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SHORT COMMUNICATIONS

Identification of 7-(2-hydroxyethyl)guanine as a product of alkylation of calf thymus DNA with Clomesone

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Studies by Gibson *et al.* on the alkylation of DNA by Clomesone, 2-chloroethyl (methylsulfonyl)methanesulfonate, a novel chloroethylating agent currently entering Phase I trials as an antitumor drug [1], led to the detection of a single purine adduct, namely 7-(2-chloroethyl)guanine [2].

In related studies, Hartley and Gibson [3] observed an increase in the cytotoxicity of Clomesone against HT-29 (Mer^+) colon carcinoma cells pretreated with methylating agents, and Dolan *et al.* [4] observed a similar effect upon pretreatment of this cell line with O^6-alkylguanines. The effects of these pretreatments on DNA–DNA cross-linking in these cells have also been investigated in an attempt to correlate the cytotoxicity of Clomesone with this DNA lesion. Pretreatment with streptozotocin induced cross-linking, and a slight increase in cross-linking was also observed upon treatment of cells with O^6-methylguanine before and after exposure to Clomesone [3, 5]. The results of these studies led to the conclusion that the cytotoxicity of Clomesone is dependent upon O^6-2-chloroethylation of guanine in DNA of these cells. However, the report by Gibson *et al.* on the detection of only 7-(2-chloroethyl)guanine upon treatment of DNA with Clomesone [2] provided no evidence that such an O^6-adduct is involved in the cytotoxicity of this new agent.

We report here the detection of a product of alkylation of calf thymus DNA by Clomesone that provides indirect evidence for O^6-alkylation of guanine in DNA and is consistent with the results of studies by Hartley and Gibson [3] and by Dolan *et al.* [4] on the effect on cytotoxicity of pretreatment of cells with methylating agents or O^6-alkylguanines.

Materials and Methods

Materials. Calf thymus DNA was purchased from the Sigma Chemical Co., and [2-chloroethyl-^{14}C] Clomesone was obtained from Dr. Rudiger Haugwitz, Drug Synthesis and Chemistry Branch, DTP, NCI, Bethesda, MD, who authorized shipment from Research Triangle Institute, NCI's contractor for synthesis of radiolabeled compounds. 7-(2-Chloroethyl)- and 7-(2-hydroxyethyl)guanine were prepared at Southern Research Institute by Dr. J. R. Piper *et al.* as described previously [6]. O^6-2-Chloroethylguanine was prepared as reported [7].

Drug treatment of DNA. Calf thymus DNA was dissolved at a concentration of 8 mg/mL in 25 mM sodium cacodylate buffer, pH 7.0, and was treated with the drug by the method of Tong *et al.* [8]. Clomesone, at a concentration of 4.68 μmol in 20 μL of 95% ethanol, was reacted with 1 mL of the DNA solution for 6 hr at 37°. Control solutions were treated with 20 μL of 95% ethanol.

Depurination of alkylated DNA. Alkylated calf thymus DNA was depurinated by the following procedures.

Acid hydrolysis: After the DNA was reacted with Clomesone for 6 hr at 37°, 50 μL of a 6 M sodium chloride solution was added; 2 vol. of 95% ethanol was added, and the DNA was wound on a stirring rod. The DNA was washed twice with 5 mL of 95% ethanol by gently agitating the stirring rod (with the DNA still wound on it) in the ethanol solution. The DNA was allowed to air dry on the rod, thereby removing any ethanol. It was redissolved in water and the process was repeated. The DNA was redissolved in 1.75 mL of water, 0.2 mL of 1 N hydrochloric acid was added, and the solution was incubated for 18 hr at 37°. After the 18-hr incubation, 0.2 mL of 1 N ammonium hydroxide was added, and the mixture was passed through a small (0.75 cc of gel) DEAE-Sephadex column to remove the oligonucleotides in preparation for HPLC analysis.

Neutral hydrolysis: The drug-treated DNA was wound, washed, dissolved and redissolved as described for the acid hydrolysis procedure to the stage where the DNA was put in solution for hydrolysis. At this point, the DNA was dissolved in 1.75 mL of 2 mM phosphate buffer, pH 6.5, and the samples were heated in a heating module for 30 min at 95°; 0.1 vol. of 1 N hydrochloric acid was added, and the samples were passed through the DEAE-Sephadex column in preparation for HPLC analysis.

