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BASE SEQUENCE SPECIFICITY OF THREE 2-CHLOROETHYLNITROSOUREAS

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Abstract—Chemical modifications of guanine are some of the most common results of interactions of DNA with many carcinogens and anti-cancer drugs, including nitrosoureas, nitrogen mustards, triazenes, polycyclic aromatics, and aflatoxins. The base sequence specificity for alkylation of guanines by three 2-chloroethylnitrosoureas has been determined. Guanines in the midst of a run of guanines are more susceptible than guanines in other base sequences. We have shown that certain 2-chloroethylnitrosoureas (BCNU, CCNU and methyl-CCNU) follow this same pattern. However, the quantitative degree of higher specificity for guanine with guanines as nearest neighbors depended on both the guanine position alkylated and the structure of the alkyl group attached. For example, when hydroxyethylation of runs of guanine occurred at N-7, a 6- to 11-fold increase of alkylation occurred compared to that found in the random base sequences of DNA, while hydroxyethylation at O-6 increased 1.2 to 3.5-fold and chloroethylation at N-7 was 2- to 4-fold higher than in DNA. Guanines with thymines on both the 3′ and 5′ sides were much less susceptible, most notably in N-7-hydroxyethylation and N-7-chloroethylation. Since guanine-rich regions are found in regulatory regions of the genome, knowledge concerning the effect of base sequence upon the production of each of the potential DNA lesions is vital to gaining an understanding of the roles of these lesions in the anti-tumor activity of a drug.

Guanine is one of the most susceptible targets in DNA for chemical modification by many carcinogens and anti-cancer drugs. Over the past several years, it has been shown that many chemical agents interact with DNA with a preference for guanines in certain base sequences. Both carcinogens and anti-tumor agents have been found to show base sequence specificity. For example, the carcinogen *N*-methyl-*N*-nitrosourea (MNU†) will methylate the N-7 position of a guanine with guanines as neighboring bases twice as frequently as a guanine with cytosines or thymines as neighboring bases [1–3]. Other carcinogens that have shown similar specificity include *N*-acetoxy-*N*-2-acetylaminofluorene [4], benzo[*a*]pyrene diol epoxide [5, 6], aflatoxin B_1 [7], and 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole [8]. The anti-tumor agents that have shown base sequence specificity include *cis*-dichlorodiammineplatinum(II) [9], 2-chloroethylnitrosoureas [10–12], nitrogen mustards [13–16], triazenes [17], and mitozolomide [12, 18]. In regard to the alkylating agents, guanines in the midst of a run of guanines are more susceptible than guanines in other base sequences. Several investigators have determined the base sequence specificity of *cis*-2-OH-CCNU [1-(2-chloroethyl)-3-(*cis*-2-hydroxy)cyclohexyl-1-nitrosourea], mitozolomide [12, 19], triazenes [17] and nitrogen mustards [13, 16] employing a gel electrophoresis method. They reported that these agents demonstrate preferential alkylation of guanines in stretches of guanines in double-stranded DNA. Hartley *et al.*[12] have demonstrated that *cis*-2-OH-CCNU shows a disproportionately strong preference for N-7 alkylation of guanine in locations of multiple adjacent guanines. Two adjacent guanines showed an average rate of N-7 alkylation per guanine 1.8 times that of a single guanine while three guanines showed a rate 4.4 times, four guanines 10.5 times and five guanines 11.8 times that of a single guanine [12]. The significance of these findings is that wherever several adjacent guanines exist in the genome, alkylation of those guanines is more likely to occur along with the possibility of subsequent mutation or inactivation of a gene. However, their methodology detected only the gross alkylation at the N-7 position of guanine. They could not differentiate between 7-hydroxyethylguanine and 7-chloroethylguanine, nor could they detect alkylation at other sites of guanine, such as the O-6 position, or cross-linked products [18]. Recently, Richardson *et al.* [20] published a most definitive report on base sequence effects of alkylation specificity. They employed synthetic oligodeoxyribonucleotides with specific guanines radiolabeled to investigate the susceptibility of specific sites to the formation of O^6-methylguanine upon

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† Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; CENU, 2-chloroethylnitrosoureas, as a class; *cis*-2-OH-CCNU, 1-(2-chloroethyl)-3-(*cis*-2-hydroxy)cyclohexyl-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(*trans*-4-methyl)-cyclohexyl-1-nitrosourea; ClEtSoSo, clomesone or 2-chloroethyl (methylsulfonyl) methanesulfonate; MNU, *N*-methyl-*N*-nitrosourea. Alkylation products of guanine: 7ClEtG, 7-(β-chloroethyl)guanine; DiGEt, 1,2-(diguan-7-yl)ethane; 7HOEtG, 7-(β-hydroxyethyl)-guanine; O^6HOEtG, O^6-(β-hydroxyethyl) guanine; and N_1HOEtG, 1-(β-hydroxyethyl)guanine. Synthetic polydeoxyribonucleotides of defined sequence: poly GG/CC, poly(dG)·poly(dC); poly GC/GC, poly(dG-dC)·poly(dG-dC); poly AC/GT, poly(dA-dC)·poly(dG-dT); and poly AG/CT, poly(dA-dG)·poly(dC-dT).

exposure to MNU. They found that the second or third guanine in a series of three adjacent guanines is 5–6 times more prone to form O^6-methylguanine as an isolated guanine.

