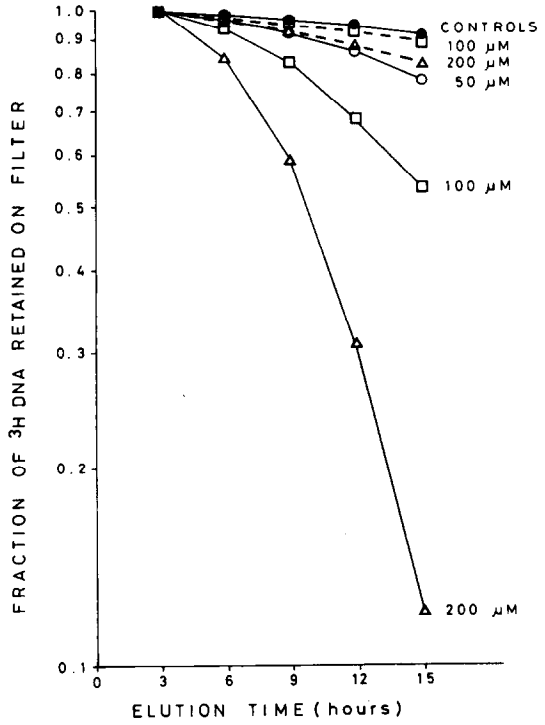

Fig. 6. Alkaline elution profile of control K562 cells (●—●) and cells treated with 50 μM (○—○), 100 μM (□—□) or 200 μM (△—△) temozolomide and 100 μM (□--□) or 200 μM (△--△) ethazolastone for 1 hr. After treatment cells were processed immediately for analysis of DNA damage by the alkaline elution method (at pH 12.6).

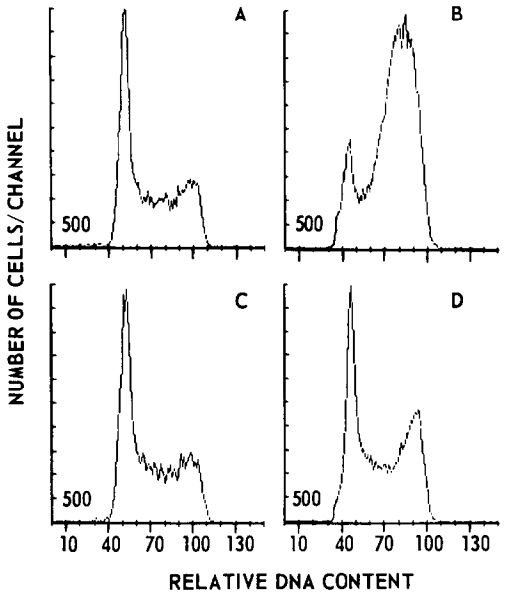

Fig. 7. Flow cytometry analysis of cell cycle phase distribution in K562 control cells (A), temozolomide (100 μM for 1 hr) treated cells (B), ethazolastone (200 μM for 1 hr) treated cells (C) and 5-aza-2'deoxycytidine (1 μM for 24 hr) treated cells (D). Analysis was done 72 hr after drug treatment for B and C, and 48 hr after drug treatment for D.

might be due to drug-induced hypomethylation. The results of our study confirm that temozolomide induces differentiation of K562 cells, but the rise in ε and γ globin genes' expression does not appear to be related to changes in their methylation pattern. Since in K562 cells most methylation sites in globin genes were already unmethylated before drug treatment it could be difficult to assess any hypomethylating effect of temozolomide. We therefore selected two other genes, c-myc and cHa-ras proto-oncogenes which presented a high frequency of methylated CCGG and GCGC sequences and in which even a weak hypomethylating effect of temozolomide could be detected. The drug caused no detectable hypomethylation in either case. In the same experiments 5-aza-2'-deoxycytidine, used as a positive control, caused hypomethylation of both c-myc and c-Ha-ras proto-oncogenes. Therefore we can exclude that the experimental conditions were inadequate for evaluation of the hypomethylating effect of temozolomide.

