Mechanism of Inactivation of Human O^6-Alkylguanine-DNA Alkyltransferase by O^6-Benzylguanine†

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ABSTRACT: Human O^6-alkylguanine-DNA alkyltransferase was rapidly inactivated by low concentrations of O^6-benzylguanine, but the alkyltransferase from the *Escherichia coli ogt* gene was much less sensitive and alkyltransferases from the *E. coli ada* gene or from yeast were not affected. O^6-Benzyl-2′-deoxyguanosine was less potent than the base, but was still an effective inactivator of the human alkyltransferase and had no effect on the microbial proteins. O^6-Allylguanine was somewhat less active, but still gave complete inactivation of both the human and Ogt alkyltransferases at 200 μM in 30 min, slightly affected the Ada protein, and had no effect on the yeast alkyltransferase. O^4-Benzylthymidine did not inactivate any of the alkyltransferase proteins tested. Inactivation of the human alkyltransferase by O^6-benzylguanine led to the formation of *S*-benzylcysteine in the protein and to the stoichiometric production of guanine. The rate of guanine formation followed second-order kinetics ($k = 600\ M^{-1}\ s^{-1}$). Prior inactivation of the alkyltransferase by reaction with a methylated DNA substrate abolished its ability to convert O^6-benzylguanine into guanine. These results indicate that O^6-benzylguanine inactivates the protein by acting as a substrate for alkyl transfer and by forming *S*-benzylcysteine at the acceptor site of the protein. The inability of O^6-benzylguanine to inactivate the microbial alkyltransferases may be explained by steric constraints at this site. The reduced effectiveness of the allyl compared to the benzyl derivative is in accord with its expected, lower rate of participation in bimolecular displacement reactions, and its ability to inactivate the Ogt alkyltransferase may be explained by its smaller size permitting access to this active site. These studies unequivocally show that the human alkyltransferase protein can act on low molecular weight substrates lacking the polynucleotide structure. In addition to their use to inactivate the protein, such substrates may also prove useful for the assay of mammalian alkyltransferase.

Specific proteins are present in virtually all organisms to prevent the accumulation of alkylated bases in DNA. A particularly important protein in this respect is O^6-alkylguanine-DNA alkyltransferase (EC 2.1.1.63) (alkyltransferase[1]) (Yarosh, 1985; Lindahl et al., 1988; Demple, 1990; Pegg, 1990; Pegg & Byers, 1992; Mitra & Kaina, 1993). This protein removes O^6-alkylguanine, a product that has been implicated in mutagenesis and carcinogenesis. Cells containing high levels of alkyltransferase show significant resistance to the cytotoxic, carcinogenic and mutagenic effects of methylating agents, and it is likely that this resistance is due to the effective repair of O^6-methylguanine by this protein. In an attempt to provide more information in support of this concept, modulations of the alkyltransferase level either by transfections with plasmid constructs expressing alkyltransferase or by applications of inhibitors of the alkyltransferase have been used [reviewed in Pegg (1990), Pegg and Byers (1992), Mitra and Kaina (1993)]. Of the available inhibitors, by far the most effective is O^6-benzylguanine (Dolan et al., 1990a, 1991a). Exposure of human cell extracts to 2.5 μM O^6-benzylguanine leads to the irreversible loss of 90% of the alkyltransferase activity within 10 min (Dolan et al., 1990a). O^6-Benzylguanine is readily taken up by mammalian cells, and exposure to 2.5 μM in the culture medium effectively removes all alkyltransferase activity (Dolan et al., 1990a, 1991a).

In the present work, we have investigated in detail, using the purified human protein, the mechanism by which O^6-benzylguanine causes the inactivation of alkyltransferase. This information will be of value in providing tools to increase knowledge of the mechanism of action of this protein and in the design of improved inhibitors that can be used to study the role of this protein in response to alkylating agents and to enhance the chemotherapeutic potential of alkylating agents. Our results show that O^6-benzylguanine is accepted as a

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[1] Abbreviations: alkyltransferase, O^6-alkylguanine-DNA alkyltransferase (EC 2.1.1.63); IPTG, isopropyl β-D-thiogalactoside; PCA, perchloric acid; RP-HPLC, reversed-phase high-pressure liquid chromatography.

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substrate at the active site of the mammalian alkyltransferase protein, leading to the formation of *S*-benzylcysteine at the active site with the production of stoichiometric amounts of guanine. Despite a close similarity in the amino acid sequences containing this cysteine at the active site, the Ada alkyltransferase from *Escherichia coli* (Lindahl et al., 1988; Bhattacharyya et al., 1988; Demple, 1990) and the alkyltransferase from *Saccharomyces cerevisiae* (Xiao et al., 1991) were not inactivated by O^6-benzylguanine and did not generate guanine from it. The Ogt alkyltransferase (Rebeck et al., 1989; Margison et al., 1990) from *E. coli* was only marginally sensitive. The Ogt alkyltransferase and the human alkyltransferase were readily inactivated by O^6-allylguanine. In contrast, the yeast alkyltransferase was not inactivated by O^6-allylguanine, and the *E. coli* Ada alkyltransferase was only slightly affected. Useful inactivators of alkyltransferase that are species-selective may be produced by taking advantage of these properties.

MATERIALS AND METHODS

Purification and Assay of Alkyltransferase. A plasmid vector for the expression of human alkyltransferase in *E. coli* was constructed as follows. Primers 1 (5′-TTGGATCTTG-GAATTCTGGACAAGGATTGT-3′) and 2 (5′-TACTG-GATCCACTCAGTTTCGGCCAGCAG-3′) that correspond to sequences at the ends of the coding region (bases 97–720) of the human alkyltransferase cDNA sequence (Tano et al., 1990; Hayakawa et al., 1990; Rydberg et al., 1990) were synthesized. Primer 1, corresponding to bases 89–111, was designed to contain an *Eco*RI restriction site, which alters the start codon sequence from ATG to CTG. Primer 2, corresponding to bases 704–732, introduces a *Bam*HI site seven bases after the termination codon. These primers were then used to isolate the alkyltransferase cDNA sequence from DNA contained in a human liver cDNA library in the Uni-ZAPXR vector (Stratagene, La Jolla, CA). A single DNA band in the 600–700-bp range was isolated and digested with *Eco*RI and *Bam*HI, and the fragment was inserted into the *E. coli* expression vector pINIII-A3($lpp^{P\text{-}5}$) (Duffaud et al., 1987) cut with the same enzymes. The plasmid was introduced into *E. coli* DH5α cells by electroporation, and six clones containing plasmids with inserts of the correct size were assayed for alkyltransferase activity. Two clones with high activity were taken, and one of these (pINAGT) was used for protein isolation. The sequence of the insert in pINAGT was determined, and it corresponded to the published human alkyltransferase sequence (Tano et al., 1990; Hayakawa et al., 1990; Rydberg et al., 1990), with the exception of a silent point mutation in the codon for histidine-86 where a C replaced the T in the third position. The method of construction changes the amino terminus of the alkyltransferase by the addition of five amino acids, giving a sequence of MKGGIL in place of M.

