Biochemical Pharmacology, Vol. 34, No. 4, pp. 515–520, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

POTENTIATION OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA (BCNU)-INDUCED CYTOTOXICITY IN 9L CELLS BY PRETREATMENT WITH 6-THIOGUANINE

WILLIAM J. BODELL,*† WILLIAM F. MORGAN,‡ JYTTE RASMUSSEN,* MARY E. WILLIAMS* and DENNIS F. DEEN*§

*Brain Tumor Research Center of the Department of Neurological Surgery; ‡Laboratory of Radiobiology and Environmental Health; and §Department of Radiation Oncology, School of Medicine, University of California, San Francisco, CA 94143, U.S.A.

(*Received* 27 *January* 1984; *accepted* 4 *June* 1984)

Abstract—9L Rat brain tumor cells were treated with 0.2 μM 6-thioguanine for 48 hr, which produced a 40% cell kill, a small (15%) inhibition of cell growth, and an accumulation of cells in S-phase. Maximum incorporation of [^{14}C]6-thioguanine into cellular DNA occurred after 24 hr of incubation; 70% of the label was incorporated into DNA as 6-thio-2′-deoxyguanosine. Pretreatment of 9L cells for 48 hr with 0.2 μM 6-thioguanine potentiated the cytotoxicity of 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) by 50% with a dose enhancement ratio of 1.5, and caused a 30% increase in the number of BCNU-induced sister chromatid exchanges (SCEs) and a 50% increase in DNA crosslinks formed, compared to treatment with BCNU alone. Used as a single agent, 6-thioguanine induced a significant number of SCEs. Results suggest that these effects may be related to the increased formation of DNA crosslinks, possibly as the result of the formation of S^6-(2-chloroethyl)-6-thioguanine in cellular DNA.

CENUs‖ are chemotherapeutic agents used in the treatment of brain tumors and a variety of other systemic tumors [1,2; for review of this topic, see Ref. 3]. Under physiological conditions, CENUs are hydrolyzed to reactive species that alkylate cellular DNA, RNA, and proteins [4, 5]. Some of the alkylation products [6] of CENUs may form DNA interstrand crosslinks in subsequent reactions [7, 8]. Currently it is believed that the formation of DNA interstrand crosslinks is responsible for the cytotoxic effects of CENUs [9].

Although the chemical structure of the crosslinks formed in cells treated with CENUs has not been determined definitely, there is evidence that the formation of O^6-(2-chloroethyl)guanine is one of the more important initial alkylation events that leads to the subsequent formation of DNA crosslinks. This hypothesis is supported by two observations: cells that can excise O^6-methylguanine from alkylated DNA are resistant to the cytotoxic effects of CENUs [9], and O^6-(2-fluoroethyl)guanine has been isolated from DNA treated with *N*-(2-fluoroethyl)-*N*′-cyclohexyl-*N*-nitrosourea [10].

Intracellularly, 6-TG is converted to 6-TG monophosphate by hypoxanthine-guanine phosphoribosyl transferase [11]; 6-TG monophosphate is then metabolized to the appropriate triphosphate and is incorporated into either RNA or DNA [12]. Because the thiol group of 6-TG is more nucleophilic than the O^6-group of guanine, incorporation of 6-TG into cellular DNA may increase the susceptibility of DNA to alkylation or increase the formation of adducts, particularly S^6-(2-chloroethyl)-6-thioguanine, that may form DNA crosslinks in subsequent reactions. Either of these mechanisms may increase the cytotoxic effects of CENUs. We report here results of experiments conducted to determine whether pretreatment of 9L cells with 6-TG would sensitize them to the effects of subsequently-administered BCNU.

MATERIALS AND METHODS

Cell culture. 9L Cells (0.5×10^6) were seeded into 75 cm^2 tissue culture flasks and grown in 15 ml of Eagle's minimum essential medium supplemented with nonessential amino acids, 10% newborn calf serum, and gentamicin (0.05 mg/ml). Before treatment, cells were incubated for approximately 24 hr at 37° in a humidified 5% CO_2:95% air atmosphere to establish early log phase growth.

