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Repair of O^6-(2-Chloroethyl)guanine Mediates the Biological Effects of Chloroethylnitrosoureas

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Chloroethylnitrosoureas (CENUs) are alkylating and crosslinking agents used for the treatment of human cancer; they are both mutagenic and carcinogenic. We compared the levels of induction of sister chromatid exchanges (SCEs) and the cytotoxicity of nitrosoureas that alkylate only with CENUs. CENUs are 200-fold more cytotoxic and induce SCEs with 45-fold greater efficiency than agents that do not crosslink; therefore, crosslinking is probably the most important molecular event that leads to cell death and induction of SCEs.

The biological and biochemical properties of both human and rat brain tumor cells that are sensitive or resistant to the cytotoxic effects of CENUs have been investigated. CENUs induce SCEs in both sensitive and resistant cells, but to induce similar levels of SCEs, resistant cells must be treated with a 5- to 14-fold higher concentration of CENUs than are used to treat sensitive cells. Resistant cells have a higher cellular level of O^6-methylguanine-DNA methyl transferase, increased repair of O^6-methylguanine, and 50% fewer DNA interstrand crosslinks formed than do sensitive cells treated with the same concentration of CENU. Based on these findings, we propose that cellular resistance to the cytotoxic effects of CENUs is mediated by O^6-methylguanine–DNA methyltransferase and that DNA repair may also modify the mutagenic and carcinogenic properties of CENUs.

Introduction

2-Chloroethyl-1-nitrosoureas (CENUs), synthesized first by Montgomery and co-workers, are cytotoxic agents effective against a wide variety of *in vitro* and *in vivo* tumor model systems (*1,2*). Currently these compounds are being used clinically to treat a variety of human tumors (*3,4*). In addition to their cytotoxic effects, these agents have been shown to be mutagenic in both prokaryotic (*5*) and eukaryotic test systems (*6*) and to induce tumors in rats (*7,8*). Consequently, in addition to their therapeutic benefit they may pose a genotoxic hazard to the treated patient (*9,10*). Therefore it is very important that a clear understanding of the biochemical processes mediating the cytotoxic, mutagenic effects, and carcinogenic effects of these compounds be obtained.

Under physiological conditions, CENUS are hydrolyzed to reactive species that alkylate cellular DNA, RNA, and proteins (*11*). Some of the monoalkylation products may form DNA interstrand crosslinks in subsequent reactions (*12,13*). DNA interstrand crosslink formation is thought to cause the cytotoxic effects of CENUs (*14–16*). One of the crosslinks formed in DNA treated with CENUs has been identified as [1-(N-3-deoxycytidyl)-2-(N-1-deoxyguanosinyl)ethane] (*13*). Tong and Ludlum (*13*) postulated that the formation of O^6-2-chloroethylguanine is the initial alkylation event that leads to the subsequent formation of this DNA crosslink. This hypothesis is supported by the observation that cells that can remove O^6-methylguanine (O^6MeG) from their DNA are resistant to the cytotoxic effects of CENUs and have fewer DNA interstrand crosslinks formed after treatment with CENUs (*14–16*).

The enzyme that repairs O^6-alkylguanine in DNA is O^6-methylguanine DNA methyltransferase (*17–19*), a receptor protein that catalyzes the dealkylation of O^6-alkylguanine in DNA to produce the alkylated protein and guanine (*17–19*). The transferase is inactivated after a single event (*17–19*). Human tumor cells have been classified as methyl excision repair-positive (Mex^+) or methyl excision repair-negative (Mex^-) based on their capability to remove O^6-MeG from DNA (*20–23*). Mex^+

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cells have 20,000–60,000 transferase molecules per cell and Mex$^-$ cells about 1200 molecules per cell (*20–23*). Because the maximum number of DNA interstrand crosslinks are formed 6 hr after treatment with CENUs (*12*), in Mex$^+$ cells there is adequate time for repair of O^6-2-chlorethylguanine adducts before they form DNA interstrand crosslinks. These results suggest that repair of O^6-alkylguanine derivatives in DNA and inhibition of DNA crosslink formation are related events and may be important factors in human tumor cell resistance to CENUs. Increased repair of DNA adducts that can form DNA crosslinks or repair of DNA crosslinks after they are formed have been implicated as major factors in the resistance of cells to the cytotoxic effects a variety of DNA crosslinking agents (*14–16,24–27*).