For purification of the alkyltransferase, the cells containing pINAGT were grown in 1–4 L of LB broth with 50 μg/mL ampicillin. Expression of recombinant alkyltransferase was induced with 0.15 mM IPTG, and the cells were harvested about 12 h later when the A_{600} was 1.7–1.8. The cells were pelleted at 4000*g* for 10 min at 4 °C, resuspended in LB broth, and pelleted again. All subsequent operations were carried out at 4 °C. The cell pellets were resuspended in 20 mM Tris-HCl (pH 8.5), 1 mM EDTA, 3 mM dithiothreitol, and 0.4 M NaCl, and the cells were broken with a French Press. Insoluble debris was pelleted at 90000*g* for 30 min, and the supernatant was saved. Polymin P (in 5% solution, pH 8.0) was added to give 0.36 μg of Polymin P/μg of DNA, and the mixture was centrifuged at 15000*g* for 15 min. The supernatant was taken and made 40% saturated in ammonium sulfate. After 30 min, the precipitated protein was removed by centrifugation at 15000*g* for 15 min, and more ammonium sulfate was added to obtain 55% saturation. After 30 min, the precipitated protein was collected at 15000*g* for 15 min and dissolved in buffer A (50 mM Hepes (pH 8.0) containing 1 mM EDTA and 3 mM dithiothreitol). After it was desalted by passage through a PD-10 desalting column (Pharmacia) equilibrated with buffer A, the solution was fractionated by cation exchange chromatography using a Mono S HR 10/10 FPLC column (Pharmacia) at room temperature. After it was loaded at a flow rate of 2 mL/min, the column was washed with buffer A at 4 mL/min until the A_{280} returned to base line. Then, the column was eluted with a gradient of 0–1 M NaCl in buffer A, such that 0.3 M NaCl was reached within 40 min. The alkyltransferase activity eluted at about 0.15 M NaCl. The fractions corresponding to this peak were pooled and concentrated.

Alkyltransferase activity was measured by assaying the loss of [^{3}H]-O^6-methylguanine from a [^{3}H]methylated DNA substrate as previously described (Dolan et al., 1991a). Protein was determined by the method of Bradford (1976). This preparation showed a single major band of MW 22 000, corresponding to the alkyltransferase, and a few minor bands that amounted to less than 5% of the total on analysis by polyacrylamide gel electrophoresis under denaturing conditions. The specific activity of the preparation was 75% of that expected if all the protein had been active alkyltransferase, and this value was used in the calculations.

Preparation of O^4-Benzylthymidine. Thymidine (1.1 g, 4.5 mmol), together with 0.23 g of NaOH (5.75 mmol), was dissolved in 25 mL of 2,2,2-trifluoroethanol. Benzyl bromide (0.55 mL, 4.5 mmol) and $AgNO_3$ (0.78 g, 4.6 mmol) were added, and the suspension was stirred vigorously for 72 h at room temperature. The resulting suspension was filtered, and the filtrate was diluted with 15 mL of methanol and 60 mL of water. This solution was loaded on a 2.8 × 71 cm Sephadex LH-20 column eluted with methanol/water (4:6) at a flow rate of 1 mL/min. The absorbance was monitored continuously at 280 nm and fractions (10 mL) were collected. Unreacted thymidine eluted in fractions 30–45. O^4-Benzylthymidine (200 mg) crystallized as fine needles in fractions 60–70: UV (nm) λ_{max} 280, λ_{min} 241; ^{1}H NMR (DMSO-d_6) δ 1.92 (s, 3H, 5-CH_3), 2.03 (m, 1H, 2′-H), 2.21 (m, 1H, 2″-H), 3.60 (m, 2H, 5′-H), 3.82 (m, 1H, 4′-H), 4.23 (m, 1H, 3′-H), 5.04 (t, 1H, 5′-OH, exchanges with D_2O), 5.21 (d, 1H, 3′-OH, exchanges with D_2O), 5.36 (s, 2H, $ArCH_2$), 6.15 (t, 1H, 1′-H), 7.30–7.47 (m, 5H, Ar), 8.06 (s, 1H, 6-H). Anal. Calcd for $C_{17}H_{20}N_2O_5$: C, 61.44; H, 6.06; N, 8.43. Found: C, 61.82; H, 5.96; N, 8.72.

Inactivation of Alkyltransferase from Different Species. Studies of the inactivation of alkyltransferase from various species by O^6-benzylguanine and related compounds were carried out using *E. coli* extracts from cells expressing the various proteins as follows. The extracts were obtained from *E. coli* GWR111, a derivative of AB1157 with Δ*ada-25* and *ogt-1::Kan*^{r} transformed with either pUC19, pDS400 that carries the *E. coli ada* gene (Shevell, 1991), pUCogt with the *E. coli ogt* gene (Rebeck & Samson, 1991), pWX1113 with the *S. cerevisiae MGT1* gene (Xiao et al., 1991), or pUCMGMT carrying the human *MGMT* cDNA open reading frame (Rebeck et al., 1989; Tano et al., 1990; Hayakawa et al., 1990); all four alkyltransferase genes were expressed from

