Chemical Structure of Carbamoylating Groups and Their Relationship to Bone Marrow Toxicity and Antiglioma Activity of Bifunctionally Alkylating and Carbamoylating Nitrosoureas

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ABSTRACT

Although the antitumor effects of chloroethylnitrosoureas have been shown to be due primarily to DNA-DNA cross-linking by the alkylating moieties of these agents, the basis of the often accompanying bone marrow toxicity has been more controversial. We report on the relative bone marrow toxicity of four model nitrosoureas with different alkylating and carbamoylating activities: 1,3-bis(2-chloroethyl)-1-nitrosourea; 1,3-bis(trans-4-hydroxy-cyclohexyl)-1-nitrosourea; chlorozotocin, (2-[3-(2-chloroethyl)-3-nitrosoureido]-2-deoxy-β-D-glucopyranose); and 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea. Inhibitions of DNA, RNA, and protein synthesis in murine bone marrow cells and of colony growth of myeloid precursor cells (granulocyte-macrophage colony-forming units) were used as in vitro end points of myelotoxicity. Further, we determined the antigenicity of the four nitrosoureas on two human gliomas in a clonogenic tumor cell assay and studied the effect of the non-nitrosourea carbamoylators potassium cyanate, chloroethyl isocyanate, cyclohexyl isocyanate, and ethyl isothiocyanate on granulocyte-macrophage colony-forming units. The results show that, at equivalent drug exposures, clonogenic glioma cell kill was significant and comparative for 1,3-bis(2-chloroethyl)-1-nitrosourea, 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea, and chlorozotocin; 1,3-bis(trans-4-hydroxy-cyclohexyl)-1-nitrosourea showed little activity. In contrast, granulocyte-macrophage colony-forming unit toxicity was low with chlorozotocin and 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea and very high with 1,3-bis(2-chloroethyl)-1-nitrosourea and 1,3-bis(trans-4-hydroxy-cyclohexyl)-1-nitrosourea. Of the isocyanates, bone marrow toxicity was highest with chloroethyl isocyanate and cyclohexyl isocyanate, intermediate with ethyl isocyanate, and lowest with KOCN and ethyl isothiocyanate. Our results indicate that (a) bifunctional alkylating is essential for antigenia activity of nitrosoureas and (b) myelosuppression is at least partly linked with carbamoylation but that structural entities in the carbamoylating isocyanate rather than a quantitative degree of carbamoylation determine the degree of potential myelotoxicity.

INTRODUCTION

The CENUs3 have been proved clinically to be effective antitumor drugs and, because of their ability to cross the blood-brain barrier (1), have been especially useful in the chemotherapeutics of central nervous system cancers. However, some nitrosoureas, especially the non-sugar-containing ones, uniquely produce dose-limiting cumulative bone marrow toxicities (2, 3). Under physiological conditions, CENUs decompose to alkylating, cross-linking chloroethyl carbonium ions as well as reactive organic isocyanates (4, 5), and there is considerable evidence (6–11) to suggest that interstrand cross-linking of cellular DNA by the bifunctionally alkylating chloroethyl carbonium ions constitutes the major cytotoxic event and consequently is responsible for the antitumor effects. However, apart from their ability to inhibit DNA ligase and other cellular enzyme activity (12–17), there is less certainty about the effects of the carbamoylating moieties of CENUs, although it has been speculated (3, 14, 18) and refuted (19–21) that the unwanted normal tissue toxicity of most CENUs might be associated with carbamoylation. The results of studies on the role of carbamoylation on bone marrow toxicity have, however, been inconclusive, because the carbamoylation models used have been either potassium cyanate or sugar-containing nitrosoureas (19, 20). The myelotoxicity of carbamoylation, non-sugar, nonalkylating nitrosoureas have thus far not been studied in this context. In order to establish whether the chemical structure of a CENU or a carbamoylating agent is related to its potential myelotoxicity, we investigated the effects of 4 structurally different nitrosoureas that differ in their alkylating and carbamoylating activities. Inhibition of DNA, RNA, and protein synthesis in murine bone marrow cells and suppression of the colony growth of myeloid precursor cells (CFU-GM) were used as in vitro end points of myelotoxicity. In vitro antiglioma effects were determined in a clonogenic cell monolayer assay using a previously described method (22). The 4 model nitrosoureas investigated were BCNU (a bifunctional alkylator and carbamoyl ator), BHCNU (a carbamoylator without alkylating activity), CHZ (a sugar-containing bifunctional alkylator with very low external carbamoylating activity), and GANU (a sugar-containing bifunc-
tional alkylator and carbamoylator). We also studied the effect of the nonalkylating, nonnitrosourea carbamoylators KOCN, CEIC, CyHIC, EIC, and ETIC on CFU-GM and compared the results with those obtained with the nitrosoureas.