Resistance of cells to the cytotoxic effects of chemotherapeutic agents appears to be one cause of the ineffectiveness of chemotherapy for human cancer (*28,29*). Approximately 60% of human brain tumors are resistant to the therapeutic effects of BCNU (*30,31*). Studies of the properties of cell lines that are resistant to chemotherapeutic agents may help define more fully the molecular mechanisms involved in cellular resistance. Currently a number of rodent derived cell lines are used to test the effects of chemotherapeutic agents. One of these, 9L, is a well characterized gliosarcoma cell line that has been used for both *in vivo* and *in vitro* cytotoxicity studies (*32*). CENU-resistant sublines have been isolated from the 9L cell line (*29*). We have begun a study of the mechanisms of resistance to CENUs using one of these sublines, 9L-2, and cell lines derived from human brain tumors (*33*).

Cell Killing and SCE Induction Caused by CENUs

The cytotoxic effect of nitrosoureas have been investigated by using a colony-forming assay, which has been described in detail (*14,33*). For our studies we have compared the cytotoxic effects in 9L cells treated with the alkylating agent 1-ethyl-1-nitrosourea (ENU) and 3-(4-amino)-2-methyl-5-pyrimidinyl-methyl-1-(2-chloroethyl)-1-nitrosourea (ACNU), an agent that both alkylates and crosslinks DNA. The structures of these nitrosoureas are shown in Figure 1. Survival plots for 9L cells treated with either ACNU or ENU for 1 hr are shown in Figure 2. To produce a 1 log cell kill required treating 9L cells with either 5 mM ENU or 25 μM ACNU. Therefore, the DNA crosslinking agent ACNU kills cells 200-fold more efficiently than the noncrosslinking agent ENU.

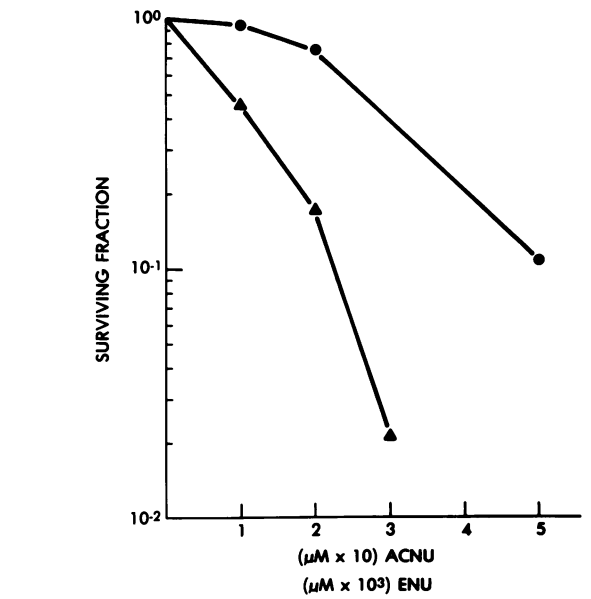

FIGURE 2. The survival of 9L cells treated for 1 hr with either (▲) ACNU or (●) ENU.

We compared the relative efficiency with which ACNU and ENU induce sister chromatid exchanges (SCEs). SCEs are reciprocal exchanges formed between sister chromatids during DNA replication (*34–36*), and a correlation between induction of SCEs and cytotoxicity has been reported (*14,37,38*). Induction of SCEs in 9L cells was measured by using the procedure of Perry and Wolff (*39*).