the *lac* promoter. Crude extracts of each transformant were made in alkyltransferase buffer (50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 0.1 mM EDTA), as described (Xiao et al., 1990), and the protein concentration was determined (Bradford, 1976). Assays were carried out using *Micrococcus luteus* DNA containing [^{3}H]-O^6-methylguanine as the predominant lesion prepared according to the method of Karran et al. (1979); DNA was alkylated with [^{3}H]methylnitrosourea from Amersham Corp. (21.5 Ci/mmol) to give a specific activity of 260 cpm/μg. Bacterial cell extracts containing 0.03 pmol of alkyltransferase were preincubated for 30 min with the four different potential inhibitors, O^6-benzylguanine (Dolan et al., 1990a), O^6-benzyl-2′-deoxyguanosine (Moschel et al., 1992), O^6-allylguanine (Moschel et al., 1992), and O^4-benzylthymidine (synthesized as described above), at 30 °C for the pUCogt- and pWX1113-transformed cells and 37 °C for the pUCMGMT and pDS400-transformed cells. Following the preincubation, 4.2 μg of *M. luteus* DNA substrate containing [^{3}H]-O^6-methylguanine was added, and the mixture was incubated for 1 h at their respective temperatures. Perchloric acid (PCA) was added to 1 M and incubated at 70 °C for 1 h to hydrolyze the DNA. Total protein was precipitated by centrifugation, washed twice with 1 M PCA, and resuspended in 0.2 mL of 10 mM NaOH, and the radioactivity was scintillation counted in 10 mL of Hydrofluor (National Diagnostics).

Formation of Guanine from O^6-Benzylguanine. O^6-Benzylguanine was synthesized as previously described (Dolan et al., 1990a). [^{3}H]-O^6-Benzylguanine was produced from it by Amersham Corporation (Arlington Heights, IL) by catalytic tritium exchange with 20 Ci of tritiated water using method TR8, as described in the manufacturer's 1992 catalog, page 143. The crude [^{3}H]-O^6-benzylguanine (340 Ci/mol) was purified by HPLC on a Beckman Ultrasphere ODS column (25 cm × 4.6 mm) using isocratic elution at a temperature of 35 °C and a buffer of equal parts methanol and 0.05 M ammonium formate (pH 4.5). Analysis of the purified material following acid hydrolysis showed that all of the ^{3}H label was in the guanine moiety, presumably at the 8-position. Measurements of guanine formation from O^6-benzylguanine were carried out using various amounts of the [^{3}H]-O^6-benzylguanine and alkyltransferase protein in an assay buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 5 mM dithiothreitol. The formation of labeled product was stopped by the addition of 0.6–0.8 mL of the same buffer containing 0.2 mM guanine and 0.2 mM O^6-benzylguanine. Aliquots were then separated by HPLC as described above. Samples containing the purified alkyltransferase were analyzed directly. Those samples containing larger amounts of protein (>0.1 mg) were freed from protein by use of Centricon 10 microconcentrators (Amicon, Beverly, MA) centrifuged at 5000*g* for about 1 h prior to separation.

The ability of alkyltransferases from various species to convert [^{3}H]-O^6-benzylguanine to [^{3}H]guanine was determined by incubating various amounts of protein (from 0.1 to 2 mg) with 4 μM [^{3}H]-O^6-benzylguanine and alkyltransferase protein in a total volume of 0.25 mL of 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 5 mM dithiothreitol. The extracts used were obtained from 500-mL cultures of *E. coli* carrying plasmids expressing the relevant enzymes, using pSM41 for the *E. coli* Ada protein (Bhattacharyya et al., 1988), pINAGT for the human protein (see above), pUCOgt (Rebeck & Samson, 1991) for the *E. coli* Ogt protein, and pWX1113 (Xiao et al., 1991) for the yeast protein. The two former plasmids express high levels of the alkyltransferase, and the crude cell extracts were used directly. The latter two plasmids express lower amounts of alkyltransferase protein, and these extracts were purified as described above for the human protein to provide extracts with an alkyltransferase activity sufficiently high that at least 10 000 cpm would have been expected to be converted to the guanine peak on the basis of the alkyltransferase activity and a 1:1 stoichiometry. Alkyltransferase activity against methylated DNA substrates present in these extracts was measured by assaying the loss of [^{3}H]-O^6-methylguanine from a [^{3}H]methylated DNA substrate as described by Dolan et al. (1991a).

Table I: Formation of *S*-Benzylcysteine in Human Alkyltransferase after Inactivation by O^6-Benzylguanine[a]

amino acid	radioactivity present (cpm): control alkyltransferase	alkyltransferase exposed to O^6-benzylguanine
experiment 1		
methionine	1730[b]	1643
cysteine	5896	4576
S-benzylcysteine	5	153 (12% of loss in cysteine)
experiment 2		
methionine	6566[b]	7328
cysteine	37298	29389
S-benzylcysteine	10	5038 (64% of loss in cysteine)
experiment 3		
methionine	19626[b]	18241
cysteine	113296	92187
S-benzylcysteine	90	14045 (66% of loss in cysteine)

[a] The experiments were carried out as described in the legend to Figure 2. The radioactivity present in each fraction was determined after subtraction of the mean base-line figure beneath the fractions forming this peak. The differences between experiments 1–3 were in the amount of alkyltransferase protein used and the amount of trypsin and carboxypeptidase added. These were as follows: experiment 1 1.25 μg of alkyltransferase, 0.125 μg of trypsin, and 0.0625 μg of carboxypeptidase; experiment 2 3.25 μg of alkyltransferase, 0.325 μg of trypsin, and 3.25 μg of carboxypeptidase; experiment 3 13.25 μg of alkyltransferase, 13.25 μg of trypsin, and 13.25 μg of carboxypeptidase. [b] Human alkyltransferase contains five cysteine and four methionine residues, but the cells from which it was isolated were grown in the presence of methionine and [^{35}S]sulfate to suppress the incorporation of radioactivity into methionine.