In the present study, we employed HPLC methodology to analyze the various guanine alkylation products resulting from the reaction of guanine-containing polynucleotides of defined sequence with a group of three 2-chloroethylnitrosoureas: BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea], CCNU [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea], and MeCCNU [1-(2-chloroethyl)-3-(*trans*-4-methyl)-cyclohexyl-1-nitrosourea]. With this method, we were able to distinguish and measure four alkylated guanine bases common to reactions with these drugs, namely, 7-(β-hydroxyethyl)guanine (7HOEtG), O^6-(β-hydroxyethyl)guanine (O^6HOEtG), 7-(β-chloroethyl)guanine (7ClEtG), and 1,2-(diguan-7-yl)ethane (DiGEt), and determine the relative susceptibility of a guanine in each of these base sequences toward the production of any of these products.

MATERIALS AND METHODS

Polydeoxyribonucleotides and other materials. The synthetic DNA homopolymer duplex poly(dG)·poly(dC) [poly GG/CC, containing the triplet sequence GGG], and the DNA alternating copolymers poly(dG-dC)·poly(dG-dC) [poly GC/GC, containing the triplet sequence CGC], poly(dA-dC)·poly(dG-dT) [poly AC/GT, containing the triplet sequence TGT], and poly(dA-dG)·poly(dC-dT) [poly AG/CT, containing the triplet sequence AGA] were obtained from LKB/Pharmacia. These synthetic polydeoxyribonucleotides of defined sequence and calf thymus DNA (Sigma) were prepared as previously described with the exception that all of the polynucleotides were sonicated to fragment sizes ranging from 50 to 2000 base pairs prior to the assay [10]. Polynucleotide double-strandedness and concentration were determined as before [10]. BCNU was obtained from Bristol Laboratories. CCNU and MeCCNU were obtained from Nancita R. Lomax, Drug Synthesis & Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. The nitrosoureas were dissolved in anhydrous ethanol immediately prior to use.

Assay. Alkylation of the polynucleotides and DNA by the alkylating agents was accomplished using an established procedure [10]. Each reaction mixture contained 0.5 μmol polynucleotide-P in 180 μL of 25 mM Tris–Cl, pH 7.0. To initiate the reaction, 2.14 μmol of the drug in 20 μL of dry ethanol was added. The mixture was incubated at 37° for 24 hr. 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 pellet of polynucleotide was collected by centrifugation. The pellet was redissolved in water, and the precipitation steps were repeated twice more to remove unreacted alkylating agent and unbound alkylation products.

Neutral hydrolysis of polymers. Dissociation of the N-7-alkylated purine bases from the polymers was accomplished by neutral hydrolysis [10, 21–23]. The ethanol-precipitated polymer was suspended in 70 μL of 25 mM sodium cacodylate buffer, pH 7, and heated in a boiling water bath for 15 min. This procedure quantitatively releases the N-7-substituted purines as well as a small amount of the unreacted adenine and guanine, while minimizing degradation of alkylated products. The polymer (partially apurinic acid) was removed by acid precipitation with HCl (pH 1), and, following centrifugation, the supernatant layer was neutralized with ammonium hydroxide for HPLC analysis.

Mild acid hydrolysis of polymers. To dissociate the O^6HOEtG from the polymer, as well as the remainder of the unmodified adenine and guanine, the acid precipitated partially apurinic acid from the neutral hydrolysis step was subjected to mild acid hydrolysis in 50 μL of 0.1 N HCl at 100° for 15 min. The hydrolysate was neutralized with ammonium hydroxide for HPLC analysis.

HPLC. We have developed and employed a gradient elution HPLC system similar to that of Ludlum and Tong [21] which yields good separation of several products formed in DNA treated with 2-chloroethylnitrosoureas [10]. The system consists of a Varian MCH-10-reverse-phase column (4.6 mm × 30 cm) and a guard column. Elution was made with 20 mM potassium formate buffer, pH 3.5, with a 5–10% acetonitrile linear gradient over 20 min at a flow rate of 1.2 mL/min. The acetonitrile concentration was then held constant for an additional 5 min. The effluent was monitored at 260 and 281 nm. Peak assignments were verified by retention times and a 281 nm/260 nm ratio of known standards. Employing these conditions, we have been able to separate and quantitate several peaks from BCNU, CCNU, and MeCCNU treated DNA including 7ClEtG, 7HOEtG, O^6HOEtG and DiGEt. Confirmation of these assignments was based on co-elution with known standards (supplied by Dr. David B. Ludlum). Additional quantities of standards have been prepared by established methods [22–28].