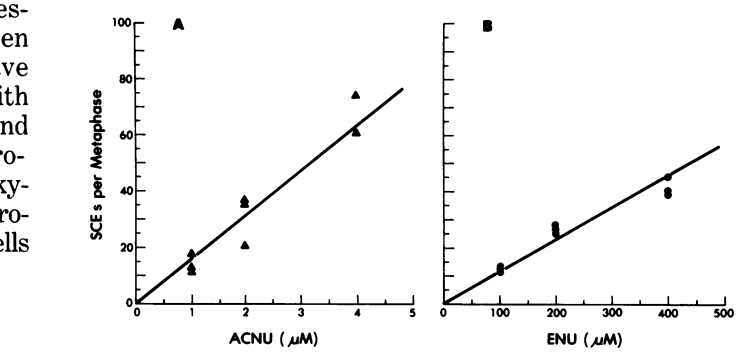

FIGURE 3. The induction of SCEs in 9L cells caused by treatment with either (*A*) ACNU or (*B*) ENU. The frequency of SCE induction was calculated by subtracting the number of SCEs in cells treated with 10 μM Budr alone (11-13 SCEs/metaphase). The dose response curves for SCE induction were calculated by linear regression analysis using the equation, $y = Ax$, where y is the number of SCEs induced, A is the slope of the line, and x is the dose of each agent. Each symbol represents the mean value for a single experiment.

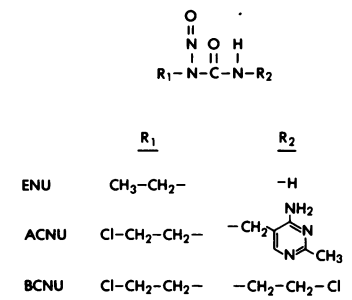

FIGURE 1. Structures of the nitrosoureas used in these studies.

Treatment of 9L cells with 2 μM ACNU induced approximately 30 SCEs/metaphase (Fig. 3*A*). A linear dose–response relationship was found, and the slope of the dose response curve was calculated to be 15.8 SCEs/μM ACNU by linear regression analysis. ENU treatment also produced a linear dose response curve (Fig. 3*B*), the slope of which was 0.11 SCEs/μM ENU. Thus, based on the ratio of the slopes for the dose–response curves, ACNU is 143-fold more efficient at inducing SCEs than is ENU.

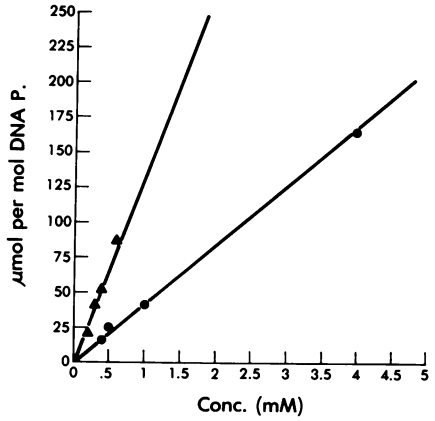

FIGURE 4. The alkylation of 9L cells DNA after treatment with (●) ^{14}C-ENU or (▲) ^{14}C-ACNU for 1 hr.

The differential induction of SCEs caused by ENU and ACNU may be the result of different levels of alkylation of cellular DNA, which may be mediated by different rates of cellular uptake or hydrolysis for the two compounds. Alkylation of cellular DNA with both ^{14}C-ENU and ^{14}C-ACNU was linear over the concentration ranges studied (Fig. 4). The slopes of the curves for the alkylation reaction are 135.7 μmole alkylation/mole DNA/mM ACNU and 42.0 μmole alkylation/mole DNA/mM ENU. Therefore, at equimolar doses ACNU alkylates DNA by a factor of approximately 3.2-fold more than ENU.

Using linear regression analysis we calculated the amount of ACNU or ENU that alkylates DNA at the doses required to induce 30 SCEs. Approximately 0.25 μmole of ACNU alkylation product per mole of DNA is required to induce 30 SCEs, while 11.4 μmole of alkylation product of ENU per mole of DNA is required to induce 30 SCEs. Therefore, after correction for the extent of alkylation of DNA, ACNU is 45-fold more efficient at inducing SCEs than is ENU.

Our studies indicate that treatment of cells with ACNU is 200-fold more cytotoxic and 45-fold more efficient at inducing SCEs than treatment with ENU. The identification of DNA alkylation produces in human fibroblasts and fetal rat brain cells treated with ENU has been reported (*40,41*). The alkylation products identified are listed in Table 1. The DNA alkylation products formed by CENU treatment of purified DNA have been extensively investigated (*13,42–46*). Even though the alkylation products in cells treated with CENUs have not been quantitated, the alkylation products of purified DNA are a good model for the reactions that occur when cells are treated with CENUs. The alkylation products of purified DNA treated with CENUs are also listed in Table 1.