Formation of S-Benzylcysteine in Alkyltransferase. E. coli containing pINAGT were grown in the presence of 0.3 mM IPTG and 0.2 mg of [^{35}S]sulfate (25 mCi/mg) in M9 medium supplemented with essential amino acids, including methionine but not cysteine, for 3.5 h. The cells were then harvested, and the alkyltransferase was purified through the Mono S step as described above. The purified, ^{35}S-labeled alkyltransferase protein (1.25–13.25 μg, depending on the experiment) was incubated with or without 200 μM O^6-benzylguanine for 60 min at 37 °C. [This represents a large excess (316–3344-fold) of O^6-benzylguanine over the amount of alkyltransferase protein.] The protein was then digested with trypsin (Boehringer-Mannheim, sequencing grade) after the addition of 5% acetonitrile and heating at 60 °C for 10 min to enhance the sensitivity to the protease. The total volume of digestion was 0.1 mL. After 24 h at 37 °C, the samples were made up to a volume of 1 mL and applied to Centricon 3 microconcentrators (MW cutoff of about 3000; Amicon), which were spun at 7500*g* until the volume was reduced to 0.5 mL. An additional 0.95 mL of water was added and the concentration repeated. The combined filtrates containing materials that pass through the concentrator were evaporated to dryness. The samples were then resuspended in 0.1 mL of 0.2 M sodium acetate (pH 5.5) containing 0.5% acetonitrile, heated at 60 °C for 20 min, and digested with carboxypeptidase Y (Boehringer-Mannheim) for 8 h at 25 °C. In experiments 2 and 3 (Table I), additional carboxypeptidase was added,

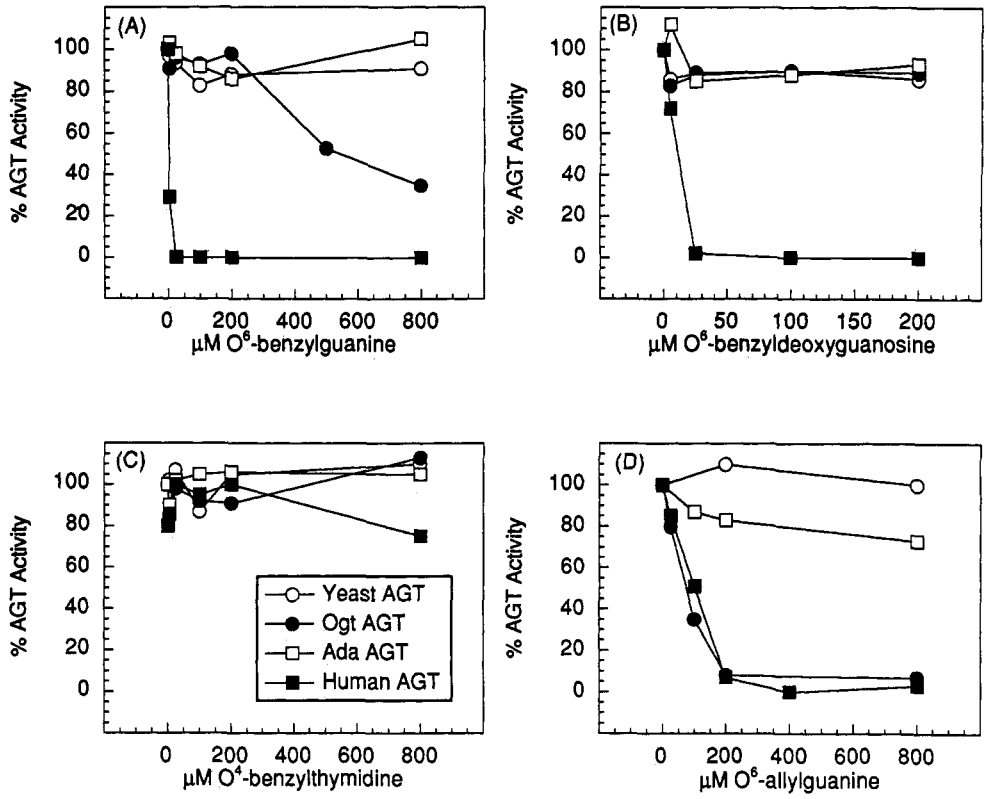

FIGURE 1: Inactivation of alkyltransferases from different organisms by O^6-benzylguanine and related compounds. Extracts from *E. coli* expressing the human (■), yeast (○), *E. coli* Ada (□), or *E. coli* Ogt (●) alkyltransferases from similar plasmid expression vectors containing 0.3 pmol of alkyltransferase were used as a source of protein. The inactivation by O^6-benzylguanine (A), O^6-benzyl-2′-deoxyguanosine (B), O^4-benzylthymidine (C), or O^6-allylguanine (D) was determined. The extracts were incubated for 30 min in 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 0. 1 mM EDTA at 37 °C for the Ada and human alkyltransferases and at 30 °C for the yeast and Ogt alkyltransferases. After the 30-min incubation, the *M. luteus* DNA substrate containing [^{3}H]-O^6-methylguanine (21.5 Ci/mmol) was added, and the mixture was incubated for 1 h at the respective temperatures. The alkyltransferase activity was determined from the transfer of radioactivity from this substrate to protein. The results are expressed as a percentage of the alkyltransferase activity present in control extracts preincubated for 30 min without a putative inhibitor. At the temperatures used, there was less a than 10% loss of activity during preincubation with no inhibitor.

and the incubation was continued for an additional 12 h. The samples were then separated by ion pair RP-HPLC as previously described for polyamine samples (Byers et al., 1992). Aliquots were collected and radioactivity was determined. The elution positions of methionine, cysteine, and *S*-benzylcysteine were determined using detection of the fluorescent products formed by post-column reaction with *o*-phthalaldehyde.

RESULTS

Ability of O^6-Benzylguanine and Related Compounds To Inactivate Alkyltransferases from Different Species. Although O^6-benzylguanine was a very potent inactivator of the human alkyltransferase, it was not effective against either the *E. coli* Ada or the yeast alkyltransferases, even when added at concentrations of up to 800 μM (Figure 1). The *E. coli* Ogt alkyltransferase was inactivated by O^6-benzylguanine but was much less sensitive than the human protein (Figure 1A). The human alkyltransferase is also inactivated by the 2′-deoxyribonucleoside of O^6-benzylguanine although, as previously reported (Moschel et al., 1992), this was less effective than the free base (Figure 1B). None of the microbial alkyltransferases were inactivated by O^6-benzyl-2′-deoxyguanosine at concentrations of up to 200 μM. Although the bacterial alkyltransferases are able to repair O^4-methylthymine in DNA relatively easily (Lindahl et al., 1988; Margison, et al. 1990; Sassanfar et al., 1991), while the human and yeast proteins are less effective (Brent et al., 1988; Koike et al., 1990; Sassanfar et al., 1991), none of alkyltransferases tested, including the human protein, was inactivated by O^4-benzylthymidine (Figure 1C). When the benzyl group of O^6-benzylguanine was replaced by an allyl moiety, the human and Ogt alkyltransferases were sensitive to inactivation, the Ada alkyltransferase was slightly affected, and the yeast protein was not inhibited (Figure 1D).