Chromatographic UV data were integrated and quantitated using standard calibration curves developed from chromatography of the known standards. The neutral and acid hydrolysates of each sample were chromatographed separately, and the quantities of each product from both chromatographic runs summed.

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

RESULTS

The results of the study are presented in Table 1. Because the polynucleotides poly GG/CC and poly GC/GC contain 50% guanine, while poly AC/GT and poly AG/CT contain 25% guanine and calf thymus DNA contains 21.7% guanine, the data are presented as "normalized" by dividing the actual millimoles of each modified base by the number of moles of guanine present in each particular assay [10]. Since each polymer consists of repeats of a single guanine-containing triplet, this normalization permits direct comparison of the relative susceptibilities of a central guanine in a triplet sequence

Table 1. Alkylated guanines in polydeoxyribonucleotides and DNA treated with CENUs*

Agent and polymer		N	7HOEtG/G	O⁶HOEtG/G	7ClEtG/G	DiGEt/G
BCNU						
poly GG/CC	GGG†	6	134.80 ± 31.26[a]	0.72 ± 0.14	4.95 ± 1.14[c]	4.23 ± 2.11
poly GC/GC	CGC	5	5.19 ± 0.76[b]	1.05 ± 0.24	1.53 ± 0.20[d]	0.24 ± 0
poly AC/GT	TGT	5	4.45 ± 0.80[b]	0.33 ± 0.13	0.15 ± 0.07[e]	0.91 ± 0.51
poly AG/CT	AGA	5	7.72 ± 1.18[b]	0.73 ± 0.58	1.41 ± 0.18[d]	0.55 ± 0
ct DNA	NGN	4	22.02 ± 1.15[b]	0.60 ± 0.09	2.19 ± 0.22[d]	0.72 ± 0.11
CCNU						
poly GG/CC	GGG	6	76.27 ± 8.10[f]	0.99 ± 0.26[h]	2.93 ± 0.17[k]	3.12 ± 0.40[n]
poly GC/GC	CGC	6	2.40 ± 0.47[g]	0.55 ± 0.28[i]	0.59 ± 0.06[l]	0.20 ± 0.09[o]
poly AC/GT	TGT	6	2.00 ± 0.22[g]	0.15 ± 0.03[j]	0.09 ± 0.02[m]	0.26 ± 0.12[o]
poly AG/CT	AGA	6	3.05 ± 0.54[g]	0.32 ± 0.17[j]	0.65 ± 0.16[l]	0.27 ± 0.13[o]
ct DNA	NGN	5	6.76 ± 1.25[g]	0.29 ± 0.07[j]	0.67 ± 0.12[l]	0.39 ± 0.09[o]
MeCCNU						
poly GG/CC	GGG	6	64.42 ± 8.44[p]	0.75 ± 0.21[r]	2.67 ± 0.28[t]	2.09 ± 0.58
poly GC/GC	CGC	6	2.14 ± 0.33[q]	0.40 ± 0.05[s]	0.61 ± 0.03[u]	0 ± 0
poly AC/GT	TGT	5	1.28 ± 0.19[q]	0.23 ± 0.07[s]	0.09 ± 0.02[v]	0.36 ± 0.10
poly AG/CT	AGA	6	2.49 ± 0.31[q]	0.26 ± 0.05[s]	0.59 ± 0.06[u]	0.44 ± 0.33
ct DNA	NGN	6	6.20 ± 1.75[q]	0.37 ± 0.07[s]	0.69 ± 0.12[u]	0.40 ± 0.20

* Results are given in millimoles modified base/mole guanine present in the assay. Each value is the mean ± SD of up to six replicate determinations. Superscript letters indicate statistical groupings; values within a column with different letters indicate that they are different at the $P < 0.01$ level (with the exception that i and j differ at the $P < 0.1$ level). The absence of superscripts in a column indicates that all the values within that column are not different at the $P < 0.1$. level.

