Formation of covalent complexes between human O⁶-alkylguanine-DNA alkyltransferase and BCNU-treated defined length synthetic oligodeoxynucleotides

T.P.Brent and J.S.Remack

Department of Biochemical and Clinical Pharmacology, St Jude Children's Research Hospital,
332 North Lauderdale, Memphis, TN 38101, USA

Received April 26, 1988; Revised and Accepted June 10, 1988

ABSTRACT

Repair of chloroethylnitrosourea (CENU)-induced precursors of DNA interstrand cross-links by O⁶-alkylguanine-DNA alkyltransferase (GAT or GATase) appears to be a factor in tumor resistance to therapy with this class of antineoplastic drugs. Since human GAT is highly specific for O⁶-guanine, yet the probable cross-link structure is N'-Guanine N³cytosine ethane, rearrangement of the initial O⁶-guanine adduct via O⁶,N¹ethanoguanine has been proposed. We suggested that GAT reaction with this intermediate would produce DNA covalently linked to protein through an ethane link from N¹-guanine to the alkylacceptor site on GAT. In preliminary studies we demonstrated a covalent complex between GAT and carmustine (BCNU)-treated DNA by a precipitation assay method. We have now developed a method for isolating the reaction product of BCNU-treated synthetic 14-mer [³²P]-labeled oligodeoxynucleotide and GAT using polyacrylamide gel electrophoresis. This approach can be used to characterize the adducts induced by CENUs that lead to complex formation with GAT. Results obtained to date are consistent with these adducts being precursors of DNA interstrand cross-links.

INTRODUCTION

The chloroethylnitrosoureas (CENUs) are clinically useful anticancer drugs (1,2). The cytotoxic antitumor effects of these bifunctional, alkylating agents are believed to be the result of their capacity to produce interstrand cross-links in cellular DNA (3). Several lines of evidence suggest that cross-link formation occurs through an initial adduct at O⁶-guanine in DNA that can be repaired by O⁶-alkylguanine-DNA alkyltransferase (GAT) (4,5). However, it appears that the final cross-link product does not involve the O⁶-position of guanine. Ludlum has proposed a scheme whereby the initial adduct undergoes an intramolecular rearrangement that ultimately yields an N¹-guanine-ethano-N³-cytosine, a product identified in DNA as the most likely interstrand cross-link structure (7) (Fig. 1). We suggested previously that GAT-mediated repair of the O⁶-N¹-ethano-guanine intermediate might yield a DNA-GAT covalent complex, as a result of cleavage of the ethano moiety from O⁶-guanine and its transfer to a cysteine residue within

Fig. 1: Scheme for induction of interstrand cross-links in DNA treated with BCNU and for GAT-mediated repair of cross-link precursors.

the GAT protein, analogous to the mechanism for repair of O^6 -methylguanine in DNA. We obtained evidence for such a reaction between BCNU-treated $[\text{}^3\text{H}]$ thymidine-labeled PM2 DNA and purified human GAT (8). The covalent complex was identified by co-precipitation of $[\text{}^3\text{H}]$ -DNA with protein under conditions where free DNA remained in solution. We report the results of a series of studies in which we have reacted BCNU-treated $[\text{}^{32}\text{P}]$ -labeled synthetic oligodeoxynucleotide of defined length with GAT and identified the covalent complex as a distinct band by polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Synthesis of Oligonucleotides

Oligodeoxynucleotides were synthesized by using cyanoethyl phosphoramidite chemistry on an Applied Biosystems Inc., Model 380A DNA Synthesizer. Four oligonucleotides of 14 residue length were produced: A) 5'-

dAATTGGGCCCTTAA-3' and its complementary sequence 5'-dTAAAGGGCCCAATT-3' and B) 5'-dAATTGCGCGCTTAA-3' and its complementary sequence 5'-dTAAAGCGCGCAATT-3'. The oligonucleotides were deprotected by addition of fresh NH_4OH and incubation at 55°C for at least 6 hours. The solution was evaporated to dryness by using a speed-vac, redissolved in 0.5 ml of water and reduced to dryness again. After being redissolved in 200 μl of 10 mM Tris-HCl (pH 7.4) 1 mM EDTA (TE) and extracted with an equal volume of 1-butanol, the aqueous layer was removed and evaporated to dryness by speed-vac. The oligonucleotide was redissolved in water, re-evaporated to dryness, and, after final redissolving in 200 μl H_2O , it was stored at -20°C. The size homogeneity of the oligonucleotides was confirmed by 20% polyacrylamide-urea DNA-sequencing gel electrophoresis.