Formation of S-Benzylcysteine in the Inactivated Human Alkyltransferase Protein. The normal reaction of alkyltransferase is to bring about the transfer of the alkyl group from DNA to a cysteine acceptor residue that is contained within the alkyltransferase amino acid sequence (Yarosh, 1985; Lindahl et al., 1988; Pegg, 1990; Demple, 1990; Pegg & Byers, 1992; Mitra & Kaina, 1993). Since the alkylcysteine formed in this reaction is not converted back to cysteine, the protein can act only once and is not really an enzyme. The most likely explanation for the irreversible inactivation of the human alkyltransferase by O^6-benzylguanine is that O^6-benzylguanine acts as an alternate substrate, forming S-benzylcysteine at the active site. In order to test this possibility, the recombinant human alkyltransferase was purified from *E. coli* cultures grown in the presence of [^{35}S]sulfate to label the cysteines in the protein. The protein was then incubated in the presence or absence of O^6-benzylguanine and digested sequentially with trypsin followed by carboxypeptidase Y. The resulting amino acids were separated by ion pair reversed phase HPLC.

In the digestions of control alkyltransferase, the radioactivity was present in the methionine and cysteine peaks (Table I, Figure 2). When the alkyltransferase was inactivated by reaction with O^6-benzylguanine, radioactivity was present in *S*-benzylcysteine and the amount of radioactivity in the cysteine peak declined (Figure 2). This experiment was carried out with three different digestion conditions, and in all cases,

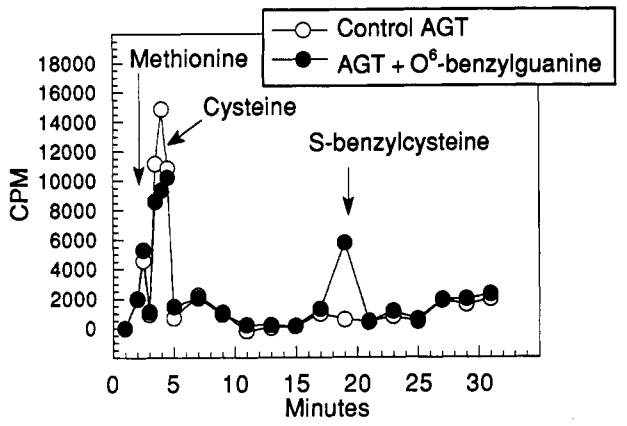

FIGURE 2: Formation of *S*-benzylcysteine in human alkyltransferase incubated with O^6-benzylguanine. The recombinant human alkyltransferase was obtained from *E. coli* grown in the presence of [^{35}S]-sulfate. After incubation for 60 min in the presence (●) or absence (○) of 200 μM O^6-benzylguanine, the protein was digested sequentially with trypsin or carboxypeptidase C as described in Materials and Methods. The amino acids were then separated by ion pair reversed-phase HPLC, and the positions of known standards of methionine, cysteine, and *S*-benzylcysteine are shown. The results shown correspond to experiment 2 of Table I.

S-benzylcysteine was detected in the inactivated alkyltransferase. Furthermore, the decline in cysteine of about 21% (22%, 22%, and 19%, respectively, in the three different conditions) is consistent with the fact that the alkyltransferase contains five cysteine residues, only one of which serves as an acceptor. However, the amount of *S*-benzylcysteine found was less than the decline in cysteine: actually 12, 64, and 66% of the expected values (Table I). It is probable that the presence of the benzyl group inhibits the digestion by carboxypeptidase, and the very low recovery was obtained in the digestion using the smallest amounts of carboxypeptidase (experiment 1). An increase in the amount of trypsin did not affect the yield of *S*-benzylcysteine (experiment 3). The HPLC system used for amino acid analysis does not separate peptides, and the slowly increasing background probably contains partially digested material, which may include the missing fraction of *S*-benzylcysteine.

Formation of Guanine from O^6-Benzylguanine. The transfer of a benzyl group from O^6-benzylguanine to the alkyltransferase protein should be accompanied by the formation of a stoichiometric amount of guanine. As shown in Figure 3, [^{3}H]guanine was produced when the purified human alkyltransferase protein was incubated with [^{3}H]-O^6-benzylguanine. The production of guanine was completely prevented if the alkyltransferase was preincubated with a methylated DNA substrate (Figure 3). A more detailed investigation of this inhibition using 150 pmol of the alkyltransferase showed that there was no effect on the formation of guanine from O^6-benzylguanine after preincubation with the control calf thymus DNA, a 52% reduction after preincubation with DNA containing 80 pmol of O^6-methylguanine, and complete inhibition when DNA containing 400 pmol of O^6-methylguanine was used (Table II).

The formation of guanine was proportional to the amount of alkyltransferase added, until amounts of alkyltransferase exceeding the amount of [^{3}H]-O^6-benzylguanine were used and all of the substrate was exhausted (Figure 4). When the O^6-benzylguanine was in excess, the amount of guanine formed was stoichiometric with the amount of alkyltransferase added. The production of guanine was time dependent (Figure 5A), and the reaction ceased when an amount of guanine equivalent to the amount of alkyltransferase added was produced (Figure 5B). Addition of more alkyltransferase led to the formation of the expected amount of additional guanine (Figure 5B). The reaction between O^6-benzylguanine and the alkyltransferase showed second-order kinetics (Figure 5A). The approximate value for the second-order rate constant was 6