HPLC analysis of purine adducts. A Waters Associates ALC 202 high-performance liquid chromatograph equipped with a Rheodyne injector, a Waters 440 UV detector, and a Perkin–Elmer LS-4 fluorescence spectrometer were used. Column effluent was monitored by UV at a wavelength of 254 nm and by fluorescence at an excitation wavelength of 250 nm and an emission wavelength of 350 nm at a sensitivity of 5.0. Slit widths were 10 nm for emission and excitation. Both UV and fluorescence detectors were monitored by Hewlett–Packard 3390A integrators.

Resolution of compounds of interest was achieved with a Spherisorb ODS (5 μm) column (250 × 4.6 mm i.d.) from Keystone Scientific (State Collete, PA). A guard column containing the same packing material preceded the analytical column. The μBondapak C18 column and Pic A reagent were obtained from Waters Associates (Milford, MA).

Analysis consisted of use of the following procedures:

Procedure A: Spherisorb ODS 5 μm column; isocratic, 25 mM KH_2PO_4 (pH 4.5):CH_3CN (95:5, v/v) 1 mL/min.

Procedure B: Spherisorb ODS 5 μm column; gradient elution (20 min), solvent A: 5 mM $NH_4H_2PO_4$ (pH 2.8):CH_3CN (95:5, v/v), solvent B: same as solvent A but 10:90, v/v, 1 mL/min. The column was reequilibrated with solvent A for 15 min between analyses.

Procedure C: μBondapak C18 column; isocratic, 25 mM $NH_4H_2PO_4$(pH 5.5):CH_3CN(95:5, v/v) with 5 mM PIC A reagent, 1 mL/min.

Procedure D: Spherisorb ODS 5 μm column; gradient elution (20 min), solvent A: 50 mM $NH_4H_2PO_4$ (pH 4.5), solvent B: 50 mM $NH_4H_2PO_4$ (pH 4.5):CH_3CN(80:20, v/v); 1 mL/min. The column was reequilibrated with solvent A for 15 min between analysis.

Results

Incubation of Clomesone with calf thymus DNA followed by acid hydrolysis gave a purine fraction that revealed the presence of 7-(2-chloroethyl)guanine, as well as guanine and adenine, upon HPLC analysis via procedure A, as reported [2].

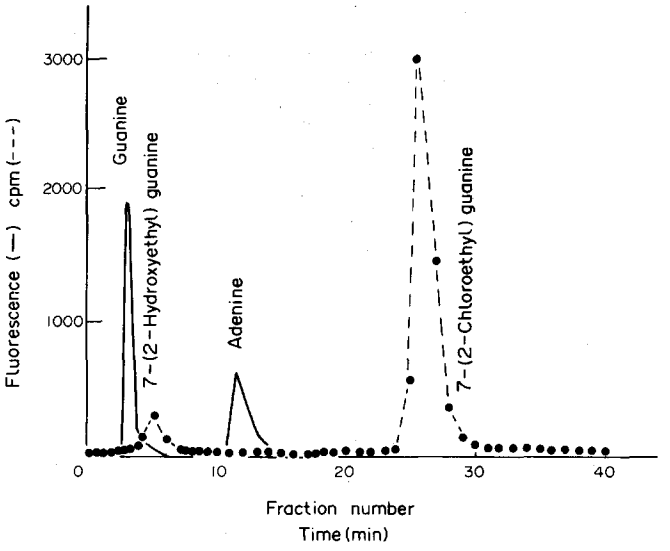

Fig. 1. HPLC of product from Clomesone (radioactive) and DNA following acid hydrolysis, using procedure A.