Treatment with 6-TG alone. Various volumes of a freshly prepared stock solution of 100 μM 6-TG (Sigma) dissolved in 10 μM NaOH were added to cell cultures. After 48 hr of incubation, medium containing 6-TG was removed, and cells were washed with MEM, trypsinized, and plated for the cell survival assay.

Sequential treatment with 6-TG and BCNU. Cells were treated with either 0.2 μM 6-TG or solvent (control). After 48 hr of incubation, both 6-TG-pretreated and control cells were treated with BCNU for 1 hr at 37°, after which the drug-containing medium was removed, and cells were rinsed with

† To whom correspondence should be addressed.

‖ Abbreviations: CENUs, chloroethylnitrosoureas; 6-TG, 6-thioguanine; SCE, sister chromatid exchange; BCNU, 1,3-bis (2-chloroethyl)-1-nitrosourea; HPLC, high performance liquid chromatography; TdR, thymidine; MEM, minimum essential medium; and FCM, flow cytometry.

MEM, trypsinized, and plated for the colony forming efficiency assay.

Cell survival assay. The cell survival assay has been described [13]. Briefly, cells were counted, diluted, and plated into 60 mm petri dishes containing 5×10^4 irradiated (40 Gy) 9L feeder cells. After incubation for 12–14 days, colonies were fixed with methanol/acetic acid, stained with crystal violet, and counted. Percent survival was calculated as the percentage of cells, relative to untreated controls, that formed colonies.

FCM analysis. Cells were treated with either 0.2 μM 6-TG or solvent (control) as described above. After 48 hr of incubation, cells were trypsinized and fixed with 70% ethanol. FCM analysis was performed as described [14].

SCE assay. Cells (0.5×10^6) were seeded into a 75 cm^2 tissue culture flask; after cells had achieved exponential growth (within 24 hr), they were treated with 0.2 μM 6-TG for 48 hr. After treatment, growth medium containing 6-TG was removed and replaced with fresh medium. Cells were then treated with various concentrations of BCNU dissolved in 100% ethanol. After 1 hr of treatment, medium was removed and replaced with medium containing bromodeoxyuridine (10 μM) and incubated for two replication cycles (approximately 28 hr). Mitotic cells were accumulated by treatment with colcemid (0.04 μg/ml) for the final 2 hr. Flasks were shaken to dislodge the mitotic cells, medium was poured off, and mitotic cells were collected by centrifugation (1000 rpm for 5 min). The pellet was treated with 2.0 ml of 0.05 M KCl for 8–10 min, fixed twice with freshly prepared glacial acetic acid–methanol (1:3), and metaphase chromosomes were spread on glass microscope slides. The method of Perry and Wolff [15] was used for differential staining of the sister chromatids. For each experiment, the frequency of SCEs was determined by counting 25 metaphase cells.

Incorporation of 6-TG into DNA. Cells (1×10^6) were plated into six to twelve (100 mm) tissue culture dishes; 24 hr later, [^{14}C]6-TG (sp. act. 56 mCi/mmole, Moravek Biochemicals) was added to cultures to give a final concentration of 0.2 μM. Cells were incubated for various periods and then trypsinized and collected by centrifugation. Pelleted cells were frozen in liquid nitrogen and stored at −40° until the DNA was isolated.

DNA was isolated and purified from the cellular pellet using the method of Bodell and Banerjee [16]. The DNA was repeatedly precipitated with sodium acetate and 100% ethanol until a constant specific activity was obtained. DNA (1 to 1.5 mg) was precipitated with sodium acetate and ethanol. The DNA precipitate was dissolved by stirring overnight in 250 μl of water.

The DNA was digested enzymatically at 37° overnight to deoxyribonucleosides using a published procedure [17] that was modified by omitting the acid phosphatase and heat-treating alkaline phosphatase to remove adenosine deaminase [18]. The digest was centrifuged through a centrifugal filter (0.2 micron pore size, Rainin Instruments) at 2000 rpm for 5 min. Fifty microliters of 0.1 M $NH_4H_2PO_4$ (pH 5.1) was added to the filtrate, and the pH was adjusted to 7 with NH_4OH. The final volume of the hydrolysate was adjusted to 510 μl with water; 10 μl of the sample was used to determine total radioactivity and 500 μl was used for HPLC analysis.