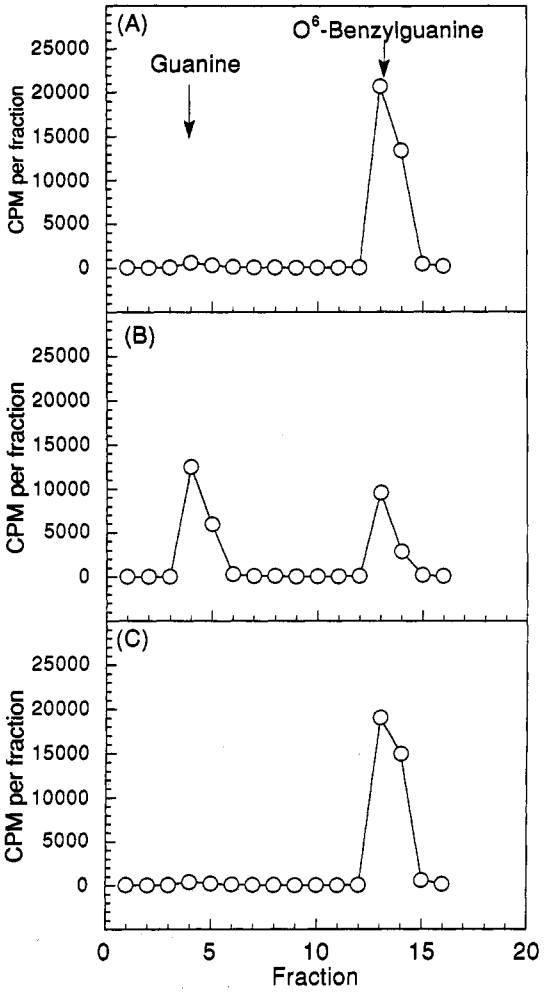

FIGURE 3: Conversion of O^6-benzylguanine to guanine by human alkyltransferase. A total volume of 0.065 mL containing 270 pmol of [^{3}H]-O^6-benzylguanine was incubated for 30 min at 37 °C with no additions (A), with 3 μg of human alkyltransferase (B), or with 3 μg of human alkyltransferase that had been preincubated for 15 min with 200 μg of DNA methylated by reaction with 5 mM *N*-methyl-*N*-nitrosourea (C). The formation of [^{3}H]guanine was then measured by subjecting the sample to HPLC on a Beckman Ultrasphere ODS column (25 cm × 4.6 mm), using isocratic elution at a temperature of 35 °C and a flow rate of 1 mL/min with a buffer of equal parts methanol and 0.05 M ammonium formate (pH 4.5). Fractions of 1 mL were collected, and radioactivity was determined as shown.

Table II: Effect of Methylated DNA on the Ability of Human Alkyltransferase To Convert O^6-Benzylguanine to Guanine[a]

addition	guanine formed (pmol)
none	141
40 μg of DNA	138
200 μg of DNA	136
40 μg of methylated DNA	66
200 μg of methylated DNA	2
no alkyltransferase added	2

[a] A total volume of 0.065 mL containing 4.2 μM [^{3}H]-O^6-benzylguanine (270 pmol) was incubated for 30 min at 37 °C with 150 pmol of human alkyltransferase that had been preincubated for 15 min with calf thymus DNA or calf thymus DNA methylated by reaction with 5 mM *N*-methyl-*N*-nitrosourea.

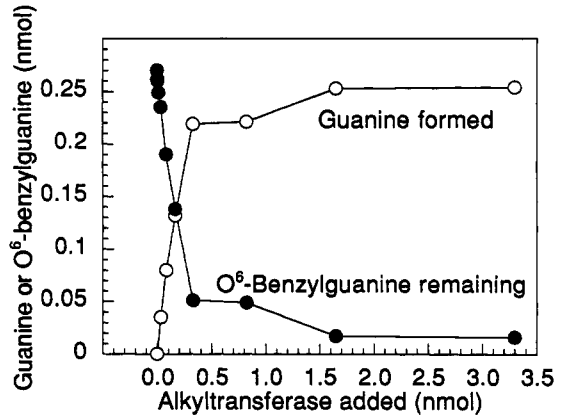

FIGURE 4: Effect of alkyltransferase concentration on guanine formation. The amount of human alkyltransferase shown was incubated for 30 min at 37 °C with 270 pmol of [^{3}H]-O^6-benzylguanine (13.5 μM) in a total volume of 0.02 mL. The amounts of guanine produced and O^6-benzylguanine remaining were then determined by HPLC, as in Figure 3.

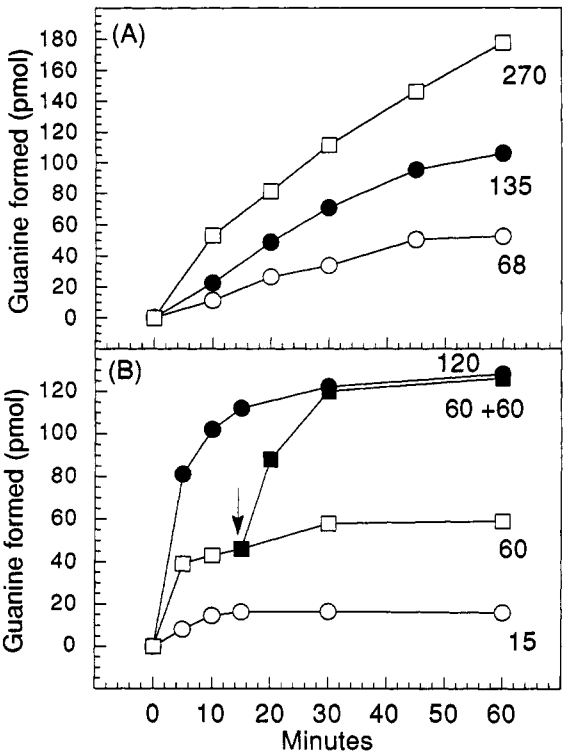

FIGURE 5: Rate of guanine formation by human alkyltransferase. In the experiment shown in A, a total volume of 0.3 mL was used containing 300 pmol of [^{3}H]-O^6-benzylguanine (1 μM) and 67.5 (○), 135 (●), or 270 (□) pmol of alkyltransferase, as indicated. In the experiment shown in B, 270 pmol of [^{3}H]-O^6-benzylguanine (13.5 μM) was incubated in a total volume of 0.02 mL with either 15 (○), 60 (□), or 120 pmol (●) of alkyltransferase. At the point indicated by the arrow, an additional 60 pmol of alkyltransferase was added to some tubes (■).

$\times 10^2$ M^{-1} s^{-1}. This is about 10 000 times less than the rate constant for double-stranded oligodeoxynucleotide substrates containing O^6-methylguanine, but only 500 times lower than that for the repair of ethyl groups in such a substrate (Scicchitano et al., 1986; Pegg & Dolan, 1989; Mitra & Kaina, 1993). The rate is several hundred times faster than that for the inactivation of alkyltransferase by O^6-methylguanine, for which a rough estimate of the second-order rate constant was about 0.2 M^{-1} s^{-1} (Dolan et al., 1985; Yarosh et al., 1986; Pegg & Dolan, 1989).