Table 1. Products of the reaction of BCNU and ENU with DNA.

	Products	Percent of total
ENU	Ethylphosphotriesters	56 ± 4
	O^6-Ethyldeoxyguanosine	9.2 ± 1.8
	O^4-Ethylthymidine	2.1 ± 0.8
	O^2-Ethylthymidine	7.1 ± 1.5
	O^2-Ethyldeoxycytidine	4.5 ± 2
	7-Ethyldeoxyguanosine	13.6 ± 1
	3-Ethyldeoxyadenosine	4.5 ± 0.6
BCNU	Monoadducts	
	Phosphotriesters	
	O^6-(2-Hydroxyethyl)deoxyguanosine	
	7-(2-Hydroxyethyl)deoxyguanosine	
	7-(2-Chloroethyl)deoxyguanosine	
	3-(2-Hydroxyethyl)deoxycytidine	
	3-N^4-Ethanodeoxycytidine	
	Crosslinks	
	1,2-(Dideoxyguanosin-7-yl)ethane	
	1-(*N*-3-Deoxycytidyl)-2-(N-1-deoxyguanosinyl)ethane	

The alkylation products formed by ENU and CENUs are very similar. The primary difference is that chloroethyl product(s) of CENUs can crosslink bases on the same or opposite strands in subsequent reactions (*13,44,45*). It has been suggested that the crosslinked base 1,2-(dideoxyguanosine-7-yl)ethane is a DNA intrastrand crosslink (*44*) and that 1-(N-3-deoxycytidyl)-2-(N-1-deoxyguanosinyl)ethane is a DNA interstrand crosslink (*13,45*). Thus, the 45-fold differential induction of SCEs and 200-fold increased cytotoxicity caused by ACNU is probably the result of DNA interstrand crosslink formation.

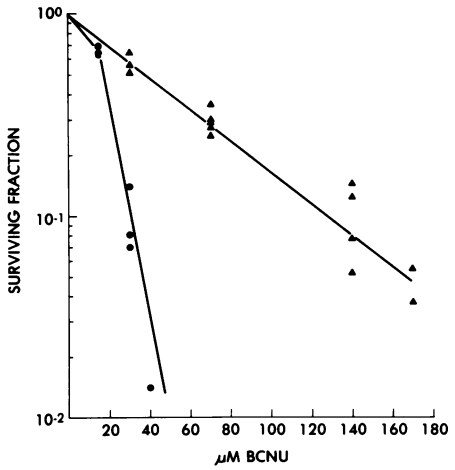

FIGURE 5. Survival curves for (●) 9L and (▲) 9L−2 cells treated for 1 hr with BCNU. Each symbol represents the mean value for a single experiment.

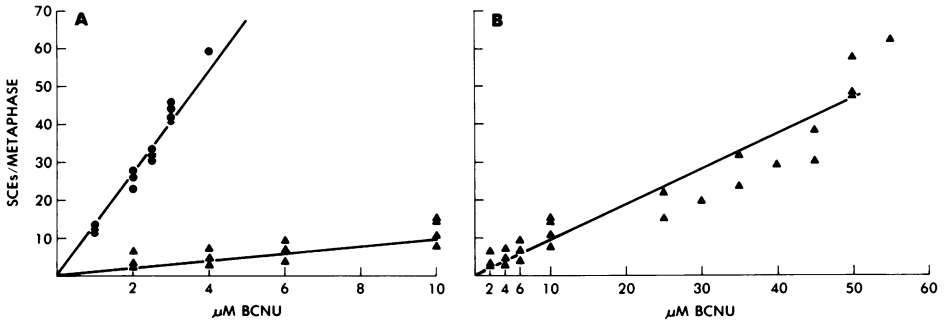

FIGURE 6. Induction of SCEs in (●) 9L and (▲) 9L − 2 cells treated with various doses of BCNU: (*A*) Doses of BCNU 0–10 μM; (*B*) dose of BCNU 0–55 μM.