† Triplet showing central guanine.

toward alkylation in each of the four sequences tested. These data were analyzed by one-way analysis of variance and grouped as determined by the Newman–Keuls test ($P < 0.01$, except for one case noted in Table 1). The central guanine in the base sequence GGG was more susceptible to the formation of 7HOEtG by all three of the nitrosoureas than any of the other triplets tested. For each alkylating agent, there was 6–10 times as much 7HOEtG/G in poly GG/CC as in calf thymus DNA, and roughly one-fifth to one-third as much 7HOEtG/G in the other polymers as in DNA. The base sequence GGG was also the most susceptible of the sequences for 7-chloroethylation by the nitrosoureas showing an enhancement of 2–33 times over the other sequences. The interesting feature of the 7ClEtG/G data is that the values for the sequence TGT from all three nitrosoureas were significantly lower (about one-seventh to one-tenth) than the values for CGC, AGA and NGN. DiGEt was difficult to locate consistently in the chromatograms, so that the results were difficult to analyze statistically. However, it appeared that the base sequence preference of the nitrosoureas to form DiGEt followed a pattern similar to that of the 7HOEtG and 7ClEtG. The susceptibility of the sequence GGG to O-6 hydroxyethylation, when treated with BCNU, was statistically indistinguishable from the other sequences. The susceptibility of GGG to O-6 hydroxyethylation by CCNU or MeCCNU may be slightly greater than the other sequences according to Newman–Keuls groupings for these agents. However, the differences among the amounts of O⁶HOEtG/G found in each of the polynucleotides were not as striking as in the case of 7HOEtG/G.

Comparison of the three alkylating agents reveals that they yielded similar distribution of products, but that BCNU was more reactive. This could be due to the increased solubility of BCNU over the other nitrosoureas tested or different reaction rates for each nitrosourea. The reported half-lives of these agents under conditions similar to those employed here are under 1 hr [29–31]; thus, it was felt that the reactions had gone to completion by the end of the 24-hr incubation period. The total alkylation of a given polynucleotide (μmol alkylated guanine/mmol alkylating agent) differed with the alkylating agent involved; however, the percent distribution amongst the alkylated products was relatively unaffected by the structure of the nitrosourea. If DiGEt was omitted because of difficulties of accurately measuring it, 7HOEtG, O⁶HOEtG, and 7ClEtG occurred at about 95.5, 1 and 3.5%, respectively, in the base sequence GGG, 67.5, 13.5 and 19%, respectively, in the base sequence of CGC, and 87, 4 and 9%, respectively, in NGN. Exceptions to this were a lesser percentage of O⁶HOEtG in GGG treated with BCNU than in GGG treated with CCNU or MeCCNU ($P < 0.01$) and a decrease in the level of 7HOEtG in TGT treated with MeCCNU with a corresponding increase in O⁶HOEtG and 7ClEtG relative to TGT treated with BCNU or CCNU ($P < 0.01$).

DISCUSSION

Base sequence had an effect upon alkylation of guanine by 2-chloroethylnitrosoureas (CENUs) and showed a different effect upon the N-7 position than on the O-6 position (see Table 1). Alkylation at the N-7 position of guanine was highly base sequence

dependent (by a factor of as much as 50-fold), but the nature of the reactive alkylating group (whether to yield a hydroxyethyl product or a chloroethyl product) played a significant role as well. Tong *et al.* [32] found that when calf thymus DNA was exposed to a number of nitrosoureas, the ratios of 7HOEtG/7ClEtG was about 2:1. Hartley *et al.* [12] found that when poly GG/CC was incubated with *cis*-2-hydroxy-CCNU, the ratio of 7HOEtG/7ClEtG was about 5:1. Our ratios for calf thymus DNA were 10:1 and for poly GG/CC about 26:1, both considerably higher than previously reported results. These higher ratios relative to Tong and to Hartley may reflect differences in methods of analysis, buffer systems, or incubation times. Differences in methods of analysis between these reports are minor. Our two-step hydrolysis of the DNA is perhaps the major difference. However, it attained the same endpoint (mild acid hydrolysis) as Tong or Hartley since all the purine bases released in both steps are included and summed in our analysis. Concerning buffer systems, it has been reported previously that increased buffer concentration results in decreased total alkylation of guanine bases due to a competition effect [3]. Various buffer anions may have differing efficiencies of competition with guanine for an alkylating intermediate.* Our scheme, outlined below, contains six intermediates which could lead to alkylation of guanine, four of which lead to hydroxyethylation. The two that are the most stable and most likely to accumulate after the parent compound is depleted are both hydroxyethylating agents. The longer incubation times we employed are sufficient to permit additional reactions with these slower reacting intermediates and it would also allow rearrangements or secondary reactions to occur [23, 33]. This can be seen in Table 1 as the conversion of approximately half of the 7ClEtG to DiGEt in the poly GG/CC, where adjacent guanines are present with which to form DiGEt. The longer incubation time is more comparable to the *in vivo* situation where tumor cytotoxicity could result from such rearranged products [33].

