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PREFERENTIAL ALKYLATION BY 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA (BCNU) OF GUANINES WITH GUANINES AS NEIGHBORING BASES IN DNA

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Abstract—The base sequence of DNA has been shown to influence the kinds and amounts of alkylation of purine bases by *N*-methyl-*N*-nitrosourea [W. T. Briscoe and L-E. Cotter, *Chem. Biol. Interact.* **56**, 321 (1985)]. In the present study, the alkylation of DNA polymers of defined sequence by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) has been investigated. The assay involved treating poly (dG)·poly(dC), poly(dG-dC)·poly(dG-dC), poly(dA-dC)·poly(dG-dT), poly(dA-dG)·poly(dC-dT), and calf thymus DNA with BCNU, followed by hydrolysis to release the modified purine bases and separation and quantitation of these by HPLC. Analysis of the results revealed that there was a 24-fold increase of 7-(β-hydroxyethyl)guanine (HOEtG) in poly(dG)·poly(dC) relative to poly(dA-dG)·poly(dC-dT). There was also a 3-fold increase in HOEtG in poly(dG-dC)·poly(dG-dC), poly(dA-dC)·poly(dG-dT) and calf thymus DNA relative to poly(dA-dG)·poly(dC-dT). A 2- to 4-fold increase of 7(β-aminoethyl)guanine (AmEtG) was observed in poly(dG-dC)·poly(dG-dC) relative to the other polymers tested. This study has determined that guanines in certain base sequences in polydeoxyribonucleotides are more susceptible to BCNU alkylation at the N-7 position than guanines in other sequences.

The 2-chloroethylnitrosoureas (CENUs)† are an important class of chemotherapeutic agents with demonstrated anti-cancer activity against a wide variety of human tumors including lymphomas, small cell carcinoma of the lung, malignant melanoma, gliomas and cancers of the gastrointestinal tract [1, 2]. One of the principal members of this class is 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU).

Under physiological conditions, CENUs decompose spontaneously to form alkylating intermediates and carbamoylating species. These can then react with DNA in a variety of ways to yield a number of products including 7-(β-chloroethyl)guanine (ClEtG), 7-(β-hydroxyethyl)guanine (HOEtG), and O^6-(β-hydroxyethyl)guanine (O^6-HOEtG) [3]. Another product, 1,2-(diguan-7-yl)ethane (diGEt), is the result of a second step modification that yields inter- and/or intrastrand cross-links [3–6], which some authors have associated with anti-tumor activity. However, the involvement of other lesions in the DNA, as well, would be expected in the anti-tumor activities of these drugs [3].

Upon decomposition, the non-alkylating portion of BCNU forms an isocyanate which will principally carbamoylate proteins but may also decompose to species such as 2-chloroethylamine which will alkylate nucleic acids [3]. An aminoethyl derivative, 7-(β-aminoethyl)guanine (AmEtG), is formed only by BCNU, of the CENUs, and is thought to be derived from the chloroethyl group attached to the carbamoylating side of the BCNU [7]. However, this portion of the drug is not thought to be required for anti-tumor activity [8–11].

Due to the very reactive nature of the alkylating species of alkylnitrosoureas, reactions with DNA were originally thought to be random in regard to the site of attack. More recent investigations have revealed a non-random attack of DNA in chromatin. In L1210 cells, CCNU, chlorozotocin, GANU and ACNU preferentially attack the DNA associated with the nucleosome core. In bone marrow, ACNU and CCNU again prefer the nucleosomal DNA, whereas chlorozotocin and GANU preferentially attack the linker DNA [11]. At another level, actively transcribing DNA shows a 1.5- to 2-fold increase in alkylation relative to non-transcribing DNA [12]. Yet, the mechanisms of actions of the 2-chloroethylnitrosoureas are poorly understood.

We developed a system to study the effects of base sequence upon the alkylation of guanine and adenine bases in DNA. In that study we demonstrated that alkylation of purine bases in DNA by *N*-methyl-*N*-nitrosourea (MNU) is not random but is significantly influenced either by the pi electron interactions of bases neighboring a purine base or by the local conformation of the DNA double helix induced by the neighboring bases [13, 14].