Cellular Resistance

Cellular resistance to the cytotoxic effects of CENUs appears to be one of the major factors for the ineffectiveness of CENUs in the treatment of human cancer. To study this process in detail, we isolated sublines of 9L by high dose treatment of rats bearing the intracerebral 9L tumor with 1,3 bis(2-chloroethyl)-1-nitrosourea (BCNU) (*29*). Established cell lines from human brain tumors were used also.

Survival plots for 9L and 9L-2 cells treated with various concentrations of the DNA crosslinking agent BCNU are shown in Figure 5. 9L-2 cells are markedly more resistant to the cytotoxic effects of BCNU than are 9L cells; 90% of the 9L cells cannot form colonies after a 1-hr treatment with 30 μM of BCNU. In contrast, 9L-2 cells must be treated with 120 μM of BCNU to achieve the same level of cell kill. Thus 9L-2 cells are at least 4-fold more resistant to the cytotoxic effects of BCNU than are 9L cells.

As found for ACNU, BCNU efficiently induces SCEs in 9L cells; the dose-response curve is linear (Fig. 6*A*) with a slope of 13.5 SCEs/μM BCNU. However, very few SCEs are induced in 9L-2 cells treated over the same dose range (Fig. 6*A*). To induce similar levels of SCEs in both cell lines, 9L-2 cells have to be treated with a 10-fold higher dose of BCNU (Fig. 6*B*). The slope of the dose-response curve for SCE induction on 9L-2 cells is 0.93 SCEs/μM BCNU. As calculated by the ratio of the slopes of the dose-response plots, 9L-2 cells are 14-fold less susceptible to the induction of SCEs than are 9L cells.

Dose-response curves for BCNU treatment in the cell lines derived from three human brain tumors are presented in Figure 7. HU-126 cells are very sensitive to the cytotoxic effects of BCNU compared to either HU-188 or HU-253-2 cells. The induction of SCEs by BCNU treatment has also been measured in these human cell lines (Fig. 7). Because the number of chromosomes per metaphase is variable from cell line to cell line, we have to make the comparison on the basis of SCEs per chromosome. The slope of the dose-response curves for BCNU treatment in HU-126 cells is 0.128 SCEs/μM BCNU for HU-188, 0.009 SCEs/μM BCNU, and for HU253-2 cells 0.023 SCEs/μM BCNU. As a ratio of their slopes for the dose–response curve HU-126 cells are 5- to 14-fold more sensitive to the induction of SCEs than are either HU-188 or HU-253-2 cells. Thus, similar results are obtained with sensitive and resistant tumor cells obtained either from rodent or human cell lines.

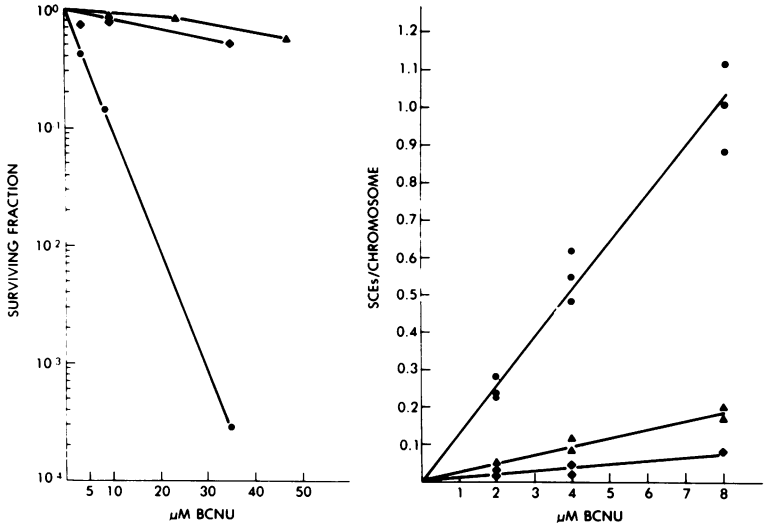

FIGURE 7. Survival curves and induction of SCEs after BCNU treatment in (●) HU-126, (♦) HU-188, or (▲) HU-253-2 cells. Survival curves were obtained after a 2 hr treatment with BCNU, and the SCE induction curves after a 1-hr treatment with BCNU.

Table 2. Analysis of DNA methylation products in cells after a 1 hr treatment with ^{3}H-MNU.