Historically, investigators have tended to explain selectivity of alkylation by CENUs in terms of potential alkylating intermediates that are generated during their spontaneous breakdown in buffered media. However, recent studies by Buckley and Brent [34–36] make it clear that one must consider the possibility of direct interaction between the parent drug and DNA.

Studies of the breakdown of CENUs in buffered media have led to the proposal of a numer of reactive intermediates that may alkylate bases of DNA. Carbenium ions [37] have been demonstrated by a number of workers [29, 38–40]. However, they are not considered important reactants with DNA because the expected kinds and quantities of rearranged alkylated bases are not generated [34, 41–43].

The site selectivity of DNA base alkylation can be explained by interactions of DNA with intermediates that involve S_N2 or concerted reactions more readily than by interactions involving S_N1 reactions. Chemical and isotopic labeling studies have indicated that there are likely several DNA alkylating intermediates formed from the breakdown of CENUs.

The scheme shown in Fig. 1 is based on reactive intermediates that have been identified in CENU breakdown. Lown *et al.* [41, 44–47] have presented evidence that CENUs break down to form the E- and Z-2-chloroethyl diazohydroxide in approximately a 4 to 1 ratio (Fig. 1, **II** and **III**). From synthesis of these compounds they have shown by ^{13}C-NMR that the Z-2-chloroethyl diazohydroxide can cyclize [44, 48] to form the 3,4-dihydro-1,2,3-oxadiazoline (**VI**) (pathway B). This intermediate had been proposed before [28, 49–51]. Parker *et al.* [52] found that in BCNU-alkylated guanine, the chloro group of 7ClEtG had migrated from its original position in the BCNU ethyl group from carbon 2 to carbon 1. They reasoned that a caged Cl^- may be involved in the rearrangement, and we have incorporated the idea in Figs. 1 and 2 (pathway D). The remaining alkylating intermediates of Fig. 1 and the E- and Z-2-hydroxyethyl diazohydroxides (**XI** and **XII**) derived from **VII** as shown in pathway C. Lown and Chauhan [54] have proposed, as have others [53], that chlorine of the CENU is displaced by the carbonyl oxygen to give the 2-alkimino-3-nitroso-2-oxazolidine (**IV**), which has been synthesized. Subsequent reopening of the oxazolidine ring by hydrolysis would give **VII** which should spontaneously decompose to give E- and Z-2-hydroxyethyl diazohydroxide (**XI** and **XII**). Thus, **II**, **III**, **VI**, **XI** and **XII** can all potentially alkylate guanines as shown. We are also proposing that ethylene oxide (**X**), which is formed [44] from the oxadiazoline (**VI**), could also react with the N-7 or O-6 positions of guanine to give rise to additional 7HOEtG(**XV**) and O^6HOEtG (**XIV**). Synthetic 7ClEtG is apparently not converted to 7HOEtG [21]. That 2-chloroethyl (methylsulfonyl) methanesulfonate (ClEtSoSo) alkylates DNA to form 7ClEtG, but not 7HOEtG confirms the lack of conversion of 7ClEtG to 7HOEtG [12].

E-2-Chloroethyl diazohydroxide (**II**) has been shown to form cross-links in DNA, but the corresponding Z-isomer does not [41, 44, 45]. Pathway A has been postulated [44, 45] in which **V**, **VIII** and **XIII** are formed. Intermediate **VIII** is unstable [55] and has been proposed to react with the N-3 of cytosine on the opposite DNA strand to yield the 1-(N^3-deoxycytidyl)-2-(N^1-deoxyguanosinyl)-ethane interstrand cross-link [33]. However, Buckley and Brent [36], using computer modeling techniques, have questioned recently the involvement of **VIII** in cross-linking reactions with cytosine. They report that in B-DNA the Watson–Crick paired cytosine would not allow **V** to assume the appropriate geometry to cyclize and form the N^1,O^6-ethanoguanine (**VIII**). Lown *et al.* [45] have proposed that OH^- competing with the N-3 of cytosine in the attack on **VIII** would result in the O^6HOEtG (**XIV**). However, Parker *et al.* [55] have synthesized O^6-chloroethylguanine (O^6ClEtG, **V**) and found it to be very unstable in aqueous buffer; it spontaneously forms N^1,O^6-ethanoguanine (**VIII**) which then breaks down to N^1HOEtG (**XIII**) rather than

* Ludlum DB, personal communication, cited with permission.