A possible reason for the difference between Tisdale's results and ours is that he determined the total number of methylcytosines present in the genome, whereas we analyzed only the methylation of cytosine in some DNA sequences (CCGG and GCGC) susceptible to the restriction enzyme used. However, Puccetti *et al.* [23] found no hypomethylating effects of methyltriazenes in rodent leukemias when they determined the percentage of total methylcytosines, and concluded that the ability of these drugs to increase tumour cell immunogenicity was not related to DNA hypomethylation.

In K562 cells, as recently reported in other leukemia cell lines [24], temozolomide causes the formation of DNA alkali-labile sites, presumably due to apurinic sites corresponding with N^7-methyl guanine. In addition it is likely that temozolomide induces alkylation of O^6-guanine, a lesion generally associated with the mutagenicity of these compounds [25, 26] and more recently also with their cytotoxicity [24]. Recently it has been found that other agents known to cause DNA damage such as doxorubicin and cisplatin can induce differentiation of K562 cells [27–29]. Since these drugs cause different types and extent of DNA damage it appears unlikely that a specific DNA lesion triggers this phenomenon. Induction of differentiation may be related to the cell cycle arrest, which is invariably produced by all compounds which cause DNA damage [30]. This hypothesis is also suggested by the finding that ethazolastone which did not induce differentiation did not cause significant DNA damage and G2-M phase arrest.

It is to note that also 5-aza-2'-deoxycytidine, which induces differentiation of different cell types [9, 10] causes alkali-labile sites in DNA [31] and an arrest in G2 phase, thus suggesting that the drug induced differentiation might be at least in part related to DNA damage. In conclusion the present study provides further evidence that K562 cells can be induced to differentiate by methyltriazenes; it is suggested that the drug-induced differentiation is not mediate by gene hypomethylation, but is more likely a result

of DNA damage and cell cycle arrest produced by this methylating agent.

Acknowledgements—This work was supported in part by a research grant from the Consiglio Nazionale delle Ricerche, Rome (Contract No. 87.01526.44) and by the Italian Association for Cancer Research.