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† Abbreviations: ACNU, 1-(4-amino-2-methylpyrimidin-5-yl)methyl-3-(2-chloroethyl)-3-nitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; CENU, 2-chloroethylnitrosourea; GANU, 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea; MNU, *N*-methyl-*N*-nitrosourea; AmEtG, 7-(β-aminoethyl)guanine; ClEtG, 7-(β-chloroethyl)guanine; diGEt, 1,2-(diguan-7-yl)ethane; HOEtG, 7-(β-hydroxyethyl)guanine; and O^6-HOEtG, O^6-(β-hydroxyethyl)guanine.

In view of the clinical importance of agents such as BCNU and its chemical relationship to MNU, it appeared desirable to delineate the role of base sequence in the alkylation of DNA by BCNU. Kohn and Gibson have suggested the importance of ascertaining how to employ design of a chloroethylating agent "to modify the selectivity for reaction with various sites, either with respect to particular positions on various bases or with respect to dependence on nucleotide sequence" [6]. In the present report, the effects on alkylation in regard to both position on the bases and dependence on nucleotide sequence are examined.

MATERIALS AND METHODS

Polydeoxyribonucleotides and other materials. The synthetic polydeoxyribonucleotides, poly(dG)·poly(dC), poly(dG-dC)·poly(dG-dC), poly(dA-dC)·poly(dG-dT), and poly(dA-dG)·poly(dC-dT), were obtained from Pharmacia Inc. (Piscataway, NJ) and calf thymus DNA was purchased from the Sigma Chemical Co. (St. Louis, MO). BCNU was purchased from Bristol Laboratories (Syracuse, NY). AmEtG, ClEtG, HOEtG and O^6-HOEtG standards were the gift of Dr. David B. Ludlum. Calf thymus DNA and each of the polymers except poly(dG)·poly(dC) were dissolved in 25 mM Tris–Cl buffer, pH 7.0, to a final concentration of approximately 5 mM DNA-phosphate (DNA-P) following repeated ethanol precipitations and dialysis to remove small molecular weight materials. The identity and concentration of each polynucleotide were confirmed by UV spectra using the following molar extinction coefficients: poly(dG)·poly(dC), $\varepsilon_{253} = 7400$; poly(dG-dC)·poly(dG-dC), $\varepsilon_{254} = 8400$ [15]; poly(dA-dC)·poly(dG-dT), $\varepsilon_{260} = 6500$; poly(dA-dG)·poly(dC-dT), $\varepsilon_{260} = 5700$ [16]; and DNA, $\varepsilon_{260} = 6178$ (1 mg/ml = 20 A_{260} units). Due to difficulty in dissolving the poly(dG)·poly(dC), this polymer was subjected to sonication as follows.* The poly(dG)·poly(dC) (100 A_{260} units) was suspended in 30–40 ml of 0.01 M EDTA, pH 8, and heated to 60° for 20 min. The polymer was then sonicated on ice for 20 min in 5-min invervals using a Heat Systems-Ultrasonics sonicator fitted with a microtip. The polymer was dialyzed against 25 mM Tris–Cl, pH 7.0, at room temperature and concentrated by molecular filtration employing an Amicon PM-10 membrane. The final concentration was approximately 5 mM DNA-P. The chain length of each polymer before and after sonication was determined by agarose gel electrophoresis employing a flat-bed 0.9% agarose gel (FMC Corp., BioProducts, Rockland, ME) in Tris/borate/EDTA (TBE) buffer containing 0.5 μg ethidium bromide/ml buffer. Samples were electrophoresed for 2 hr at 50 V [17]. Polymer lengths in number of base pairs were determined by comparison with a Hind III digest of lambda phage DNA (International Biotechnologies, Inc., New Haven, CT).

To ascertain the degree of double-strandedness of each polymer, thermal denaturation curves were made in 7.5 mM sodium phosphate, 1 mM sodium EDTA, pH 6.75. The hyperchromic shifts were compared with literature values which are: for poly(dG)·poly(dC), 1.23; poly(dG-dC)·poly(dG-dC), 1.20; poly(dA-dC)·poly(dG-dT), 1.41; and poly(dA-dG)·poly(dC-dT), 1.28 [17, 18].

Bis(2-chloroethyl)-1-nitrosourea was obtained from Bristol Laboratories and dissolved in anhydrous ethanol immediately prior to use.