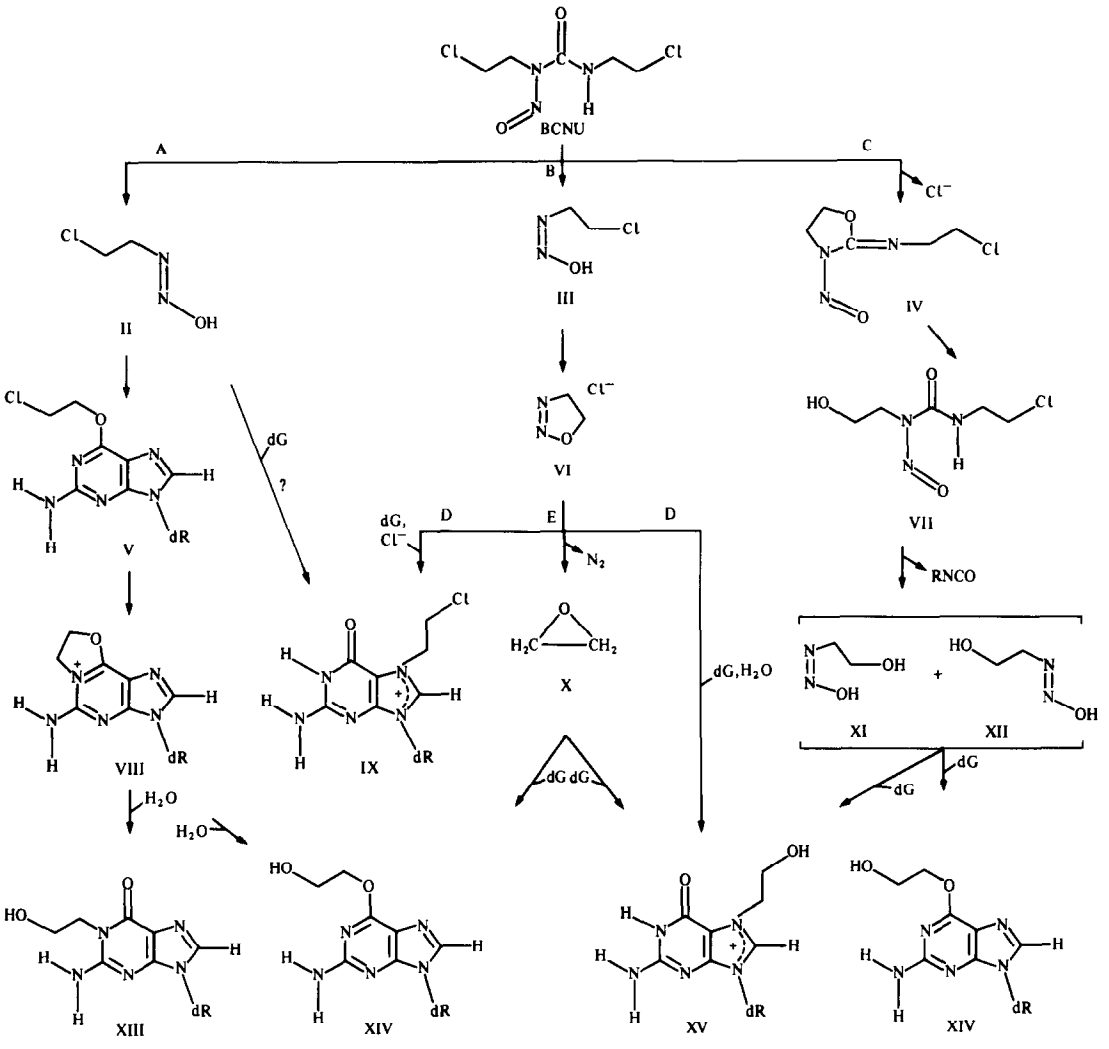

Fig. 1. Proposed pathways of aqueous breakdown of BCNU and reactions with 2′-deoxyguanosine (dG). See text for details.

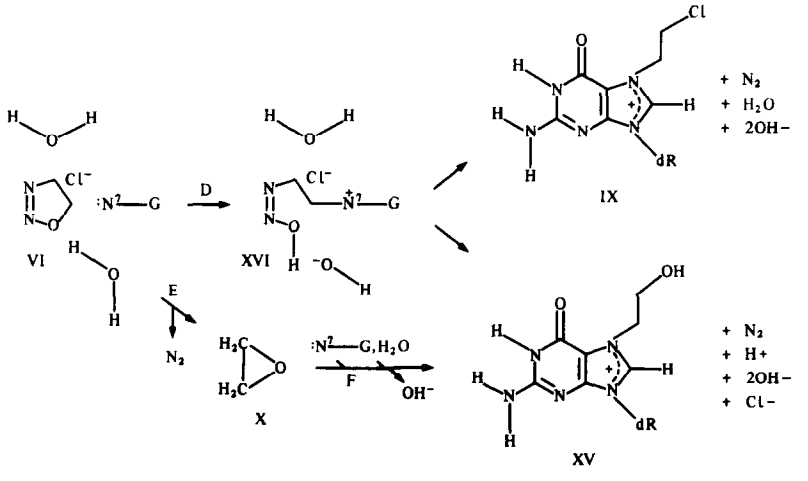

Fig. 2. Proposed possible mechanisms of the reactions of 3,4-dihydro-1,2,3-oxadiazoline with 2′-deoxyguanosine (dG).