Strand Annealing and ^{32}P -end Labeling

Equal amounts of complementary strands of the 14-mer were mixed and the solution adjusted to 100 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (STE). The strands were annealed by heating to 65°C for 5 min before cooling to 20°C over a period of several hours. The annealed 14-mers were 5'-end-labeled by incubation with T_4 polynucleotide kinase using the 5'DNA terminus labeling system from Bethesda Research Laboratories. Typically, 400 ng of 14-mer were incubated at 37°C for 30 min with 100 μCi [γP^{32}]ATP (3000 Ci/mmol; New England Nuclear) and 50 units of polynucleotide kinase in 250 μl of buffer containing 60 mM Tris-HCl, 10 mM MgCl_2 and 33 nM ATP. The reaction was stopped by cooling to 0°C and the labeled oligonucleotide was separated from unreacted ATP by centrifuging through a Quick-Spin Sephadex G-25 column (Boehringer-Mannheim) that was pre-equilibrated with STE.

BCNU Treatment of 14-mer

Typically ^{32}P -5' end-labeled 14-mer (in 125 μl STE) was treated with BCNU (Bristol Laboratories) (5M in ethanol) at 37°C for various times at concentrations up to 500 mM. Oligonucleotide was separated from the drug by passage through a Quick-Spin Sephadex G-25 column.

Alkyltransferase Purification

O^6 -alkylguanine-DNA alkyltransferase (GAT) was purified from cultured human lymphoblasts (CEM-CCRF line) as described previously (9). A unit of GAT is defined as the amount that transfers 1 pmol of methyl from DNA to protein (9). Fractions 3, 4 and 5 were purified approximately 10-, 30-, and 500-fold respectively. GAT was inactivated by incubation with an excess of methylnitrosourea-treated calf thymus DNA. GAT (Fraction 4) was reacted with [^3H]-methylnitrosourea-treated calf thymus DNA for use as a radiolabeled marker in SDS-PAGE.

Fig. 2: SDS-PAGE of partially purified human GAT after reaction (60 min 37°C) with [^{32}P]-labeled double-stranded 14-mer (sequence A) -0.1 pmole.

A) Protein banding pattern visualized by silver staining.

B) Autoradiograph of the gel in (A).

Lanes 1 and 12 are molecular weight marker proteins; lanes 2 and 11 are [^3H -methyl]-labeled GAT, Fraction 4 (6 units); lanes 3, 4, 5 are Fraction 3 (1 unit); lanes 6, 7, 8 are Fraction 4 (1 unit); lanes 9, 10 are Fraction 5 (4 units). Lanes 3 and 6: BCNU-treated 14-mer was reacted with inactivated GAT; lanes 4, 7, and 9: BCNU-treated 14-mer was reacted with active GAT; lanes 5, 8, and 10: untreated 14-mer was reacted with active GAT.

Polyacrylamide-SDS Gel Electrophoresis

Proteins in the various purified preparations of GAT were analyzed by electrophoresis through 1.5 mm thick 10% polyacrylamide SDS gels according to the method of Laemmli (10) using a Protean II Slab Gel apparatus (Bio-Rad). Gels were silver stained (Bio-Rad) and dried onto Whatman filter paper before exposure at -70°C to Kodak X-OMAT AR film for autoradiographic detection of ^{32}P -labeled protein bands.

The intensity of bands on the film were quantitated by using a densitometer (E-C Apparatus Corp) interfaced with a Shimadzu C-R3A integrating recorder.

RESULTS

Different fractions of GAT purified to various degrees from CEM-CCRF cells were incubated with ^{32}P -end-labeled double stranded 14-mer that had been pre-treated with BCNU for 60 min at 37°C. Figure 2 shows the reaction

Fig. 3: Formation of 30 Kd complex with time of reaction by GAT (Fraction 4, 1 unit) with BCNU-treated 14-mer (0.1 pmole) in 30 μ l reaction volume. The 14-mer (sequence A) was pretreated with BCNU (500 mM) for 60 min at 37°C before incubation with GAT for the following intervals: 0 min, lane 2; 15 min, lane 3; 30 min, lane 4; 45 min, lane 5; 60 min, lane 6; 90 min, lane 7; 120 min, lane 8; lane 1 was [3 H-methyl]-labeled GAT. The radiolabeled 30 Kd bands identified in the autoradiograph on the left were quantitated by determining peak areas from densitometric scans of the film, and the time course is plotted on the right.