HPLC was performed using a Chromatronix 3500 pump coupled to a Rheodyne 7120 sample injector valve with a 500-μl sample loop. A 5 micron C-18 reverse phase column (Alltech 605-RP) was used for the separation. Ultraviolet absorbance at 280 nm was monitored with a Perkin Elmer model 55 detector. The mobile phase was 0.01 M $NH_4H_2PO_4$, pH 5.1, with 5% methanol. The system was operated at a flow rate of 1.2 ml/min. The average retention times were: deoxycytidine, 8.8 min; deoxyguanosine, 24.8 min; TdR, 26.4 min; 6-thio-2′-deoxyguanosine, 28 min; and deoxyadenosine, 68 min.

Fractions (1.2 ml) were collected and mixed with 10 ml of Aquasol. The level of radioactivity was counted on a Beckman LS-250 liquid scintillation counter. The counting efficiency for ^{14}C-radioactivity was 88%, and the average overall recovery of the sample was 92%.

Alkaline elution assay. Cells (0.5×10^6) were seeded into 100 mm dishes. The following day they were incubated with 0.2 μM 6-TG and 0.01 μCi/ml [^{14}C]TdR (60 mCi/mmole) for 48 hr. Control cells were incubated with 0.01 μCi/ml of [^{14}C]TdR only, and cells used as internal standards were incubated with 0.1 μCi/ml of [^{3}H]TdR (82.7 Ci/mmole). Before treatment, medium containing labeled compound was removed and replaced with fresh medium. The ^{14}C-prelabeled cells were treated with various concentrations of BCNU for 1 hr at 37°. After the treatment period, medium was replaced with fresh medium, and cells were incubated for an additional 6 hr, after which the medium was replaced with cold Hanks' balanced salt solution. The ^{3}H-labeled cells that served as an internal control were irradiated with 300 rads, and the ^{14}C-labeled cells were irradiated with 600 rads of X-rays. X-irradiation was performed with a General Electric Maxitron 300 (300 kVP; 20 mA: nominal half-value layer 2.0 mm Cu). A modification of the procedure described by Kohn *et al.* [19] was used to determine the number of DNA crosslinks formed. Approximately 5×10^5 treated ^{14}C-labeled cells and a similar number of control ^{3}H-labeled cells were filtered onto 25 mm diameter, 2 micron pore size polyvinyl chloride filters (Millipore Corp., Bedford, MA) and washed twice with cold calcium- and magnesium-free phosphate-buffered saline (5 ml). Cells were lysed on the filter with 5 ml of Sarkosyl–NaCl–EDTA lysis solution (pH 10.0) containing 0.5 mg/ml proteinase K. The lysate was digested with proteinase K for 1 hr, and then the lysis solution was allowed to flow through by gravity. Filters were then rinsed with 4 ml of 0.02 M disodium EDTA (pH 10.0). No suction was applied before or after lysis. Elution was carried out in the dark with a solution of tetrahydryl-EDTA plus tetrapropylammonium hydroxide (Eastman Kodak, Rochester, NY), 2% in water (pH 12.2), at a flow rate of 2 ml/hr. Fractions were collected at 1-hr intervals for 13–15 hr. Samples were mixed with 4 ml of Aquasol (New England Nuclear, Boston, MA) for scintillation counting. Radioactivity remaining on the filter was determined by treating filters with 0.4 ml of 1 N HCl at 70° for 1 hr, followed by 2.5 ml of

0.4 N NaOH at room temperature for 30 min and then 6 ml of Aquasol. The remaining radioactivity in the column and pump tubing was recovered by passing 2.5 ml of 0.4 N NaOH through the funnel into the tube. The solution was left in the tubing for 1 hr and then collected; 0.4 ml of 1 N HCl was added and the neutralized solution was mixed with 6 ml of Aquasol. Radioactivity was counted either on a Packard Tri-Carb or a Beckman LS-330 liquid scintillation spectrometer. Results were calculated as the fraction of [^{14}C]DNA and [^{3}H]DNA retained on the filter at each fraction.