MATERIALS AND METHODS

Bone Marrow Cells. DBA/2 mice (8 to 12 weeks old; Charles River, Portage, MI) were sacrificed by cervical dislocation, the femurs were removed and cleaned of adhering muscle, and the contents of each femur was flushed out with 1 ml of MEM plus EBS. In order to lyse contaminating erythrocytes, the cells were pelleted by centrifugation at 200 x g for 10 min and resuspended in sterile 0.09% NaCl solution for 60 s. Double-strength MEM was added, and the cells were washed twice and adjusted to the required density.

GM-CSF. GM-CSF was prepared by a modification of a previously described method (23). Each of 10 male C57BL mice was given i.p. injections of 0.1 ml of lipopolysaccharide solution (100 µg/ml; Sigma Chemical Co., St. Louis, MO) and sacrificed after 2 h. The lungs were removed, minced, and incubated for 72 h with MEM containing 10% FCS. After centrifugation at 3000 x g for 30 min, the supernatant-conditioned medium was incubated at 56°C for 30 min and dialyzed (4°C) at a molecular weight cutoff of 12,000 to 14,000 (Spectrapor membrane tubing; Spectrum Medical Industries, Los Angeles, CA) against 0.9% NaCl solution buffered with 20 mM Tris-HCl (pH 7.4). Before use, each CSF batch was filtered through a 0.2-µm filter (Millipore Corp., Bedford, MA) and tested for CFU-GM activity.

Drugs and Chemicals. BCNU, BHCMU, CHZ, and GANU were all obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD. KOCN, CEIC, CyHIC, EIC, and ETIC were purchased from Aldrich Chemical Co., Milwaukee, WI. The required amounts were dissolved in minimum quantities of sterile double-distilled water or ethanol and diluted with ice-cold Hanks' balanced salt solution to the desired stock concentrations. All drug solutions were freshly prepared and used immediately. [methyl-3H]thymidine (specific activity, 49 Ci/mmol), [5,6-3H]uridine, and L-[4,5-3H]leucine, (specific activity, 54 Ci/mmol) were all obtained from ICN Pharmaceuticals, Irvine, CA.

Drug Exposure and Plasma Clot CFU-GM Assay. For a 2-h drug exposure, 50 µl each of stock drug solutions were added for 950 µl of a suspension containing 2 x 10⁶ nucleated bone marrow cells, 20% FCS, and 10% CSF to yield final concentrations of 0 to 50 µM BCNU, 0 to 250 µM BHCMU, 0 to 200 µM CHZ, and 0 to 150 µM GANU, respectively. After 2 h at 37°C, the cells were washed twice with fresh medium and plated at 2 x 10⁵ cells/ml as described below.

We have previously reported (24) the optimization of the plasma clot assay for murine and human CFU-GM used in this study. Briefly, a 2-ml cell suspension was prepared containing bone marrow cells (2 x 10⁶/m) and 20% FCS, 10% CSF, and 10% citrated bovine plasma (Sigma). MEM supplemented with EBS and l-asparagine (20 µg/ml) was used. After thorough mixing, 0.5-ml aliquots of the suspension were transferred to each well of a 4-well tissue culture dish (1.6 cm in diameter; Nunc Intermed, Roskilde, Denmark) and allowed to coagulate. The plates were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 7 days. Colonies consisting of more than 50 cells were counted using an Olympus inverted microscope at x40.