O^6HOEtG (**XIV**). Whether this occurs in double-stranded DNA remains to be shown. It is also uncertain whether **III** can attack at the N-7 of guanine to give rise to 7ClEtG.

All three of the nitrosoureas studied here showed a marked increase in N-7 alkylation, particularly hydroxyethylation, of the poly GG/CC compared to the other polymers. Why is this so? The three most obvious effects by guanines as nearest neighbors are (a) steric effects, (b) activating effects on the nucleophilicity of certain guanine sites, and (c) direct chemical participation by neighboring bases.

Consideration of steric effects by base neighbors in double-stranded DNA was based on CPK space-filling models of the pertinent base-paired DNA triplets. Five positions on guanine in single-stranded DNA could be alkylated. However, in double-stranded DNA only N-7 and O-6 in the major groove and N-3 and N-2 in the minor groove were available and those in the minor groove were relatively hindered.

The N-7 and O-6 positions of the central G of GGG and AGA are very exposed to attack, and there was no apparent steric reason why AGA was less reactive than GGG (see Table 1). Although the 5′-pyrimidines of CGC and TGT are closer to the N-7 of G, and could hinder alkylation, it appears that this steric effect is not a factor because CGC and AGA showed about the same degree of alkylation. On the other hand, the 5-methyl group of thymine in TGT sterically hindered the N-7 of G and may explain the reduced chloroethylation observed for TGT compared to GGG. Table 1 shows that the 7ClEtG for poly AC/GT had the lowest value (0.15) of the set of polynucleotides and compares to a range of 1.41 to 4.95 mmol 7ClEtG/mole G for the other polymers. However, the value of 7HOEtG for this polymer was about the same as for other synthetic polymers except for poly GG/CC and may indicate that hydroxyethylation was not sterically hindered. There do seem to be more hydroxyethylating than chloroethylating species for N-7, and some of the hydroxyethylating species are structurally more compact and may be less hindered. It appears, however, that steric effects by base sequence, at best, give only a partial explanation of the observed quantitative results reported here.

Another possible explanation of our results is that the nucleophilic sites on guanine are activated toward alkylation as a consequence of the base sequence. The DNA backbone structure is the same for all DNA-like polymers and a number of bonds intervene between bases. Thus, neither inductive effects nor resonance effects are likely to account for the activating effects observed, since both are through bond phenomena and are ordinarily only effective one or two bonds away [56, 57]. The other known phenomenon that can produce activating effects is field effects, which are transmitted through space rather than through bonds. In double-stranded DNA the bases in the α-helix are stacked in close proximity to each other and, therefore, field effects may be expected. The activating effects may be due to base sequence effects on the electronegativity of the N-7 position of guanine in the GGG polymer. This "electrostatic model" has been proposed previously [12, 14, 58]. The contributions of neighboring bases upon the molecular electrostatic potentials at both the N-7 and O-6 positions of guanine have been calculated by Pullman and Pullman [58]. Their calculations reveal that, for the N-7 position, the electronegativity of guanine in the sequences we tested is GGG $\gg$ AGA > TGT > CGC. This order correlates fairly well with the values for 7HOEtG/G in Table 1, and correlates moderately well with values for 7ClEtG/G except for the value for the sequence TGT. The values of Pullman and Pullman for the electronegativities at the O-6 position of guanine, for the sequences we tested, are in the order: GGG > AGA > CGC = TGT. The values for O^6HOEtG/G in Table 1 do not follow the same order. Thus, changes in molecular potentials alone cannot account for the base sequence selectivity of CENUs that we have observed, but it is likely that they are a contributing factor, especially for N-7 hydroxyethylation.