The crosslink index was calculated using the formula of Ewig and Kohn [7]:

$$[(1 - R_0)/(1 - R_1)]^{\frac{1}{2}} - 1$$

where R_0 and R_1 are the relative retention for untreated and BCNU-treated cells respectively. Relative retention was defined as the fraction of the [^{14}C]DNA remaining on the filter when 50% of the [^{3}H]DNA remained on the filter.

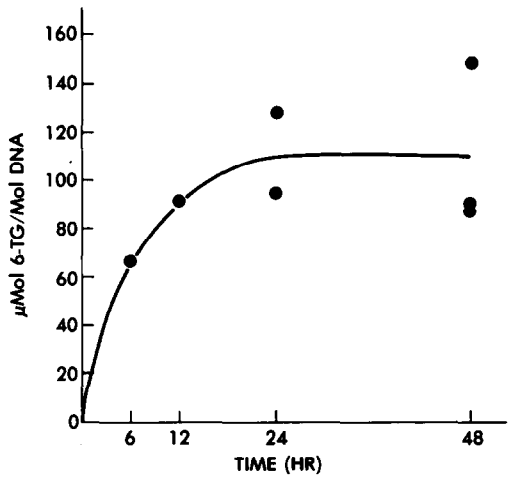

Fig. 2. Plot of the incorporation of [^{14}C]6-TG into 9L cellular DNA as a function of incubation time.

RESULTS

Previous studies from this laboratory have shown that cells in G_1 and G_2/M phases of the cell cycle are more sensitive to the cytotoxic effects of BCNU than are cells in other phases of the cycle [20]. For the studies reported here, it was necessary to determine a concentration of 6-TG that was minimally cytotoxic and did not significantly perturb the cell cycle when used as a single agent. Low concentrations of 6-TG were found to be very cytotoxic to 9L cells (Fig. 1); treatment of 9L cells with 0.6 μM 6-TG produced a cell kill of more than 1 log. Therefore, we chose to perform experiments with 0.2 μM 6-TG, a concentration at which approximately 60% of treated cells survived and cell growth was inhibited by approximately 15% (Fig. 1).

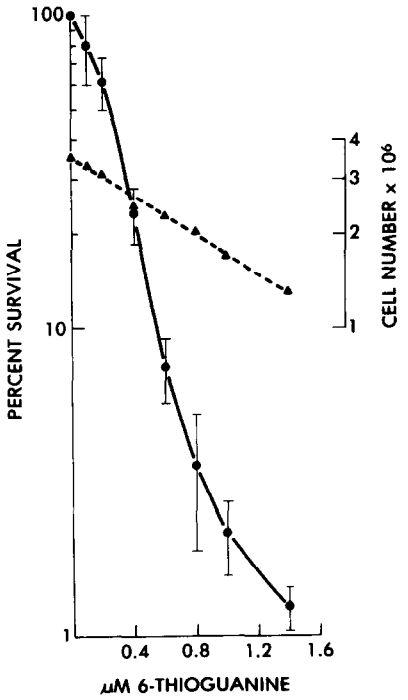

Fig. 1. Survival plots (●) and cell numbers (▲) for 9L cells grown for 48 hr with various concentrations of 6-TG. Effects of 6-TG on growth were determined by counting the number of cells in each flask for both control and 6-TG-treated cells.

The effect of a 48-hr treatment with 0.2 μM 6-TG on the distribution of 9L cells in the cell cycle was determined by FCM. Percentages of cells in G_1, S, and G_2/M were 58.2, 36.1, and 5.6, respectively, for control cells, and 41.2, 57.1, and 1.5, respectively, for 6-TG-treated cells. Treatment with 6-TG caused 9L cells to accumulate in S phase.