products after analysis by SDS-PAGE. The silver-stained protein banding patterns in Fig. 2A indicate the relative purity of Fractions 3, 4, and 5. Fraction 5 (lanes 9 and 10) has a dominant band of protein at about 25,000 daltons which is consonant with the molecular size of GAT from these human cells. Although fraction 5 was purified only about 500-fold with respect to alkyltransfer activity, it appears to be much purer on the gel suggesting that it contains a substantial amount of the inactive protein. This is consistent with the lability and low yield of the activity in fraction 5. The band observed for [3 H]-methylated GAT in lanes 2 and 11 of the autoradiograph (Fig. 2B) confirms the identity of the 25 Kd band. Figure 2B shows the 32 P-labeling patterns corresponding to the protein banding patterns shown in Fig. 2A. A radiolabeled band at about 30,000 daltons is seen in all the lanes where active GAT was incubated with BCNU-treated 14-mer. This 30 Kd band is absent in those lanes where BCNU-treated 14-mer was

Fig. 4: Formation of 30 Kd complex by reaction of GAT (Fraction 4, 1 unit) with 14-mer pretreated with BCNU for different lengths of time. 14-mer (Sequence A, 0.1 pmole) was pretreated with BCNU (500 mM) for the following times: 0 min, lane 2; 30 min, lane 3; 60 min, lane 4; 90 min, lane 5; 2 hr, lane 6; 3 hr, lane 7; lane 1 was [^3H -methyl]-labeled GAT. After incubating the alkylated DNA with GAT (37°C for 60 min in a reaction volume of 30 μl) reaction products were analyzed by SDS-PAGE and autoradiography. The radiolabeled 30 Kd bands were quantitated by densitometry and 30 Kd peak areas were plotted against the indicated times of 14-mer exposure to BCNU.

incubated with inactivated GAT, or where active GAT was reacted with 14-mer that was not treated with BCNU. Fraction 3, the crudest preparation of GAT, displays several radiolabeled bands other than at 30 Kd; however, these bands are present even where inactivated GAT or untreated 14-mer were used in the reaction, hence these bands probably reflect DNA binding proteins other than GAT. Figure 3 depicts the time course for reaction between BCNU-treated 14-mer and GAT to yield the 30 Kd band on SDS-PAGE analysis. The extent of reaction was quantitated by densitometry of the bands on the x-ray film and shows that the reaction occurs rapidly, being essentially complete by 60 min.

The time course for induction by BCNU of adducts in the 14-mer that can form the 30 Kd complex when reacted with GAT was next determined. Figure 4 shows the autoradiograph of SDS-PAGE analysis and a densitometric plot that depicts the time course for induction of such adducts in the 14-mer. The reaction was rapid and complete within 90 min.

Fig. 5: Dose response for BCNU-induction of adducts in 14-mer that yield the 30 Kd complex when reacted with GAT. 14-mer (Sequence B) was treated with the indicated concentrations of BCNU for 60 min at 37°C. After drug removal the 14-mer (0.2 pmole) was incubated with GAT (Fraction 4, 2 units) for 60 min at 37°. Reaction products were analyzed by SDS-PAGE, autoradiography and densitometry of the 30 Kd band.

The dose response for BCNU-induction of these adducts in 14-mer is shown in Figure 5. The minimum dose where detectable 30 Kd complex formed with GAT was about 10 mM BCNU. The induction of adducts yielding the 30 Kd complex increased with dose up to 200-300 mM. The conditions, arrived at from these studies for obtaining maximum yield of 30 Kd complex, were treat-

Fig. 6: Stability in the 14-mer of BCNU-induced adducts that yield 30 Kd complex with GAT. 14-mer (Sequence B, 0.2 pmoles) was treated with 500 mM BCNU for 60 min at 37°C. After drug removal the alkylated 14-mer was incubated for the indicated times before reaction with GAT (Fraction 4, 2 units) for 60 min at 37°C and analysis of reaction products by SDS-PAGE, autoradiography and densitometry of the 30 Kd band.

ment of 14-mer for 90 min at 37°C with 250-500 mM BCNU and subsequently reacting alkylated 14-mer for 60 min at 37°C with GAT.

We determined the half-life for the adduct that yield the 30 Kd complex since this might provide a clue to its identity. Figure 6 shows the decay of capacity to form the complex by 14-mer that had been pulse-treated with BCNU (90 min, 37°, 250 mM). After one or two hours during which the adduct appeared to increase, there was a progressive loss of capacity to form the complex, the half-life being about 5 hours.

DISCUSSION

In these studies we have used SDS-PAGE to identify and quantitate the complex formed between BCNU-treated defined-length synthetic oligodeoxynucleotides and human GAT. This approach has distinct advantages over the previous technique where we determined co-precipitation of radiolabeled viral DNA with total proteins precipitated by addition of SDS-KCl. The new method has enabled us to identify the complex as a single molecular species with a molecular weight of 30 Kd. This is exactly the size we would predict for the 25 Kd GAT protein coupled to the 4.5 Kd 14-mer. The formation of this 30 Kd species is absolutely dependent on GAT activity further strengthening the conclusion that it arises from the GAT protein. This method has also enabled us to segregate the oligonucleotide-GAT complex from non-GAT protein-DNA complexes and also from contaminating free DNA or protein. Thus we have been able to identify the complex even with relatively crude cell extracts (eg Fraction 3, Fig. 2). The elimination of nonspecific noise from the assay has enabled us to quantitate the complex, cleanly, and to characterize with some confidence the reaction for complex formation and the kinetics for formation and persistence of the adducts that lead to complex formation.