Assay. Alkylation of the polynucleotides and DNA was accomplished using a procedure modified from Ludlum and Tong [3]. The alkylation reaction consisted of the following components: 1.1 μmol DNA-P or polydeoxyribonucleotide-P in 289 μl 25 mM Tris–Cl, pH 7.0, and 5.35 mg BCNU in 32 μl ethanol added to initiate the reaction. The mixture was immediately mixed by vortexing and incubated for 24 hr at 37°. The reaction was stopped by addition of 0.1 vol. of 2 M potassium acetate and 2 vol. of cold 95% ethanol, and the precipitated polynucleotide was pelleted by centrifugation. The pellet was redissolved in water, and the precipitation steps were repeated twice more to remove unreacted BCNU and unbound BCNU breakdown products.

Dissociation of the alkylated purine bases from the polymers was accomplished by neutral hydrolysis [3–5]. The ethanol-precipitated polymer was suspended in 80 μl of 25 mM sodium cacodylate buffer, pH 7, and heated in a boiling water bath for 15 min. This procedure quantitatively released the substituted purines as well as some of the unreacted adenine and guanine, while minimizing degradation of the alkylated products.† The polymer was removed by acid precipitation with HCl (pH 1), and, following centrifugation, the supernatant layer was neutralized with ammonium hydroxide for HPLC analysis.

High performance liquid chromatography. A gradient elution HPLC system was developed in our laboratory similar to that described by Ludlum and Tong [3]. The system consists of a Varian MCH-10 reverse-phase column (4.6 mm × 30 cm) and guard column. Elution was with 0.02 M potassium acetate buffer, pH 5.0, with a 3–8% acetonitrile linear gradient over 30 min at a flow rate of 1.25 ml/min. The acetonitrile concentration was then held at 8% for an additional 5 min. The effluent was monitored at 254 nm. Peak assignments were verified by employing known standards. A typical chromatogram is shown in Fig. 1.

Statistical analysis. Data were analyzed by one-way analysis of variance and grouped as determined by Newman–Keuls test ($P < 0.01$).

RESULTS

Analysis by HPLC of the synthetic polydeoxyribonucleic acids treated with BCNU was able to separate and detect AmEtG, HOEtG, and ClEtG, as well as guanine and adenine.

* S. L. Broitman, A. G. Letai and J. R. Fresco, personal communication, cited with permission.

† W. T. Briscoe and S. P. Duarte, unpublished observation.

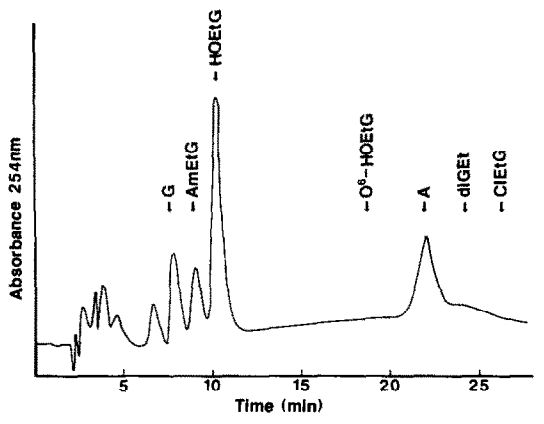

Fig. 1. HPLC of calf thymus DNA (1.1 μmol phosphate) incubated with 5.35 mg BCNU for 24 hr at 37°. The DNA was precipitated 3 times with 2 vol. of ethanol and 0.1 vol. of 2 M potassium acetate. Modified purines were released from the DNA by neutral hydrolysis in 25 mM sodium cacodylate buffer, pH 7, at 100° for 15 min. The partially apurinic DNA was removed by cold acid precipitation with HCl, pH 1, and the supernatant layer was neutralized with ammonium hydroxide. The purine bases were separated by HPLC consisting of a Varian MCH-10 reverse-phase column (4.6 mm × 30 cm) and guard column. Elution was with 0.02 M potassium acetate buffer, pH 5.0, with a 3–8% acetonitrile linear gradient over 30 min at a flow rate of 1.25 ml/min. The acetonitrile concentration was then held at 8% for an additional 5 min. The effluent was monitored at 254 nm. Peak assignments were verified from known standards: A, adenine; AmEtG, 7-(β-aminoethyl)guanine; ClEtG, 7-(β-chloroethyl)guanine; diGEt, 1,2-(diguan-7-yl)ethane; G, guanine; HOEtG, 7-(β-hydroxyethyl)-guanine; and O^6-HOEtG, O^6-(β-hydroxyethyl)guanine. Also shown are the solvent front at 2–4 min and the BCNU decomposition product at 6.6 min.