Cell type		Methylated products, μmole methylated purine/mole DNA			Ratio O^6-MeG
		N-3-MeA	N-7-MeG	O^6-MeG	N-7 MeG
9L	E-1	1.2	18.3	2.3	0.125
	E-2	1.8	19.2	2.2	0.114
	E-3	1.1	21.6	2.5	0.115
9L-2	E-1	1.8	22.9	2.3	0.100
	E-2	1.3	21.4	1.9	0.088
HU-126	E-1	1.7	44.2	5.4	0.122
	E-2	2.15	39.9	5.6	0.141
HU-188		0.83	11.1	0.76	0.067
HU-253-2	E-1	3.3	42.0	2.1	0.05
	E-2	0.75	13.2	0.48	0.036

Table 3. O^6-Methylguanine-DNA-methyl transferase activity of human brain tumor cells.

Cell line	O^6-MeG demethylated, pmole/mg protein
HU-126	0.07
HU-188	0.37
HU-253-2	0.22

DNA Repair and Resistant Cells

Recently published data (*14–16*) suggest that a relationship exists between the repair of O^6-alkylguanine derivatives and cellular resistance to CENUs in human tumor cells. We have examined the possibility that a similar mechanism is responsible for the observed resistance of 9L-2, HU-188, and HU-253-2 cells to SCE induction and cytotoxicity. For our studies we treated these cells with 100 μM ^{3}H-MNU, a dose of MNU that does not saturate the methyltransferase activity in Mex$^+$ cells (*21*). The extent of methylation of DNA in all of the cell lines after a 1-hr treatment with 100 μM ^{3}H-MNU was very similar (Table 2). The formation of N-3-MeA and N-7-MeG was also the same in these cell lines. The O^6-MeG/N-7-MeG ratio in 9L and HU-126 cells was similar to values reported for other cell lines that do not repair O^6-MeG (*20*). In contrast, the O^6-MeG/N-7 MeG ratio in 9L-2 cells was 0.094, approximately 20% lower than in 9L. In HU-188, and HU-253-2 cells the O^6-MeG/N-7 MeG ratio was 0.067, and 0.043, respectively. Because O^6-MeG is a stable alkylation product of DNA, the lower O^6-MeG/N-7-MeG ratio in 9L-2, HU-188, and HU-253-2 cells represents repair of O^6-MeG by these cells during the 1-hr treatment period.

These results indicate that cells resistant to CENUs have an increased capacity for removal of O^6-alkylguanine derivatives from DNA than do the corresponding sensitive cells. Comparison of the results in 9L-2, HU-188, and HU-253-2 show that O^6-MeG was removed to a greater extent during the 1-hr interval in the resistant human lines compared to 9L-2. This may indicate that resistant human cells have a higher level of O^6-methylguanine DNA methyltransferase than resistant rodent cells. More extensive studies of resistant human and rodent cell lines must be conducted before a definitive conclusion can be drawn.

We have measured the level of O^6-methylguanine DNA methyl transferase activity in some of our human cell lines. For this assay, cellular extracts of sonicated cells are incubated at 37°C for 60 min with DNA containing 1 pM of O^6-MeG. After the incubation period, samples are acid hydrolyzed, the alkylation products are separated by HPLC, and the residual O^6-MeG is determined by liquid scintillation counting. HU-126 cells have low levels of methyltransferase activity (Table 3). In contrast, HU-188 and HU-253-2 cells have 3- to 5-fold higher levels of this enzyme. These results correlate very well with the removal of O^6-MeG in the human cell lines treated with ^{3}H-MNU (Table 2).

Because results of earlier studies indicate that DNA interstrand crosslinks formed after CENU treatment are responsible for both cell killing and SCE induction, we compared the number of DNA interstrand crosslinks formed in sensitive and resistant cells after BCNU treatment. We used the technique of alkaline elution developed by Kohn and co-workers (*17*) to estimate the number of interstrand crosslinks formed in treated cells. The relative number of DNA interstrand crosslinks formed in cells after treatment with either 50 or 100 μM BCNU for 1 hr, followed by a 6-hr incubation period,

Table 4. Formation of DNA interstrand crosslinks in cells treated for 1 hr with BCNU.[a]

BCNU treatment	Crosslink index × 10^3				
	9L	9L-2	HU-126	HU-188	HU-253-2
50 μM	109 ± 15	50 ± 10	37	8	24
100 μM	214 ± 19	96 ± 30	80	30	57

[a] The number of proteinase K-resistant DNA crosslinks was determined as described (*14*).