REFERENCES

1. Luce JK, Thurman WG, Isaacs BL and Talley RW, Clinical trials with the antitumour agent 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388). *Cancer Chemother Rep* **54**: 119–123, 1970.
2. Kingra GS, Comis R, Olson KB and Horton J, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) in the treatment of malignant tumors other than melanoma. *Cancer Chemother Rep* **55**: 281–283, 1971.
3. Comis RL, DTIC (NSC-45388) in malignant melanoma: a perspective. *Cancer Treat Rep* **60**: 165–176, 1976.
4. Gerulath AH and Loo TL, Mechanism of action of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide in mammalian cells in culture. *Biochem Pharmacol* **21**: 2335–2343, 1972.
5. Mizuno NS, Decker RW and Zakis B, Effects of 5-(3-methyl-1-triazeno) imidazole-4-carboxamide (NSC-407347), an alkylating agent derived from 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388). *Biochem Pharmacol* **24**: 615–619, 1975.
6. Stevens MFG, Hickman JA, Langdon SP, Chubb D, Vickers L, Stone R, Baig G, Goddard C, Gibson NW, Slack JA, Newton C, Lunt E, Fizames C and Lavelle F, Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (CCRG 81045; M & B 39831), a novel drug with potential as an alternative to dacarbazine. *Cancer Res* **47**: 5846–5852, 1987.
7. Bonmassar E, Bonmassar A, Vadlamudi S and Goldin A, Immunological alteration of leukemic cells *in vivo* after treatment with an anti-tumor drug. *Proc Natl Acad Sci USA* **66**: 1089–1095, 1970.
8. Tisdale MJ, Antitumour imidazotetrazines—X. Effect of 8-carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4-(3H)-one (CCRG 81045; M& B 39831; NSC 362856) on DNA methylation during induction of haemoglobin synthesis in human leukemia cell line K652. *Biochem Pharmacol* **35**: 311–316, 1986.
9. Jones PA and Taylor SM, Cellular differentiation, cytidine analogs and DNA methylation. *Cell* **20**: 85–93, 1980.
10. Creusot F, Acs G and Christman JK, Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2′-deoxycytidine. *J Biol Chem* **257**: 2041–2048, 1982.
11. Lozzio CB and Lozzio BB, Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* **45**: 321–334, 1975.
12. Wanda PE, Lee LT and Howe C, A spectrophotometric method for measuring hemoglobin in erythroleukemic cells (K562). *J Histochem Cytochem* **29**: 1442–1444, 1981.
13. Waalwijk C and Flavell RA, MspI, an isoschizomer of hpaII which cleaves both unmethylated and methylated hpaII sites. *Nucleic Acids Res* **5**: 3231–3236, 1978.
14. Bird AP and Southern EM, Use of restriction enzymes to study eukaryotic DNA methylation—I. The methylation pattern in ribosomal DNA from *Xenopus laevis*. *J Mol Biol* **118**: 27–47, 1978.
15. Southern EM, Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**: 503–517, 1975.
16. Wahl GM, Stern M and Stark GR, Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc Natl Acad Sci USA* **76**: 3683–3687, 1979.
17. Hatch CL and Bonner WM, Direct analysis of RNA in whole cell and cytoplasmic extracts by gel electrophoresis. *Anal Biochem* **162**: 283–290, 1987.
18. Maniatis T, Fritsch EF and Sambrook J, *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratories, Cold Spring Harbor, 1982.
19. Kohn, KW, Ewig RAG, Erickson LC and Zwelling LA, Measurement of strand breaks and cross-links by alkaline elution. In: *DNA Repair. A Laboratory Manual of Research Producers*, Vol. 1 pt. B (Eds. Friedberg EC and Hanawalt PC), pp. 379–401. Marcel Dekker, New York, 1981.
20. Cheah MSC, Wallace CD and Hoffman RM, Hypomethylation of DNA in human cancer cells: A site-specific change in the c-myc oncogene. *JNCI* **73**: 1057–1065, 1984.
21. Borrello MG, Pierotti MA, Bongarzone I, Donghi R, Mondellini P and Della Porta G, DNA methylation affecting the transforming activity of the human Ha-ras oncogene. *Cancer Res* **47**: 75–79, 1987.
22. Chandler LA, Ghazi H, Jones PA, Boukamp P and Fusenig NE, Allele-specific methylation of the human c-Ha-ras-1 gene. *Cell* **50**: 711–717, 1987.
23. Puccetti P, Fuschiotti P, Dominici P, Borri-Voltattorni C, Romani L and Fioretti MC, DNA methylating activity in murine lymphoma cells xenogenized by triazene derivatives. *Int J Cancer* **39**: 769–773, 1987.
24. Catapano CV, Broggini M, Erba E, Ponti M, Mariani L, Citti L and D'Incalci M, *In vitro* and *in vivo* methazolastone-induced DNA damage and repair in L-1210 leukemia sensitive and resistant to chloroethylnitrosoureas. *Cancer Res* **47**: 4884–4889, 1987.
25. Eadie JS, Conrad M, Toorchen D and Topal MD, Mechanism of mutagenesis by O^6-methylguanine. *Nature* **308**: 201–203, 1984.
26. Doniger J, Day RS and DiPaolo JA, Quantitative assessment of the role of O^6-methylguanine in the initiation of carcinogenesis by methylating agents. *Proc Natl Acad Sci USA* **82**: 421–425, 1985.
27. Jeannesson P, Ginot L, Manfait M and Jardillier J-C, Induction of hemoglobin synthesis in human leukemic K562 cells by adriamycin. *Anticancer Res* **4**: 47–52, 1984.
28. Tonini GP, Parodi MT, Bologna R, Persici P and Cornaglia-Ferraris P, Cisplatin induces modulation of transferring receptor during cellular differentiation *in vitro*. *Cancer Chemother Pharmacol* **18**: 92, 1986.
29. Tonini GP, Radzioch D, Gronberg A, Clayton M, Blasi E, Benetton G and Varesio L, Erythroid differentiation and modulation of c-myc expression induced by antineoplastic drugs in the human leukemic cell line K562. *Cancer Res.* **47**: 4544–4547,
30. Tobey RA, Different drugs arrest cells at a number of distinct stages in G_2. *Nature* **254**: 245–247, 1975.
31. D'Incalci M, Covey JM, Zaharko DS and Kohn KW, DNA alkali-labile sites by incorporation of 5-aza-2′-deoxycytidine into DNA of mouse leukemia L1210 cells. *Cancer Res* **45**: 3197–3202, 1985.