A rate plot for the incorporation of [^{14}C]6-TG into cellular DNA as a function of incubation time is shown in Fig. 2. Maximum incorporation of 6-TG occurred by 24 hr after treatment was begun. Radioactivity was incorporated into cells primarily as 6-thio-2′-deoxyguanosine (as determined by HPLC analysis). Approximately 70% of the total radioactivity coeluted with unlabeled 6-thio-2′-deoxyguanosine that was added as a marker; the remaining 30% eluted as several smaller peaks, the identities of which were not determined. Similar levels of incorporation of 6-TG into DNA have been reported for other cell lines [21, 22].

Survival plots for BCNU-induced cytotoxicity in untreated 9L cells and in 9L cells pretreated with 0.2 μM 6-TG are shown in Fig. 3. Levels of survival have been normalized to account for the cytotoxic effects of treatment with 6-TG alone. Pretreatment of 9L cells with 6-TG potentiated BCNU cytotoxicity; the dose enhancement ratio at the 10% survival level was 1.5 (average of three experiments).

Plots of the number of SCEs induced by BCNU alone and for cells treated with 0.2 μM 6-TG before treatment with BCNU are shown in Fig. 4. Comparison of the curves shows that approximately 4-fold more SCEs were induced in cells treated with 6-TG before BCNU treatment. Control cells have a frequency of SCEs of 11–13 SCEs/metaphase, while

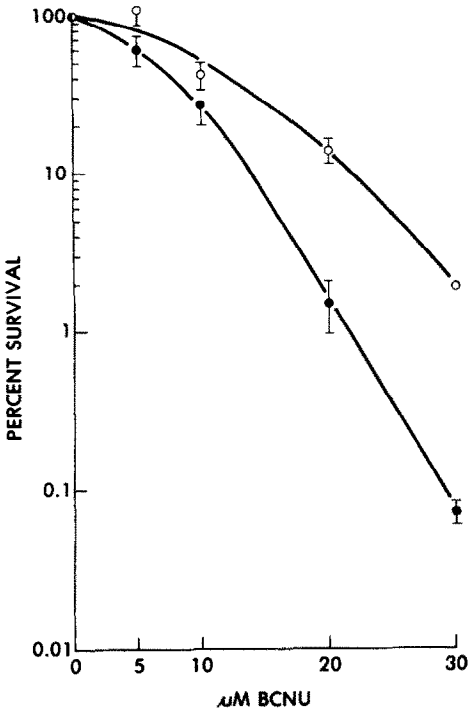

Fig. 3. Survival plots for 9L cells treated with BCNU alone (○) or treated with BCNU after a 48-hr pretreatment with 6-TG (●).

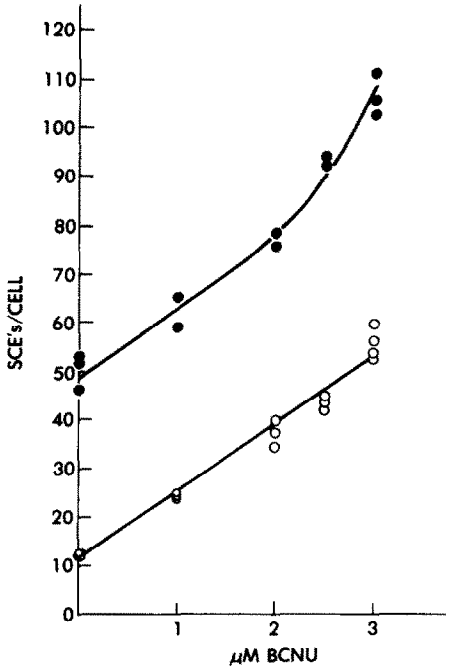

Fig. 4. Total number of SCEs per metaphase in 9L cells treated with BCNU alone (○) or BCNU after pretreatment with 0.2 μM 6-TG (●). Each symbol represents the mean value of SCEs per experiment. For each experiment, SCEs in 25 metaphases were counted.