The time course for reaction between BCNU-treated 14-mer and GAT (Fig. 3) is similar to that previously reported for PM2 DNA (8). The reaction is 90% complete within 60 min (Fig. 3) which is somewhat slower than with PM2 DNA (11). The yield of 30 Kd complex increased with BCNU dose (Fig. 5) up to about 300-500 mM; above this concentration no further increase could be obtained, either by increasing BCNU concentration or by repeated applications at 500 mM, suggesting that all available sites have been alkylated. Since a maximum of a few percent of the 14-mer in the reaction became bound to GAT (data not shown), we infer that the adducts leading to the complex represent a minor fraction of the total adducts. Although we used very high

doses of BCNU so that we would obtain the best yield of the complex, we did observe complex formation after treatment with doses as low as 10 mM BCNU (Fig. 5). Moreover, with the much larger PM2 DNA target (10,000 base pairs), previously we observed extensive complex formation using 10 mM BCNU (11). We consider therefore, that the adducts formed at the higher doses are likely to reflect the situation at lower more pharmacological doses. Both 14-mer sequences (A) and (B) were used in these studies. (A) has a middle sequence GGGCCC while (B) has an alternating sequence GCGCGC. The terminal AATT sequences were added to favor double-strand formation without formation of hairpin or aggregate structures. The reason for testing both sequences (continuous vs. alternating) stem from evidence that BCNU reacts more actively with runs of Gs than with single G's (12); however, we found no difference between the two sequences in the yields of 30 Kd complex.

The time course for reaction of BCNU with the 14-mer discloses that the adducts which are substrates for GAT are induced to a maximum level within about 1 hour (Fig. 4). This is similar to the rate we have determined for induction of interstrand cross-link precursors in BCNU-treated calf thymus DNA (13) and is consistent with the stability of BCNU in aqueous solution at 37°C (14).

The adducts that yield the 30 Kd complex disappear at 37°C, after drug removal, with a half-life of 4-5 hours (Fig. 6). This value is of the same order of magnitude as that for decay of BCNU-induced interstrand cross-link precursors in calf thymus DNA (3 hours at 37°C) (13), hence supports the model illustrated in Fig. 1 where the DNA interstrand cross-link precursor and the substrate for DNA-GAT complex are one and the same.

ACKNOWLEDGEMENTS

This work was supported by grants CA 14799, CA 36888 and CA 21765 from the National Institutes of Health and by ALSAC. We thank Mr. Kim Lee of the St. Jude Molecular Resource Center for synthesizing the oligodeoxynucleotides and Linda Daniels for editing the manuscript.

REFERENCES

1. Carter S.K. (1976) Proceedings of the Seventh New Drug Seminar: Nitrosoureas. *Cancer Treat. Rep.* 60:645-811
2. Kornblith P.L., Smith B.H. and Leonard L.A. (1981) *Cancer (Phila.)* 47:255-265
3. Bodell W.J., Aida T., Berger M.S. and Rosenblum M.L. (1985) *Environ. Health Perspect.* 62:119-126
4. Erickson L.C., Laurent G., Sharkey N.A. and Kohn K.W. (1980) *Nature (Lond.)* 288:727-729

5. Yarosh D.B., Hurst-Calderone S., Babich M.A. and Day R.S. III (1986) *Cancer Res.* 46:1663-1668
6. Brent T.P. (1984) *Cancer Res.* 44:1887-1892
7. Ludlum D.B. and Tong W.P. (1985) In: F.M. Muggia (ed.) *Cancer Chemotherapy*, Vol. 2, pp. 141-154, Boston: Martinus Nijhoff
8. Brent T.P., Smith D.G. and Remack J.S. (1987) *Biochem. Biophys. Res. Commun.* 142:341-347
9. Brent T.P. (1985) *Pharmacol. Therap.* 31:121-140
10. Laemmli U.K. (1970) *Nature* 227:680-685
11. Brent, T.P., Remack, J.S. and Smith, D.G. (1987) *Cancer Res.* 47:6185-6188
12. Hartley J.A., Gibson N.W., Kohn K.W. and Mattes W.B. (1986) *Cancer Res.* 46:1943-1947
13. Brent T.P. Submitted for publication
14. Weinkam R.J. and Deen D.F. (1982) *Cancer Res.* 42:1008-1014