For the continuous drug exposure, stock drug solution was added to the culture mixture such that, after adding all the culture constituents, a final concentration of 0 to 300 µM was achieved for CEIC, CyHIC, KOCN, EIC, and ETIC. Culture mixture (500 µl) was then transferred to each well of the 4-well culture dish, allowed to coagulate, and incubated as described above.

Colonies were scored, representative cultures were transferred to clean glass slides and allowed to air dry. The slides were fixed in 4% paraformaldehyde solution (pH 7.4) and stained with Giemsa. Another set of cultures were similarly transferred to glass slides, fixed, and stained for α-naphthyl acetate esterase using a modification (24) of a previously described technique (25). Utilizing morphological and cytochemical criteria, 3 types of colonies surviving drug treatment were observed and quantified: type I, pure granulocytes; type II, pure macrophages; and type III, a mixture of granulocytes and macrophages.

DNA, RNA, and Protein Synthesis. The incorporation of [3H]thymidine, [3H]uridine, and L-[3H]leucine in acid-insoluble material was used as a quantitative measure of de novo cellular synthesis of DNA, RNA, and protein, respectively. The duration of preincubation of bone marrow cells with CSF and the radiouclide pulse required to achieve maximum precursor incorporation have been optimized and reported previously (24). In this study, 2 x 10⁶ bone marrow cells in 1 ml MEM containing 20% FCS were preincubated with 10% CSF for 6 h. Stock drug solution was added to achieve the final concentrations as in the CFU-GM assay, and the mixture was incubated for an additional 2 h. The cells were washed twice with medium and resuspended in 1 ml fresh medium containing 10% FCS and 5% CSF. Stock solutions of [3H]thymidine, [3H]uridine, and L-[3H]leucine were diluted with MEM and added to the cell suspension to achieve final concentrations of 10 and 5 µCi/ml, respectively. The suspensions were then incubated for 4 h at 37°C in 5% CO₂. Aliquots (250 µl in quadruplicate) of each cell suspension were transferred to each well of a 96-well microtiter plate (Nunc Intermed). The cells were immediately harvested on glass fiber filters using a cell harvester (Skatron, Sterling, VA) and washed with cold 5% trichloroacetic acid. After drying, the filters were placed in scintillation vials, scintillation liquid [4 g of a mixture of 2 parts p-bis(o-methoxyphenyl)benzene and 98 parts of PPO in 1 liter of toluene] was added, and the radioactivity was counted in a Beckman LS 330 liquid scintillation counter.

Human Glioma Cytotoxicity Studies. The clonogenic monolayer assay used has been described previously (22). Two human malignant glioma cell lines (HU407 and Hu126) in early in vitro passage (HU407:2; Hu126:8) were trypsORIZED, washed twice with medium, and plated at various cell numbers (500 to 10,000 cells) in T25 tissue culture flasks (Coming, NY). 9L rat gliosarcoma cells were X-irradiated (40 Gy) and added to each flask at 5 x 10⁶ cells/flask. The flasks were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 24 hr. Stock BCNU, BHCMU, CHZ, and GANU solutions were prepared as described previously and aliquots were added to achieve final concentrations as described above. The cultures were incubated at 37°C in 5% CO₂ for 2 to 3 weeks, and the colonies were counted after fixation and staining with 3% crystal violet-methanol.

Evaluation of Cytotoxicity. In order to compare the cytotoxicities of the 4 agents tested, the surviving fraction (CFU-GM and gliomas) and the percentage of inhibition of DNA, RNA, and protein synthesis relative to untreated control bone marrow cells were plotted against the drug dose and/or the integrated drug exposures [(c) x (r)] for each drug over the entire incubation period. The decay kinetic values of the nitrosoureas in MEM supplemented with EBS and 20% FCS were determined in a biocopy assay and were all shown to follow first kinetics. Thus, the in vitro half-life (t½) data in MEM plus 20% FCS (Table 1) were used to determine the in vitro decay rate constants (k)

\[ k = 0.693/t_{\text{½}} \]
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Table 1
Decay kinetic constants for nitrosoureas in MEM supplemented with EBS and 20% FCS