The DNA alkylation procedure was repeated with [2-chloroethyl-^{14}C]Clomesone, and the DNA was similarly depurinated by neutral hydrolysis. HPLC analysis using procedure A gave the results shown in Fig. 1 and indicated the presence of 7-(2-chloroethyl)guanine (*ca.* 90%) and a minor radioactive component (*ca.* 10%) with a retention time identical to that of 7-(2-hydroxyethyl)guanine upon HPLC analysis in the same system. Alkylation of DNA with [^{14}C]Clomesone was repeated twice, once followed by neutral hydrolysis and once followed by acid hydrolysis, as a means of determining whether the amount of the hydroxyethylated derivative would change, based on the hydrolysis conditions, and indicate that the hydroxyethylguanine was an artifact of the depurination procedures. HPLC analysis of both purine fractions yielded identical results, providing support for the hydroxyethyl derivative as a *bona fide* product of the alkylation reaction and not of the depurination step. The radiolabeled Clomesone was also analyzed by thin-layer chromatography (TLC) on silica gel in ethyl acetate in an attempt to determine whether the sample was contaminated with any impurities that might be able to produce the 2-hydroxyethylguanine adduct directly. However, radioactivity attributable only to Clomesone (R_f *ca.* 0.8) and a band that remained at the origin was observed, and these two bands accounted for greater than 99% of the radioactivity on the TLC plate, as revealed by radioassay of the entire plate by scintillation counting.

Confirmation of the presence of the 2-hydroxyethyl derivative in the minor radioactive product (Fig. 1) was sought by subjecting the purine fraction to additional HPLC analysis using a different procedure. Using gradient elution with a reverse-phase column (procedures B and D), we were able to resolve the minor product into two radioactive components, one of which corresponded by retention time (13 min) in both systems to that of authentic 7-(2-hydroxyethyl)guanine. For confirmation, the sample was spiked with unlabeled standards to facilitate identification of the radioactive components and analyzed by procedure D, resulting in co-elution of both standards with two of the radioactive components as shown in Fig. 2. Another radioactive component eluted at a retention time of 7 min, and this unidentified component (*ca.* 70% of the minor component found using procedure A) was collected for further analysis by paired ion HPLC analysis (procedure C) because of its short retention time under the conditions used previously (procedures A, B and D). The unknown component was also not retained under these conditions, leading us to conclude that it was likely not an alkylated purine but was rather a minor radioactive impurity. As further confirmation, radiolabeled Clomesone was separated by TLC as described, and the Clomesone band

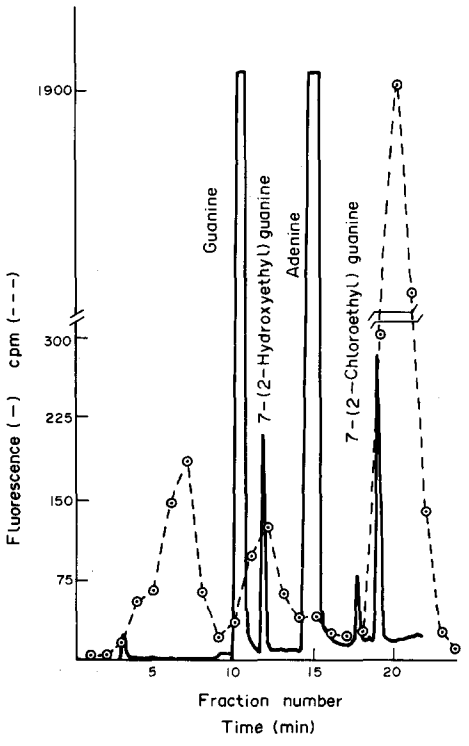

Fig. 2. Co-HPLC of 7-(2-hydroxyethyl)- and 7-(2-chloroethyl)guanine and the product from [^{14}C]Clomesone and DNA following acid hydrolysis, using procedure D.

and the band at the origin were collected and incubated with DNA as described, yielding purine fractions following acid hydrolysis. HPLC analysis (procedure D) of the purine fraction from the Clomesone band was identical to that observed previously (Fig. 2), whereas no radioactivity was present in fractions from the band at the origin (data not shown). HPLC analysis included co-HPLC with 1- and 7-(2-hydroxyethyl)guanine and 7-(2-chloroethyl)guanine.