Table III: Production of Guanine from O^6-Benzylguanine by Extracts from *E. coli* Expressing Recombinant AGTs[a]

strain	alkyl-transferase expressed	alkyltransferase activity (pmol/mg)	guanine formed (pmol/mg)
DH5α/pIN	none	1[b]	<0.1
DH5α/pINAGT	human	750	630
pSM41	Ada	327	<0.3
pUCOgt	Ogt	57	2
pWX1113	yeast	22	1

[a] Extracts from *E. coli* cultures expressing recombinant alkyltransferase from the species shown were partially purified, and the alkyltransferase activity was measured (Dolan et al., 1991b). Sufficient protein was then used for assay of the production of [^{3}H]guanine from 4 μM [^{3}H]-O^6-benzylguanine so that at least 10 000 cpm would have been expected to be present as guanine if guanine formation was stoichiometric with alkyltransferase activity. [b] May contain some endogenous Ogt and Ada activity.

Table IV: Purification of Recombinant Alkyltransferase[a]

stage	protein (mg)	yield (%)	activity (pmol/mg)	guanine formation (pmol/mg)
crude extract	1218	100	420	422
ammonium sulfate	362	53	750	602
Mono-S	3.6	23	32700	30550

[a] The recombinant human alkyltransferase was purified from 2-L cultures of pINAGT in DH5α cells. The activity in a standard alkyltransferase assay and in the guanine formation assay was measured at each stage as shown.

Ability of Recombinant Alkyltransferases To Produce Guanine from O^6-Benzylguanine. No guanine was formed when [^{3}H]-O^6-benzylguanine was incubated with crude extracts from *E. coli* cells, but when extracts from cells containing the pINAGT expression vector that produces the human alkyltransferase were used, there was substantial formation of guanine (Table III). This amounted to 80–100% of the value expected on the basis of the alkyltransferase activity. Little, if any, guanine was formed when extracts from *E. coli* containing expression plasmid vectors producing the Ada, Ogt, or yeast alkyltransferases were tested. The sensitivity of this assay was lower with the assays for the yeast or Ogt alkyltransferases, which were expressed from vectors giving a lower level of expression, but even in these experiments, the guanine formation was less than 5% of the value expected on the basis of the alkyltransferase activity. These results therefore confirm that these microbial alkyltransferases do not readily accept O^6-benzylguanine as a substrate.

The formation of [^{3}H]guanine from [^{3}H]-O^6-benzylguanine was a convenient way to monitor the purification of the recombinant human alkyltransferase (Table IV). This assay is very rapid since the reaction goes to completion within a short time, and the separation by HPLC of the substrate and product shown in Figure 1 takes only about 15 min. There was an exact correlation between the activity of the alkyltransferase measured in this way and that measured by the standard assay using methylated DNA.

DISCUSSION

Although there are some minor differences in the substrate specificities of the alkyltransferases isolated from different species, the repair of alkylated DNA by these proteins is quite similar. The alkyltransferase protein has a unique mechanism for the repair of O^6-methylguanine-DNA adducts, in that the alkyl group is transferred to a cysteine acceptor site and the cysteine at this site is not regenerated. Thus, the protein is

irreversibly inactivated after transferring a single methyl group (Yarosh, 1985; Lindahl et al., 1988; Demple, 1990; Pegg, 1990; Pegg & Byers, 1992; Mitra & Kaina, 1993). There is good evidence that the alkyltransferase is not specific for methyl adducts and can remove ethyl, *n*-propyl, 2-chloroethyl, and even *n*-butyl groups in a similar manner (Lindahl et al., 1988; Pegg et al., 1985; Morimoto et al., 1985; Pegg, 1990). Although the rate of repair decreases as the size of the alkyl group increases, the final result is the same with the protein inactivated by the addition of an alkyl group at the cysteine acceptor site and the DNA restored to its normal state.

O^6-Methylguanine in double-stranded DNA is repaired at the fastest rate by the alkyltransferase, but it is well established that O^6-methylguanine in short, single-stranded oligodeoxynucleotides can be demethylated (Yarosh et al., 1986; Dolan et al., 1985; Pegg & Dolan, 1989; Spratt & de los Santos, 1992). In fact, even the free base, O^6-methylguanine, appears to be a substrate on the basis of experiments that have shown the inactivation of the alkyltransferase protein by incubation with O^6-methylguanine and the apparent production of guanine concomitant with this inactivation (Yarosh et al., 1986; Dolan et al., 1985; Pegg & Dolan, 1989; Spratt & de los Santos, 1992). However, this process has not been studied in detail because the rate is extremely slow and is bimolecular, necessitating a relatively large amount of the alkyltransferase protein. For these reasons, definitive experiments to establish that the O^6-methylguanine free base is a substrate for the alkyltransferase, forming *S*-methylcysteine in the protein, have not been reported.

Expression of the recombinant alkyltransferase protein in large amounts and the more rapid inactivation of the mammalian protein by O^6-benzylguanine have allowed us to study this reaction in more detail. The results clearly show that O^6-benzylguanine is a substrate for the human alkyltransferase and that the inactivation of the protein is brought about by the formation of *S*-benzylcysteine at the active site. This mechanism is in agreement with the original rationale for the synthesis of this compound as a putative alkyltransferase inactivator (Dolan et al., 1990a), in that the benzyl group would be expected to more readily enter into such a bimolecular displacement reaction than a methyl group. The ability of O^6-allylguanine to inactivate the mammalian alkyltransferase much more effectively than O^6-methylguanine, but less rapidly than O^6-benzylguanine (Figure 1; Moschel et al., 1992), is also consistent with this hypothesis. The parallel formation of guanine and inactivation of the human alkyltransferase protein rule out the possibility that the inactivation occurs merely by the tight binding of O^6-benzylguanine in some hydrophobic pocket that blocks the active site.

Our results provide good evidence that, within the limits of experimental accuracy, the expected stoichiometry occurs, with one molecule of guanine being formed for every molecule of alkyltransferase that is inactivated. In order to calculate this value, the results were corrected assuming that 75% of the protein in the alkyltransferase preparation was active. This 75% value was that obtained by comparing the measured specific activity of the human alkyltransferase preparation with the theoretical expected value of 46 000 pmol/mg. A fraction of the difference is due to the presence of minor contaminating protein bands seen after analysis of the alkyltransferase by polyacrylamide gel electrophoresis under denaturing conditions, but these did not amount to more than 10% of the total protein. Therefore, it is likely that a fraction of the alkyltransferase as purified was inactive. The presence of similar amounts of inactive alkyltransferase in purified preparations and in cells has been reported by others (Koike et al., 1990; Gonzaga et al., 1992; Zhukovskaya et al., 1992). The inability of the inactivated alkyltransferase to produce guanine from O^6-benzylguanine is clearly shown in Figure 3.