<table>
<thead>
<tr>
<th>Agent</th>
<th>$t_{1/2}$ (half-life) (h)</th>
<th>$k$ (drug decay rate constant) (h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCNU</td>
<td>0.54</td>
<td>1.28</td>
</tr>
<tr>
<td>BHCNU</td>
<td>0.13</td>
<td>5.33</td>
</tr>
<tr>
<td>CHZ</td>
<td>0.52</td>
<td>1.33</td>
</tr>
<tr>
<td>GANU</td>
<td>0.77</td>
<td>0.9</td>
</tr>
</tbody>
</table>

RESULTS

CFU-GM. Chart 1 shows the in vitro survival curves of committed murine myeloid stem cells (CFU-GM) at various drug exposures of the 4 nitrosoureas tested. BCNU inhibited CFU-GM the most. The inhibition was dose dependent with a log kill of 1.0 at 22 $\mu$M-h and a total elimination of colony growth corresponding to >2 logs kill at 30 $\mu$M-h. BHCNU was also dose dependently highly inhibitory to CFU-GM, although to a lesser degree than BCNU. At an exposure of 35 $\mu$M-h BHCNU, CFU-GM growth was reduced by 1 log, and at 48 $\mu$M-h growth was totally abolished (>2 log kill). CHZ and GANU were least toxic to CFU-GM. Even at the relatively high exposure of 50 $\mu$M-h CHZ and GANU, CFU-GM growth was inhibited by only 0.4 and 0.3 log, respectively. CFU-GM sensitivities to the nonalkylating, non-nitrosourea carbamoylators are summarized in Chart 2. KOCN and ETIC were relatively nontoxic to CFU-GM, producing only a 0.87 and 0.83 log kill after continuous exposure of bone marrow cells to 150 $\mu$M. In contrast, CEIC and CyHIC demonstrated high CFU-GM toxicity with a >2-log kill at 50 and 100 $\mu$M, respectively.

Table 2 summarizes the results of the morphological and cytochemical staining of the cells of CFU-GM colonies surviving treatment by BCNU, BHCNU, CHZ, and KOCN. Compared to controls, there was no significant difference in the distribution of surviving colony types for the representative agents.

DNA, RNA, and Protein Synthesis in Bone Marrow Cells. This study was performed using BCNU, BHCNU, CHZ, and GANU. As shown in Chart 3, BCNU, BHCNU, and CHZ inhibited DNA synthesis in CSF-pretreated bone marrow cells in the same rank order as they inhibited CFU-GM colony growth. The inhibition was dose dependent. At 40 $\mu$M-h BCNU and BHCNU, DNA synthesis was reduced by 92 and 70%, respectively. CHZ and GANU were least inhibitory on DNA synthesis with only 53% (CHZ) and 57% (GANU) inhibition at 100 $\mu$M-h. Charts 4 and 5 show that both RNA and protein synthesis were inhibited by all 4 drugs to a much lesser degree than DNA synthesis.

At 40 $\mu$M-h, inhibition of RNA synthesis was 52% for BCNU,
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Chart 3. Inhibition of DNA synthesis in murine bone marrow cells by the nitrosoureas BCNU, BHCNU, CHZ, and GANU. Bone marrow cells were pretreated for 2 h with 10% GM-CSF before exposure to the nitrosoureas for 2 h. Percentage of inhibition was calculated with reference to untreated controls.

Chart 4. Inhibition of RNA synthesis in CSF-pretreated murine bone marrow cells by BCNU, BHCNU, CHZ, and GANU after a 2-h drug exposure. Percentage of inhibition was expressed relative to untreated controls.