The results of these experiments are summarized in Table 1. From these data it is apparent that HOEtG was present in approximately the same amount in poly(dG-dC)·poly(dG-dC), poly(dA-dC)·poly(dG-dT) and calf thymus DNA which was a 3-fold increase over that found in poly(dA-dG)·poly(dC-dT) ($P < 0.01$). The amount of HOEtG was increased about 24-fold in poly(dG)·poly(dC) relative to poly(dA-dG)·poly(dC-dT) ($P < 0.01$). Other notable differences included a 2- to 4-fold increase in the levels of AmEtG in the poly(dG-dC)·poly(dG-dC) relative to the other polynucleotides ($P < 0.01$), and ClEtG was detected only in the poly(dG)·poly(dC).

The data also were "normalized" by dividing by the relative number of guanine moieties present in each polymer, and are shown in Table 2. This is a very useful way to consider these data because the polymers poly(dG)·poly(dC) and poly(dG-dC)·poly(dG-dC) contain 50% guanine (550 nmol guanine/assay), whereas the poly(dA-dC)·poly(dG-dT) and poly(dA-dG)·poly(dC-dT) contain 25% guanine (275 nmol guanine/assay) and calf thymus DNA contains 21.7% guanine (239 nmol guanine/assay). The normalized values in Table 2 were obtained from the raw data values and represent the number of alkylated bases per 1000 guanines present. As shown by this normalization, the central guanine in the triplet GGG is 10–11 times more likely to form HOEtG as the guanine in the triplets CGC or AGA and almost 4 times as likely as the guanine in the triplet TGT or a guanine in calf thymus DNA. Similarly, the guanine in CGC is from 1.5 to 2 times as likely to form AmEtG as guanine in the other triplets studied. Calf thymus DNA contains 28.5% adenine, 21.6% cytosine, 21.7% guanine, and 28.2% thymine [19]. It is interesting to note that the weighted average of the amounts of HOEtG in the four synthetic polymers was 37.24, which compares favorably with the experimental value of 35.09 for calf thymus DNA. Similarly, the weighted average of the amounts of AmEtG in the polymers was 13.30 which also is close to the experimental value of 11.07 for calf thymus DNA.

To eliminate conformational changes resulting from sonication as a factor in the increased production of HOEtG in poly(dG)·poly(dC), the following experiment was performed. Three of the polymers, poly(dG)·poly(dC), poly(dG-dC)·poly(dG-dC), and poly(dA-dG)·poly(dC-dT), were suspended at approximately 0.15 mg/ml in 0.01 M EDTA, pH 7.8, and subjected to sonication as described in Materials and Methods. DNA ther-

Table 1. Alkylguanine products of synthetic polynucleotides and DNA treated with BCNU

Polynucleotide	HOEtG (nmol)	AmEtG (nmol)	ClEtG (nmol)
Poly(dG)·poly(dC)	60.6 ± 2.0* (6)	5.3 ± 2.3 (4)	0.9 ± 0.2 (5)
Poly(dG-dC)·poly(dG-dC)	6.4 ± 1.7 (6)	9.9 ± 1.1 (5)	ND†
Poly(dA-dC)·poly(dG-dT)	8.0 ± 1.4 (4)	3.8 ± 1.0 (3)	ND‡
Poly(dA-dG)·poly(dC-dT)	2.5 ± 0.1 (5)	3.3 ± 0.5 (4)	ND
Calf thymus DNA	8.4 ± 2.0 (6)	2.6 ± 1.2 (4)	ND

In this assay, each polydeoxyribonucleotide, 1100 nmol of DNA-phosphate, was treated with 25 μmol BCNU. Abbreviations: HOEtG, 7-(β-hydroxyethyl)guanine; AmEtG, 7-(β-aminoethyl)guanine, and ClEtG, 7-(β-chloroethyl)guanine.