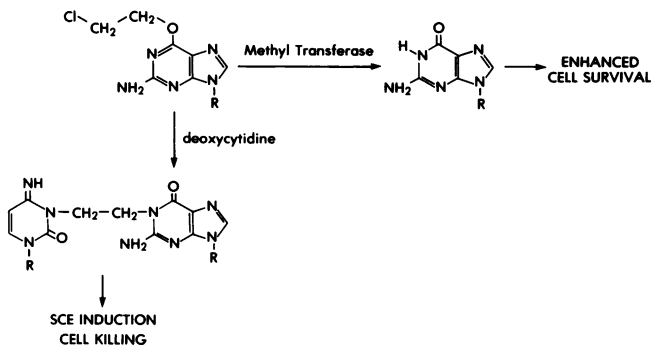

FIGURE 8. Proposed molecular mechanism for the mediation of SCE induction and cell kill by O^6-methylguanine DNA methyltransferase.

are listed in Table 3. The number of DNA interstrand crosslinks formed in the resistant cells 9L-2, HU-188, and HU-252-2 are approximately 50% that of the corresponding sensitive cell lines 9L and HU-126.

A possible mechanism through which cellular resistance to the cytotoxic effects of CENUs may be mediated by DNA repair is shown in Figure 8. Resistant cells have an increased capacity to remove O^6-(2-chloroethyl)guanine in DNA. Enzymatic removal of this alkylation product will reduce DNA crosslink formation and therefore should reduce both SCE induction and the cytotoxic effect of CENU treatment. In contrast, sensitive cells have a very poor capacity to remove O^6-(2-chloroethyl)guanine derivatives. Therefore, in these cells, DNA crosslink formation will occur that leads to SCE formation and cell kill. In agreement with this hypothesis, Robbins et al. (*48*) and Brent (*49*) have reported that O^6-methylguanine-DNA methyltransferase removes a chloroethylation product that forms DNA crosslinks. Our results showing that there are fewer DNA crosslinks formed in cells that actively remove O^6 methylguanine is consistent with this mechanism.

Mutagenic and Carcinogenic Effects of CENUs

Methyl and ethyl nitrosourea have been shown to be potent mutagens in a variety of test systems (*50*). Recently, the mutagenicity of CENUs has been investigated. These compounds have been shown to induce mutations in both prokaryotic (*5*) and eukaryotic test systems (*6*). The molecular mechanism for the mutagenic effect of alkyl nitrosoureas has been attributed to their capacity to form the O-alkylpurine and O-alkylpyrimidine derivatives listed in Table 1. These alkylation products have been shown to cause misincorporation of non-Watson-Crick base pairing partners in *in vitro* systems (*51–54*). CENUs have been shown to form O^6-(2-hydroxyethyl)deoxyguanosineand3,N^4-ethanodeoxycytidine in DNA. These alkylation products probably induce mutations by a mechanism that leads to misincorporation.

CENUs have been shown to induce tumors, primarily lung tumors, leukemias, and lymphosarcomas, when administered to either mice or rats (*7,8*). Patients undergoing chemotherapy receive doses up to 1.5 g/m^2. Clinical evidence has been presented that treatment of patients with CENUs may cause secondary leukemias (*9,10*).

There is very good evidence that DNA repair plays a major role in organ-specific carcinogenesis. Investigations with nitrosoureas and nitrosoamines have shown that the cellular capacity to remove O^6-alkylguanine and O^4-alkyl thymidine is inversely related to the susceptibility of the organ to the tumorigenic effect of these compounds (*55–60*). Therefore the cellular capacity to remove the O^6-alkylguanine derivatives produced by CENU treatment should modify the carcinogenic effects of these agents. Cellular repair of O^6-alkylguanine derivatives formed by CENUs may have two major effects: it prevents the formation of a DNA crosslink and hence reduces the cytotoxic effect of CENU treatment, and it removes the promutagenic base and reduces the carcinogenic effect of the compounds.

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