Table 1. Induction of SCEs*

BCNU concn (μM)	Number of SCEs induced BCNU alone	BCNU + 6-TG
1	12.2 ± 2.0 (75)	13.2 ± 2.8 (50)
2	25.5 ± 3.2 (75)	28.6 ± 5.3 (50)
2.5	31.8 ± 2.5 (75)	41.2 ± 2.7† (50)
3.0	43.0 ± 3.3 (100)	56.5 ± 5.9† (70)

* The number of SCEs induced by BCNU alone was calculated by subtracting the number of background SCEs (11–13) or, for cells pretreated with 6-TG, by subtracting the sum of the background SCEs and the number of SCEs induced by 6-TG pretreatment alone, from the experimental value. Values are means and standard deviations of the mean. The number of metaphases counted for the determination is listed in parentheses.

† Significant at P = 0.001 by Student's *t*-test.

treatment with 6-TG alone increased this frequency to approximately 50 SCEs/metaphase (data not normalized for background). Therefore, 6-TG effectively induces SCEs when used as a single agent.

The background frequency and number of 6-TG-induced SCEs were subtracted to estimate the frequency of BCNU-induced SCEs (Table 1). BCNU treatment gave a linear dose response for the induction of SCEs (Fig. 4). Pretreatment of 9L cells with 6-TG increased the number of SCEs induced by BCNU at the two higher doses (Table 1). The dose–response curve for cells treated with 6-TG and then treated with BCNU is nonlinear, and the dose–response curves for BCNU treatment of both 6-TG pretreated and nonpretreated cells have a greater divergence with increasing BCNU dose. Pretreatment with 6-TG caused a 30% increase in the number of SCEs induced by treatment with 2.5 and 3.0 μM BCNU; the difference in induction of SCEs between control and pretreated cells was significant at both BCNU doses (Table 1).

The crosslinking indices for 9L cells treated with 50 or 100 μM BCNU are listed in Table 2. Pretreatment of 9L cells with 6-TG increased the number of BCNU-induced crosslinks by 50% for both BCNU doses. The amount of potentiation of DNA crosslink formation caused by pretreatment with 6-TG is similar to the amount of potentiation found for cell kill and SCE induction.

DISCUSSION

We have shown that pretreatment of 9L cells with 0.2 μM 6-TG increased the cytotoxic response, the induction of SCEs, and the formation of DNA crosslinks to subsequent BCNU treatment. These results are in agreement with those of Fujimoto and coworkers [23, 24] who found that 6-TG treatment increases the cytotoxicity of 3-((4-amino-2-methyl-5-pyrimidiniyl)methyl) - 1 - (2 - chloroethyl) - 1 - nitrosourea. Maybaum and Mandel [25, 26] have shown that treating cells with 6-TG increases damage to chromatids. The results obtained in this study show that 6-TG effectively induces SCEs in 9L cells. We are currently extending our studies on the induction of SCEs using other thiopurines and thiopyrimidines.

Wotring and Roti Roti [27] found that treatment

Table 2. Number of DNA crosslinks formed*

Treatment	Crosslink index × 10^3
50 μM BCNU	109 ± 15 (5)
50 μM BCNU + 0.2 μM 6-TG	163 ± 29 (5)
100 μM BCNU	214 ± 19 (4)
100 μM BCNU + 0.2 μM 6-TG	335 ± 90 (4)

* Values are the means and standard deviations of the mean. The number of determinations made is given in parentheses.

of L1210 cells with 10 μM 6-TG caused cells to accumulate in the G_2/M phase. FCM analysis showed that 9L cells treated with 0.2 μM 6-TG for 48 hr accumulated in S phase compared to controls. 9L cells in G_1 or G_2/M phases are more sensitive to the cytotoxic effects of BCNU than are cells in S phase [20]. These results suggest that the potentiation of BCNU cytotoxicity caused by pretreatment with 6-TG was not the result of an accumulation of cells in BCNU-sensitive phases of the cell cycle.