% for BHCNU, and less than 3% for GANU and CHZ, even at 80 \( \mu \text{M} \cdot \text{h} \) (Chart 4). The levels of inhibition of protein synthesis in bone marrow cells (Chart 5) were similar to that of RNA synthesis; at 40 \( \mu \text{M} \cdot \text{h} \), inhibition was 38% for BCNU, 30% for BHCNU, 2% for CHZ, and 10% for GANU. Even at 100 \( \mu \text{M} \cdot \text{h} \) CHZ, inhibition of bone marrow cell RNA and protein synthesis was only 12 and 10%, respectively.

Gliomas. Charts 6 and 7 summarize the in vitro cytotoxic activity of BCNU, CHZ, BHCNU, and GANU on the 2 early-passage glioma cell lines, Hu407 and Hu126. At 25 \( \mu \text{M} \cdot \text{h} \), approximately 1.8 logs (BCNU), 1.88 logs (CHZ), and 1.91 logs...
CENUs have both bifunctional alkylating as well as carbamoylating properties (4, 5), and strong evidence (6-11, 18) has been provided to show that cellular DNA-interstrand cross-linking caused by the CENUs is probably the principal cytotoxic and antitumor lesion. However, the role of carbamoylation in cytotoxic events has been less clear. Using nitrosoureas with differing chemical reactivity, Kann et al. (15) showed that carbamoylation contributed to the cytotoxicity of ionizing radiations in L1210 cells by inhibiting repair of DNA damage. This was further supported by the observation of Fornace et al. (12) that CEIC, the carbamoylating moiety of BCNU, was a potent inhibitor of DNA ligase inhibition. However, it has been reported recently (27) that, although different levels of DNA cross-linking were produced by BCNU and CHZ in rat bone marrow cells after in vitro treatment with both agents, the rate of cross-link removal was essentially the same. Thus, despite the large number of studies aimed at understanding the possible molecular mechanisms that account for the differences in bone marrow toxicity of different CENUs, there is still controversy as to the relative roles of carbamoylation and alkylation-cross-linking in causing normal tissue toxicity. In earlier studies of the role of carbamoylation per se in causing bone marrow toxicity, potassium cyanate was used as the model for pure carbamoylation, and observations made regarding its bone marrow toxicity or lack thereof were projected to the nitrosoureas. In contrast to such earlier investigations, we report in this study the antitumor and bone marrow toxicity of 4 model nitrosoureas which differed from each other in (a) the presence or absence of carbamoylating or bifunctional alkylating activity and (b) the structure of the carbamoylating moiety in the parent molecule. BHCNU, the model nitrosourea with pure carbamoylating and no alkylating activity, lacks a sugar in its carbamoylating moiety and was shown to have potent dose-dependent inhibitory activity on CFU-GM as well as on DNA synthesis in CSF-pretreated bone marrow cells similar to BCNU which both alkylates and carbamoylates. The poor carbamoylator, CHZ, despite its higher alkylating potential relative to BCNU, and the potent alkylator and carbamoylator, GANU, were the nitrosoureas least toxic to CFU-GM and bone marrow cell DNA synthesis. The latter 2 CENUs contain glucopyranose in their isocyanate moieties. These observations are consistent with other in vivo and in vitro findings (19-21, 28, 29). The moderate effects of the nitrosoureas on RNA and protein synthesis that we observed are also consistent with other published reports (30). At doses as high as 300 μM, KOCN showed little CFU-GM toxicity even at continuous exposure. This lack of toxicity of potassium cyanate to the bone marrow was also observed by Panasci et al. (20), who showed no measurable bone marrow suppression after treating mice with a single dose of 750 μmol/kg of KOCN for 1 week. In contrast, CEIC and CyHIC, the respective carbamoylating moieties of the clinically myelotoxic agents, BCNU and CCNU, demonstrated in vitro CFU-GM toxicity similar to that observed with BCNU and BHCHU.