Since O^6-2-chloroethylguanine has been reported [7], a specimen was synthesized and subjected to conditions employed for both acid and neutral hydrolysis of DNA. Co-HPLC analysis (procedure D) of these fractions with 1- and 7-(2-hydroxyethyl)guanine and O^6- and 7-(2-chloroethyl)guanine produced 1-(2-hydroxyethyl)guanine, as reported [7], but no 7-(2-hydroxyethyl)guanine.

Discussion

Studies by Hartley and Gibson [3] and by Dolan *et al.* [4] on the influence of methylating agents and of 6-alkylguanines on the cytotoxicity of Clomesone provided indirect evidence for O^6-alkylation of DNA of HT-29 (Mer^+) cells as an important event in this cytotoxicity. However, analysis of purine adducts of calf thymus DNA treated with Clomesone identified only 7-(2-chloroethyl)guanine [2]. Because of the very low level of production in our experiments of the minor radioactive component, which on subsequent HPLC analysis was also shown to be contaminated with an unidentified impurity, it is understandable that 7-(2-hydroxyethyl)guanine was not detected in the previous investigations by Gibson *et al.* [2]. It is also possible that DNA alkylation may have proceeded somewhat more efficiently in our experiments.

Since concern over whether the hydroxyethyl adduct was an artifact was justifiable, repeating the depurination step under two sets of conditions was necessary. It seems reasonable that some variability would have been observed in the production of the minor adduct, if it was indeed artifactual. It was also possible that the labeled Clomesone was contaminated with an impurity that might have yielded the hydroxyethylguanine. However, TLC analysis of the labeled agent indicated *ca.* 93% purity for Clomesone with the bulk of the remaining activity failing to migrate (>6%), suggesting the unlikely presence of contaminants that would produce the hydroxyethylated guanine upon incubation with DNA. Also, incubation of TLC-purified, radiolabeled Clomesone and of the immobile impurity with DNA and identical isolation of purine fractions followed by HPLC analysis gave for the Clomesone fraction results identical to those obtained from Clomesone that had not been purified by TLC but no detectable radioactivity in HPLC fractions of the purine fraction from the immobile band.

We propose that the 7-hydroxyethyl adduct arises by initial O^6-guanine 2-chloroethylation of calf thymus DNA followed by intramolecular alkylation of N^7 to generate an unstable, 1,4-oxazinium intermediate, which subsequently undergoes hydrolytic ring opening at the O^6 position to produce a 6-hydroxypurine and a hydroxyalkyl moiety at the site of hydrolysis. A subsequent, typical lactim–lactam rearrangement would yield a 7-(2-hydroxyethyl)guanylium unit in the DNA, and depurination would yield 7-(2-hydroxyethyl)guanine. Although generation of 7-(2-hydroxyethyl)guanine from synthetic O^6-2-chloroethylguanine was not observed, the results do not preclude its generation from O^6-2-chloroethylguanyl moieties in DNA in contrast to O^6-2-chloroethylguanine itself.

These observations are consistent with a mechanism of action of Clomesone that includes O^6-alkylation of guanine in DNA and that can be influenced by agents that affect the efficiency of guanine O^6-alkyltransferase to protect cells by removal of lesions produced by O^6-alkylation. Consequently, our observations provide a molecular basis for the effects of methylating agents and 6-alkylguanines on the cytotoxicity of Clomesone to HT-29 cells as observed by others [3, 4].

Because of the reported instability of O^6-2-chloroethylguanine [7], it may be difficult to isolate this expected, direct-alkylation product of Clomesone from drug-treated DNA. However, it may be possible to identify a related product by use of Clomesone analogs that would yield more stable O^6-alkylated products, and such attempts are in progress in our laboratory.

In summary, evidence at the molecular level is presented in support of alkylation of O^6-guanine moieties of DNA as the mechanism of cytotoxicity of Clomesone to HT-29 cells and consists in the isolation and identification of a product resulting from alkylation of calf thymus DNA with Clomesone, followed by depurination to yield 7-(2-hydroxyethyl)guanine, whose formation is reasonably explained by O^6-guanine chloroethylation followed by intramolecular alkylation at N^7 of guanine and subsequent hydrolysis to the hydroxyethylguanine.

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