The formation of crosslinked bases is probably more important for the cytotoxic effects of BCNU than is the formation of base alkylation products. In cells treated with ethylnitrosourea, a nitrosourea that alkylates but cannot crosslink DNA, cytotoxicity is reduced to 1/100th that caused by BCNU [28]. Erickson *et al.* [9] found that human cells resistant to the cytotoxic effects of CENUs have fewer DNA interstrand crosslinks after treatment with CENUs than cells that are sensitive to these agents. Additional evidence indicates that the number of DNA crosslinks formed in BCNU-treated cells affects cellular survival. Pretreatment of 9L cells with alpha-difluoromethylornithine, a polyamine biosynthesis inhibitor that decreases intracellular levels of polyamines and thereby changes the conformation of DNA and makes it more susceptible to subsequent crosslink formation, increases the number of BCNU-induced SCEs and DNA crosslinks and potentiates the cytotoxic effect of BCNU [29–31].

In 9L cells pretreated with 6-TG, both the number of SCEs induced and the number of crosslinks formed by subsequent treatment with BCNU were increased. Large numbers of SCEs are induced by low doses of BCNU; therefore, treatment with relatively high doses of BCNU produces too many SCEs to count. On the other hand, the alkaline elution assay can detect crosslinks only in cells treated with high doses of BCNU. Because of this, it was necessary to use 30-fold different concentrations of BCNU to measure SCEs and DNA crosslinks. The dose–response curve for SCE induction caused by BCNU in 9L cells is linear, and we assume that the curve remains linear over large dose ranges. Therefore, data for SCE induction and crosslink formation obtained with two very different concentrations of BCNU can be compared. We have suggested that DNA crosslinks induce SCEs at least 45-fold more efficiently than do base alkylation products (W. J. Bodell, T. Aida and J. Rasmussen, manuscript submitted for publication). We believe that the increase in SCEs in 9L cells pretreated with 6-TG reflects the increase in crosslinks formed, and that the SCE assay is a more sensitive measure of the increase in crosslinks than is the alkaline elution assay.

6-TG incorporation into DNA could change the reactivity of DNA. We found that, in alkylation reactions with dimethylsulfate, 6-thio-2′-deoxyguanosine is more reactive than deoxyguanosine, and that the S^6 position is alkylated more frequently than the O^6 position (W. J. Bodell, unpublished observation). Based on these results, we propose that the potentiation caused by 6-TG is the result of an increase in the formation of S^6-(2-chloroethyl)-6-thioguanine, which subsequently reacts to form DNA crosslinks, and thereby increases the number of crosslinks formed by BCNU alone. However, further work is required to establish this mechanism.

The concentration of 6-TG used in these studies was relatively low. Administration of 3.4 mg/kg of 6-TG to humans results in a plasma level of 1.79 μM 8 hr after injection [32]. The level of 6-TG used in this study is 11% of the plateau level in plasma. Therefore, relatively low doses that cause potentiation of BCNU cytotoxicity *in vitro* can be achieved clinically.

Studies are in progress to determine whether 6-TG pretreatment potentiates the cytotoxicity of other alkylating agents such as nitrogen mustard and *cis*-platinum, and whether it can enhance cytotoxicity in cells already resistant to BCNU.

Acknowledgements—This research was supported by NIH Grants CA-13525, CA-31867, and CA-31868, the Andres Soriano Cancer Research Fund, and the Department of Energy through Contract DE-AM03-76-SF01012. We thank Dr. T. Hoshino and T. Nagashima for performing the FCM analysis, and Neil Buckley for editorial assistance.