Activation of the N-7 position of guanine relative to other positions by nearest neighbor effects may cause it to react more efficiently with all alkylating intermediates including less reactive intermediates that are not normally a factor. The 3,4-dihydro-1,2,3-oxadiazoline (**VI**) is a possible candidate as one such intermediate. In our experiments, the amount of alkylation achieved incorporated from 0.01 to 1.7% of the CENU used. This yield is low enough that either Brundrett's estimate of 5% oxadiazoline at pH 7 [50] or Lown's 20% Z-2-chloroethyl diazohydroxide [47] would accommodate the results. The results of Parker *et al.* [52] suggest that 7HOEtG and 7ClEtG may come from the same intermediate, and Fig. 2 shows a two-step reaction (pathway D) that could account for it. A set of concerted reactions can also be written. Once the initial attack by N-7 occurs (Fig. 2), the ratio of 7HOEtG to 7ClEtG would be determined by the relative efficiency of displacement of nitrogen from carbon by chloride ion or water/hydroxyl ion, which we would assume is independent of the nearest neighbor base. This could account for 7HOEtG/7ClEtG ratios for those polymers whose ratios are similar and low [3–5]. The increased absolute amount of 7HOEtG and 7ClEtG in poly GG/CC may be due to activation of G by its nearest neighbors, making it more effective in the ring opening reaction (Fig. 2, step 1, pathway D) relative to the competing formation of ethylene oxide (**X**) (Fig. 2, step 1, pathway E). The net effect of this would be to raise the amounts of 7HOEtG and 7ClEtG relative to their quantities in other polymers. However, this alone would not explain why the 7HOEtG/7ClEtG ratio in poly GG/CC is different from that ratio in the other polymers. From Table 1, the 7HOEtG/7ClEtG ratio for GGG treated with BCNU was 27 while the values of the 7HOEtG/7ClEtG ratio for CGC, TGT, AGA, and DNA treated with BCNU were 3.4, 30, 5.5, and 10 respectively. Clearly, there is an effect by the base sequence upon this ratio. Thus, the data indicate that the activating effect by base sequence for 7HOEtG formation is higher than for 7ClEtG. It would seem to require at least one additional intermediate to account for this result. Any Z- or E-2-hydroxyethyl diazohydroxide (**XI** and **XII**) present could also form

7HOEtG (**XV**) (Fig. 1). Perhaps one (or both) of these isomers is more reactive with activated G in poly GG/CC than in other polymers. Another possibility is that activated G in poly GG/CC reacts with ethylene oxide (Fig. 2, reaction F). Ethylene oxide (**X**) is probably more stable than any of the diazohydroxides and, therefore, may be a better candidate for increased nucleophilic attack by activated N-7 of guanines. It is of interest that ethylene oxide is the alkylating agent used to synthesize the 7HOEtG standard from guanosine [27].

A third general way to account for the base sequence effects on the alkylation of G in runs of G in DNA has been proposed recently by Buckley and Brent [34–36]. Based on kinetic analyses of the alkylation of DNA and studies of the spontaneous decomposition of nitrosoureas, they have concluded that the alkylation of two adjacent guanines (dG_1dG_2) occurs by the direct addition of the imido form of the nitrosourea to the O^6 or N-7 position of dG_1 to form a tetrahedral addition complex. The chloroethyl part of the complex is transferred to the O^6 or N-7 group of the adjacent dG_2 by a displacement reaction to produce alkylated-dG_2 [34, 36]. This is an attractive proposal to account for the base sequence specificity for alkylations by runs of guanine in DNA, and it should be taken into consideration in the experimental designs of future studies. We cannot rule out this mechanism in our studies, but we do not think it accounts for all of the results. For example, the ratios of 7HOEtG/7ClEtG are not easily explained this way unless one envisions a hydroxyethyl nitrosourea being involved in hydroxyethylation. This would involve the additional set of reactions such as formation of **IV** and then **VII** in our scheme (Fig. 1). It seems unlikely that a pathway which requires two extra steps for hydroxyethylation would be favored over the more direct chloroethylation by the parent CENU.

In summary, the results of these studies indicate that base sequence has an effect on both the total alkylation by CENUs and base site selectivity, but the precise reason for this awaits further investigation with model systems. It was also shown that, for the three alkylating nitrosoureas studied, there was little difference between them in their attraction to a given site of alkylation except for the improved solubility and reactivity of BCNU, as demonstrated by the higher overall alkylation by BCNU.

Many oncogenes are known to have regions of high guanine content within them or associated with their flanking regions [15]. Such oncogenes are comprised of specific base sequences that are crucial for the expression of a transformed or metastatic phenotype in a tumor. For example, the SV_{40} genome contains six copies of the regulatory sequence GGGCGG important to its transforming function. Related GC containing elements are found in the flanking regions of many oncogenes and in retroviruses [13, 14, 59–61]. In view of the preponderance of stretches of two, three, or more adjacent and/or nearby guanines in these oncogenes, it would appear that these guanines might be the most likely targets for chemical modification by 2-chloroethylnitrosoureas or other agents. Therefore, future work necessitates the identification of the lesion(s) which is(are) responsible for the cytotoxicity of a drug. It is also imperative that the base sequence specificity of each lesion (and especially the cytotoxic ones) be determined so that each drug or combination of drugs with their characteristic specificities can be properly employed to modify and inactivate oncogenes characteristic of specific tumors.

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