* Nanomoles of product reported as mean ± SD (N), where N is the number of samples assayed.

† In one of six samples, 0.55 nmol ClEtG was detected; however, in the remaining samples, none was detected.

‡ ND = not detected.

Table 2. Nanomoles of HOEtG and AmEtG formed per micromole of guanine present in BCNU-treated polynucleotides

Polynucleotide	Triplet	HOEtG (nmol/μmol guanine in assay)	AmEtG
Poly(dG)·poly(dC)	GGG	110.26 ± 3.73	9.66 ± 3.60
Poly(dG-dC)·poly(dG-dC)	CGC	11.63 ± 3.09	18.00 ± 1.96
Poly(dA-dC)·poly(dG-dT)	TGT	29.10 ± 5.03	13.78 ± 3.70
Poly(dA-dG)·poly(dC-dT)	AGA	9.10 ± 0.53	12.04 ± 1.77
Calf thymus DNA		35.12 ± 8.40	11.07 ± 5.19

mal denaturation curves of poly(dG-dC)·poly(dG-dC) before and after sonication were performed and showed no difference in hyperchromicity (i.e. no increase in single-strandedness). The degree of hyperchromicity (A_{260} post-denaturation/A_{260} pre-denaturation) was compared with literature values and confirmed that poly(dG-dC)·poly(dG-dC) was >70% double-stranded. The other polymers were likewise demonstrated to be >80% double-stranded. Agarose gel electrophoresis of these polymers before and after sonication revealed that sonication sheared the polymers to lengths of 300–800 base pairs compared with the 2,000–100,000+ base pairs in the unsonicated polymers. Alkylation by BCNU followed by HPLC analysis of these polymers demonstrated no significant difference between the sonicated and unsonicated forms of these polymers in the quantities of the HOEtG formed ($P > 0.35$). This eliminates sonication as an explanation of the increased hydroxyethylation found in poly(dG)·poly(dC).

DISCUSSION

The sizable increase in the occurrence of HOEtG in the poly(dG)·poly(dC) could have resulted from one of three sources. First, the reaction could be enhanced by the sequence of bases in the DNA, that is, by the neighboring guanine bases. Second, it could be a function of some unique conformational arrangement of the poly(dG)·poly(dC). However, under the reaction conditions employed, all of the polymers are double-stranded and do not assume the Z-DNA conformation such that conformation should not have an effect upon one polymer over another [20]. Third, the increase could have arisen from the sonication of this polymer with the increased hydroxyethylation being a result of BCNU reacting with guanines near the ends of the polymer molecules or with single-stranded regions. The lack of significant change in the degree of total alkylation or of formation of HOEtG in poly(dG-dC)·poly(dG-dC) or poly(dA-dG)·poly(dC-dT) before and after sonication is inconsistent with this third explanation. The high degree of double-strandedness of the polymers as determined by hyperchromic shift upon thermal denaturation also argues against this explanation.

From these data, it is clear that a guanine in poly(dG)·poly(dC) is almost four times more susceptible to attack by BCNU than is a typical guanine in DNA or in the sequence TGT and almost 11 times more susceptible to attack than a guanine in the sequence CGC or AGA. BCNU contains two chloroethyl groups. The one in the N-1 position is involved in the alkylation of bases and accounts for HOEtG, ClEtG, O^6-HOEtG and diGEt. The chloroethyl group in the N-3 position is normally involved in carbamoylation of proteins *in vivo*. In addition, this group can alkylate nucleic acids via formation of chloroethyl isocyanate during BCNU decomposition [3]. It is presumed that aminoethyl alkylation to yield AmEtG occurs either via chloroethyl isocyanate followed by decarboxylation or via chloroethyl amine, a breakdown product of chloroethyl isocyanate [3]. In view of this, it appears that the alkylation of guanine, as in the case which yields HOEtG, is increased in situations where it has guanines as neighbors and is decreased where it has adenines or cytosines as neighbors. In previous work from this laboratory, it was demonstrated that a guanine flanked on the 3′- and 5′-sides by other guanines was 1.5 to 2 times as susceptible to methylation at the N-7 site by *N*-methyl-*N*-nitrosourea as a guanine flanked by adenines, cytosines or thymines [13, 14]. Thus, the results of the current report are consistent with the earlier findings that N-7 alkylation of guanine is preferential for guanines surrounded by other guanines. The results reported here are also consistent with similar findings involving alkylation by 1-(2-chloroethyl)-2-(*cis*-2-hydroxy)cyclohexyl-1-nitrosourea [21]. In that paper, Hartley *et al.* reported a 6-fold increase of HOEtG in poly(dG)·poly(dC) relative to poly(dG-dC)·poly(dG-dC). They also observed a 4.5-fold increase in ClEtG production in the homopolymer relative to the alternating copolymer. The observations are in good agreement with those reported in this paper, especially considering the differences in alkylating agents and reaction conditions employed. In other experiments reported in the same paper, they used a modification of the Maxam–Gilber DNA sequencing procedure to determine the relative degrees of N-7 alkylation of guanines in a DNA fragment of known sequence. While their method does not differentiate between the various alkylguanine products, HOEtG, AmEtG, and ClEtG, it was demonstrated that 1-(2-chloroethyl)-2-(*cis*-2-hydroxy)cyclohexyl-1-nitrosourea preferentially alkylates the middle guanines in runs of three or more guanines. Similar preferences were found for a series of nitrogen mustards, and the authors suggest that similar reacting intermediates may be involved in each of these instances [22].