REFERENCES

1. V. A. Levin and C. B. Wilson, *Cancer Treat. Rep.* **60**, 719 (1976).
2. V. A. Levin, in *Nitrosoureas in Cancer Treatment* (Eds. B. Senou, P. S. Schein and J. L. Imbach), p. 171. Elsevier/North Holland, Amsterdam (1981).
3. B. Senou, P. S. Schein and J. L. Imbach (Eds.), *Nitrosoureas in Cancer Treatment.* Elsevier/North Holland, Amsterdam, (1981).
4. C. J. Cheng, S. Fijimura, D. Grunberger and I. B. Weinstein, *Cancer Res.* **32**, 22 (1972).
5. K. D. Tew, S. Sudhakar, P. S. Schein and M. E. Smulson, *Cancer Res.* **38**, 3371 (1978).
6. D. B. Ludlum and W. P. Tong, in *Nitrosoureas in Cancer Treatment* (Eds. B. Senou, P. S. Schein and J. L. Imbach), p. 21. Elsevier/North-Holland, Amsterdam (1981).
7. R. A. G. Ewig and K. W. Kohn, *Cancer Res.* **38**, 3197 (1978).
8. K. W. Kohn, *Cancer Res.* **37**, 1450 (1977).
9. L. C. Erickson, G. Laurent, N. A. Sharkey and K. W. Kohn, *Nature, Lond.* **288**, 727 (1980).
10. W. P. Tong, M. C. Kirk and D. B. Ludlum, *Biochem. Pharmac.* **32**, 2011 (1983).
11. T. A. Krenitsky, R. Papaioannou and G. Elion, *J. biol. Chem.* **244**, 1263 (1969).
12. D. M. Tidd and A. R. P. Paterson, *Cancer Res.* **34**, 746 (1974).
13. D. F. Deen, P. M. Bartle and M. E. Williams, *Int. J. Radiat. Oncol. Biol. Phys.* **5**, 1663 (1979).

14. T. Hoshino, D. F. Deen, M. E. Williams and Y. Sano, *Cancer Res.* **41**, 4404 (1981).
15. P. Perry and S. Wolff, *Nature, Lond.* **251**, 156 (1974).
16. W. J. Bodell and M. R. Banerjee, *Nucleic Acids Res.* **3**, 1689 (1976).
17. B. Singer, W. J. Bodell, J. E. Cleaver, G. H. Thomas, M. F. Rajewsky and W. Thon, *Nature, Lond.* **276**, 85 (1978).
18. K. C. Kuo, R. A. McCune and C. W. Gehrke, *Nucleic Acids Res.* **8**, 4763 (1980).
19. K. W. Kohn, R. A. Ewig, L. C. Erickson and L. A. Zwelling, in *DNA Repair: A Laboratory Manual of Research Procedures* (Eds. E. C. Friedberg and P. C. Hanawalt), p. 279. Marcel Dekker, New York (1981).
20. R. Bjerkvig, S. M. Oredsson, L. J. Marton, M. Linden and D. F. Deen, *Cancer Res.* **43**, 1497 (1983).
21. R. D. Armstrong, R. Vera, P. Snyder and E. Cadman, *Biochem. biophys. Res. Commun.* **109**, 595 (1983).
22. B. H. Herbert, S. Drake and J. A. Nelson, *J. Liquid Chromat.* **5**, 2095 (1982).
23. S. Fujimoto, M. Ogawa and Y. Sakurai, *Cancer Res.* **42**, 4079 (1982).
24. S. Fujimoto and M. Ogawa, *Gann* **71**, 659 (1980).
25. J. Maybaum and H. G. Mandel, *Cancer Res.* **42**, 3852 (1983).
26. J. Maybaum and H. G. Mandel, *Expl. Cell Res.* **135**, 465 (1981).
27. L. L. Wotring and J. L. Roti Roti, *Cancer Res.* **40**, 1458 (1980).
28. P. J. Tofilon, M. E. Williams and D. F. Deen, *Cancer Res.* **43**, 473 (1983).
29. D. T. Hung, D. F. Deen, J. Seidenfeld and L. J. Marton, *Cancer Res.* **41**, 2783 (1981).
30. P. J. Tofilon, S. M. Oredsson, D. F. Deen and L. J. Marton, *Science* **217**, 1044 (1982).
31. P. J. Tofilon, D. F. Deen and L. J. Marton, *Science* **222**, 1132 (1983).
32. K. Lu, J. A. Benvenuto, G. P. Bodey, J. A. Gottlieb, M. G. Rosenblum and T. L. Loo, *Cancer Chemother. Pharmac.* **8**, 119 (1982).