The in vitro cytotoxic activity of the 4 model nitrosoureas on the glioma cell lines contrasted with those obtained with the bone marrow cells. At drug exposures within clinically achievable ranges, BCNU, GANU, and CHZ demonstrated significant and comparable clonogenic cell kill in 2 early-passage human glioma cell lines. Although BHCNU showed some cytotoxic activity in both glioma cell lines, the degree of cytotoxicity was considerably less than was observed with BCNU, GANU, and CHZ. We have previously shown (24) that human glioma cell lines resistant to BCNU were cross-resistant to CHZ while demonstrating a low level of sensitivity to BHCNU similar to that of BCNU-sensitive tumors. Furthermore, treatment of rats bearing i.e. 9L gliosarcoma with BCNU and BHCHU at approximate 10% lethal doses resulted in no increased survival of the BHCHU-treated group, whereas in the BCNU-treated group there was a median increased life span of 6 weeks over the controls. These in vitro and in vivo observations demonstrate the relative lack of anti-glioma activity of BHCHU.

The cytotoxicity data on both glioma and bone marrow cells indicate, as has also been suggested by others (9, 11, 18, 31), that, for a nitrosourea, bifunctional alkylating activity (with or without carbamoylation) is essential for antitumor activity. However, our results with the model nitrosoureas and isocyanates clearly demonstrate that bone marrow toxicity may be linked, at least partly, with the carbamoylating potential of the agent but that the manifestation of myelotoxicity by an isocyanate depends upon the presence or absence of other structural groups in the moiety. Other clinical and experimental studies (21, 28, 29, 32-34) have also shown that structural variables such as the presence of a glucose in position 2 or 3 of a CENU, as in CHZ or GANU, can reverse or reduce the myelosuppressive potential of...
the agent even if high carbamoylating activity is maintained. The exact mechanism by which sugar-containing CENUs, irrespective of their carbamoylating activity, spare the marrow is still not fully understood. However, Green et al. (33) found that GANU and CHZ alkylate DNA in L1210 cells to a higher degree than they alkylate DNA of bone marrow cells. In contrast, DNA alkylation by the myelotoxic CCNU was higher for bone marrow cells than for L1210 cells. Furthermore, they showed that, while all 3 CENUs preferentially bound to nucleosomal core DNA in both marrow and L1210 cells, CHZ and GANU was preferentially bound to core DNA in L1210 cells and to internucleosomal linker DNA in bone marrow cells. Similar observations were made by Tew et al. (35) for HeLa cells. If these results are confirmed for human tumors, then in light of evidence (36) showing a high level of methyl excision repair of methylated products in linker DNA, it may be postulated that repair of alkylation coupled with a reduced bone marrow-tumor cell DNA alkylation ratio (probably as a consequence of the presence of a group such as a 2-glucose) might explain, at least partly, the reduced myelotoxicity of some CENUs such as CHZ and GANU over others, e.g., BCNU and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.

The results discussed above and the data that we newly present in this study suggest that quantitative and qualitative differences in bone marrow-tumor cell alkylation and/or carbamoylation may account for some of the observed differences in toxicity of the structurally different nitrosoureas. The presence of carbamoylation, especially in the absence of a glucose moiety, will induce or potentiate bone marrow toxicity without necessarily increasing antitumor activity significantly. The comparative results with the structurally different carbamoylators (Table 1) BHCNU, CEIC, CyHIC, EIC, ETIC, and KOCN show that rather than a quantitative degree of carbamoylation there might be a structural prerequisite of an isocyanate necessary to produce myelotoxicity. Since a potential cytotoxic lesion of an isocyanate may be associated with the carbamoylation of key enzymes such as DNA ligase (12, 13, 15) and other cellular proteins (12, 14, 37), we may speculate that a stereochemical specificity present in BHCNU, CEIC, and CyHIC but absent in EIC, ETIC, glucose-linked isocyanates or in isocyanic acid (the active carbamoylator produced upon hydrolysis of KOCN) is required for interaction with and inactivation of active sites of such proteins. These putative qualitative differences in carbamoylation could further explain the varying levels of bone marrow-tumor cell toxicity observed with structurally different carbamoylating CENUs and other organic isocyanates.

We are currently investigating the effects of some of these model nitrosoureas and isocyanates on the activity of target enzymes such as methyltransferase and glutathione reductase in bone marrow as well as in gioma cells and how these relate to in vivo and in vitro myelotoxic and antitumor effects.

REFERENCES

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