Additionally, it appears that the reaction involving

the other chloroethyl group which yields AmEtG is enhanced in the case of guanine with cytosines as neighbors, but to a lesser degree. Since this involves a different reacting intermediate than in the HOEtG case, the fact that these results are different is not surprising.

The apparent absence of ClEtG from all the polymers except poly(dG)·poly(dC) indicates the levels, if present, were below our detection limit (0.2 nmol). As mentioned previously, ClEtG is stable under the hydrolysis conditions employed and does not break down to HOEtG or guanine to any appreciable degree. It is probable that the quantity of ClEtG formed parallels that of HOEtG and, as such, is detectable only in the poly(dG)·poly(dC).

Whether the effect seen in these polymers is due to the nearest neighbors at the 5′- and/or 3′-position adjacent to a guanine or whether it is due to the conformation of the double-stranded polymers induced by the repeating nature of these polymers is unclear. In "normal" DNA containing all possible combinations of bases, the local conformation around a given guanine flanked by certain bases will closely approximate the conformation found in the corresponding synthetic repeating polymer since "it is largely these neighbor interactions that determine the local three-dimensional shape imparted by the sequence" [23]. Thus, the neighboring base sequences determine the conformation. On the other hand, because of the close association of the pi electrons of neighboring bases, and since these are mobile and respond to the electron distribution of neighboring bases, the availability of the pi electrons of a guanine to partake in a nucleophilic substitution will be modulated by the nature of the neighboring bases [23]. The electrostatic potential at the N-7 position of guanine has been shown to be dependent upon the neighboring bases and guanines with guanine neighbors have the most negative potentials [22]. Mattes *et al.* [21, 22] have pointed out that the increased electronegativity at that site would increase its affinity for a positively charged species such as a chloroethylaziridinium or a chloro- or a hydroxyethyldiazohydroxide ion. Since BCNU alkylation is a nucleophilic substitution reaction very likely involving such ions, it would be attracted by and sensitive to the availability of electrons on the guanine, making electron distribution a more attractive explanation of the results obtained in these experiments.

In any case whether the increased alkylation is due to electronic effects of one guanine on another or whether there is a physical structural change due to multiple guanines in the sequence, the results reported here and those of other experiments from both this laboratory and elsewhere [13, 14, 21, 22, 24, 25] open the possibility for the existence of "hot spots" for alkylation of DNA by specific mutagens. This perhaps means that certain base sequences in DNA may be much more selectively alkylated than previously thought. We have shown that the sequence GGG is the most susceptible to hydroxyethylation by BCNU of the sequences tested. It follows that any gene containing a GGG within a critical region would have increased susceptibility to modification and/or inactivation by BCNU alkylation. Other drugs have proven to be preferential or specific for other sequences [24, 25]. Thus, as we gain a better understanding as to what specific gene or genes need to be modified or inactivated to kill a particular tumor type, and as the nucleotide sequences of those genes are determined, we will be better able to design drugs targeted for certain tumor types.

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