The inability of O^4-benzylthymidine to inactivate the human alkyltransferase is, at first sight, slightly surprising since it has now been shown unequivocally that this protein is able to repair O^4-methylthymine in DNA substrates (Koike et al., 1990; Pegg & Byers, 1992; Mitra & Kaina, 1993). However, the activity of the mammalian and yeast alkyltransferases toward O^4-methylthymine seems to be much less than that toward O^6-methylguanine (Koike et al., 1990; Sassanfar et al., 1991). Even with the *E. coli* Ada alkyltransferase, careful kinetic studies with oligodeoxynucleotide substrates have shown that O^4-methylthymine is repaired 10 000 times more slowly than O^6-methylguanine (Graves et al., 1989). Furthermore, the 2′-deoxyribonucleoside of O^6-benzylguanine was a considerably less active substrate for the mammalian alkyltransferase than the free base (Figure 1; Moschel et al., 1992), so it is possible that the two factors result in such a low reaction rate for O^4-benzylthymidine that no reaction can be detected. Even the *E. coli* Ogt protein, which is relatively more active against O^4-methylthymine in DNA (Margison et al., 1990; Sassanfar et al., 1991), was not affected by O^4-benzylthymidine. Synthesis of O^4-allylthymine may provide a good inactivator for this protein.

The striking difference between the mammalian and the yeast and *E. coli* alkyltransferases in their sensitivity to inactivation by O^6-benzylguanine and related compounds confirms and extends previous findings with the *E. coli* Ada alkyltransferase (Dolan et al., 1991b), but is unexpected in view of the considerable similarity in these proteins in general and, particularly, in the sequence surrounding the cysteine acceptor site of these proteins, which is I(V)PCHRV(I) in all cases (Pegg & Byers, 1991; Mitra & Kaina, 1993). It seems highly probable that all of the proteins act by a similar mechanism, and the most probable explanation for the results is that there is a steric restriction on the size of the substrate that can be accommodated at the active site. If the relative order of decreasing size of this site is human > Ogt > Ada ≥ yeast, then these results would also be consistent with the findings that the Ogt protein is slightly sensitive to O^6-benzylguanine and that O^6-allylguanine is able to inactivate the *E. coli* Ogt alkyltransferase as effectively as it acts on the human protein. There is some evidence that the site in the Ada protein may be sterically hindered since there is a large difference in the relative rate of repair of methyl- compared to ethyl- and larger alkyl-DNA adducts (Dolan et al., 1985; Pegg et al., 1985; Lindahl et al., 1988). This difference is considerably smaller with the human protein. It may also be relevant that the *E. coli* Ada protein differs from the mammalian alkyltransferase in that the second-order rate constant for repair of O^6-methylguanine in DNA is considerably higher (Bhattacharyya et al., 1988, 1990; Mitra & Kaina, 1993). It is possible that there is a conformational shift in the alkyltransferase protein when it binds to DNA. Such a change could alter the size of the active site. However, the Ada and yeast alkyltransferases remained resistant to O^6-benzylguanine, even when DNA was added.[2]

Another possibility that is worthy of consideration is that the hydrophobic nature of the O^6-benzylguanine is needed for binding to a hydrophobic pocket in the human alkyltransferase

[2] T. Crone and A. E. Pegg, unpublished observations.

prior to reaction and that the other alkyltransferase proteins do not have such a region. This seems less probable, but it is noteworthy that the 2'-deoxyribonucleoside (Table I) and other modifications that make the molecule more hydrophilic (Moschel et al., 1992) decrease the activity of O^6-benzylguanine as an inactivator.

The use of low molecular weight substrates/inhibitors such as O^6-benzylguanine will greatly facilitate the investigation of the mechanism of transfer brought about by the alkyltransferase. At present, relatively little is known about this reaction. Model reactions, in which the demethylation of O^6-methylguanine and O^4-methylthymine derivatives in the presence of thiophenol and triethylamine was studied, have not provided any clear evidence of the relevance of these reactions to that brought about by the alkyltransferase (Kohda et al., 1991). It has been suggested that the transfer is mediated via the generation of a thiolate anion from the cysteine acceptor and that the formation and stabilization of this anion are facilitated by the adjacent arginine and histidine residues (Demple, 1990; Kohda et al., 1992). However, studies by site-directed mutagenesis have indicated that these basic residues are not absolutely essential for the activity of the protein, although the rate of transfer has not yet been measured (Ling-Ling et al., 1992).[2] The use of such mutants and model low molecular weight substrates such as O^6-benzylguanine will allow the mechanism to be evaluated in more detail. It is also possible that peptide fragments of the alkyltransferase may be able to interact with O^6-benzylguanine, and attempts to define the minimal peptide sequence needed for this reaction are in progress.

There is current interest in the potential use of O^6-benzylguanine as a therapeutic agent to improve the antineoplastic action of chloroethylating agents. Several studies have shown that the response to such agents of human tumor xenografts carried in nude mice is dramatically improved by the administration of O^6-benzylguanine (Dolan et al., 1990b; Mitchell et al., 1992; Friedman et al., 1992; Gerson et al., 1992). Although the therapeutic index was increased in these studies, there was an increased toxicity that presumably relates to the depletion of alkyltransferase in sensitive normal tissue sites such as the bone marrow. The transgenic expression of a microbial alkyltransferase in such tissues, which can be achieved by the use of appropriate vectors (Dumenco et al., 1991, 1993), would render them resistant to O^6-benzylguanine. Either the Ada gene product or the yeast alkyltransferase might be used in this way, provided that the proteins are sufficiently stable and are adequately directed to a nuclear location. If these latter considerations prevent the use of these alkyltransferases, further work to investigate the molecular basis for the sensitivity to O^6-benzylguanine may allow a modified mammalian protein to be designed that could be expressed in normal cells to provide protection. However, the present results also raise the possibility that widespread therapeutic use of O^6-benzylguanine combined with alkylating agents could lead to the formation of mutants of the human alkyltransferase resistant to inactivation by the base. It is, therefore, very important that more information on the features that render the human alkyltransferase capable of using O^6-benzylguanine be obtained, and such studies are in progress.

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