DNA Adducts of the Antitumor Agent Diaziquone

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ABSTRACT

We have studied adduct formation of the antineoplastic agent diaziquone (AZQ, NSC 182986) with DNA and nucleotides in vitro. The aziridine moieties of AZQ can be expected to interact covalently with DNA which, in turn, presumably elicits the antitumor activity. We analyzed AZQ-DNA adducts by a modified 32P-postlabeling assay involving purification of the nucleoside P1-enriched labeled adducts by high-salt C8 reversed-phase thin-layer chromatography and separation of the eluted adducts on a polyethyleneimine-cellulose layer using non-urea salts solutions. Modification of calf thymus DNA with AZQ produced two major (22% and 40%) and at least eight minor adducts. At equal concentrations of AZQ and DNA (1 μg/μl each), peak binding was observed in about 2 h [1926 ± 378 (SD) fmol/μg of DNA] with the binding levels remaining practically unchanged through 4 h. However, incubation for 24 h resulted in over 40% decline, indicating adduct instability. AZQ was found to be highly reactive in vitro as evidenced by its substantial binding (49 ± 14 fmol/μg of DNA) even at a DNA:AZQ ratio of 100:1. When incubated with mononucleotides, AZQ reacted extensively with adenine, guanine, and cytosine but only slightly with thymine. Cochromatography of the modified DNA and nucleotides revealed that one of the major adducts and several minor adducts were guanine derived. The aziridine rings of AZQ were found to be the main reactive sites as its monoaminooalcohol derivative showed as much DNA reactivity as did the parent compound, but no activity was observed when both aziridine groups were hydrolyzed to diaminoalcohols. The improved 32P-postlabeling assay seems capable of detecting relatively polar adducts such as those formed with AZQ at a level of one adduct/108 nucleotides.

INTRODUCTION

Chemotherapeutic drugs that are genetically active when used at high-dose levels in controlled clinical settings are ideal genotoxic agents to study the mechanism of interaction with DNA. The potential antitumor activity of aziridinylquinones has been recognized for sometime, and of the 1500 quinones tested for antitumor activity, a large number of the active compounds were found to exhibit antitumor activity through aziridine moieties (1). AZQ1 (Fig. 1) is a rationalized synthetic lipophilic benzoquinone derivative with limited ionization which was intended to promote its efficacy to cross the blood-brain barriers (2).

AZQ is active against a number of animal and human tumors. This drug was introduced in preclinical trials about 15 yr ago and, since then, Phase I, Phase II, and Phase III clinical trials have investigated its antitumor activity against a variety of neoplasms, such as recurrent primary anaplastic CNS tumors (3), CNS leukemia (4), refractory small cell lung carcinoma (5), advanced large bowel carcinoma (6), and head and neck cancer (7), among others. Although its mechanism of action is uncertain, AZQ is a highly genotoxic agent. It is an alkylating agent inducing DNA strand breaks, interstrand DNA-DNA cross-links, and DNA-protein-cross-links in several mammalian cells in culture (8, 9). This drug also induces sister chromatid exchanges in mouse and human PBLs in vitro and in vivo (10, 11) and micronuclei in cytogenes B-blocked mouse PBLs in vivo (12). AZQ is mutagenic in the Ames test (13). It induces lung adenomas in strain A mice (14) and induces anchorag indep- dependent colony formation in skin fibroblasts in vitro (15).

In this study, we have investigated the potential of AZQ to interact covalently in vitro with DNA and mononucleotides by a substantial variation of the 32P-postlabeling assay (16, 17) and shown formation of multiple AZQ-DNA adducts.

MATERIALS AND METHODS

Chemicals

AZQ, NSC 182986 (CAS 800-24-8), was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. TFE (99%) was from Fluka. Calf thymus DNA and 2′-deoxyxynucleoside 3′-monophosphates (dGp, dAp, dCp, and dTp) were from Sigma, St. Louis, MO, and Pharmacia LKB, Piscataway, NJ, respectively. The DNA was freed of the contaminating RNA by incubation with RNases A and T1, (18). Materials required for the 32P-postlabeling assay were essentially as described (16, 19). [γ-32P]ATP (>3000 Ci/mmol) was synthesized in the laboratory from 32P (ICN, Irvine, CA) (16, 19, 20).

Instruments

Melting points were taken on a Thomas-Hoover capillary apparatus and are uncorrected. IR spectra were determined on a Perkin-Elmer Model 1420 spectrophotometer, and UV spectra, with a Beckman Model DU-70. 1H-NMR spectra were recorded on a GE Model GN500 spectrometer at 500 MHz using tetramethylsilane as the reference. Electron ionization mass spectra (70 eV) were obtained by HPLC-MS using an Extrel Model ELQ-400-2 mass spectrometer interface to a DuPont 8800 Series HPLC via an Extrel Thermabeam interface. The control temperature of the interface was 151°C. Exact mass determinations were recorded on a VG70-250SER mass spectrometer. Semi-preparative HPLC separations were performed with a DuPont Model 8800 HPLC. Peak detection was achieved using a DuPont Model 860 absorbance detector with a 313-nm filter. Analytical TLC and preparative TLC were carried out on precoated fluorescent silica gel plates (AnaTech, Newark, DE). Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

Preparation of AZQMAA and AZQDAA

AZQ (1.5 g) was first dissolved in N,N-dimethylacetamide (75 ml) and diluted with water (750 ml). The pH of the solution was adjusted to 4.0 with 0.1 N HCl and stirred at room temperature for 24 h. The
resulting solution (pH 4.6) was freeze-dried. The brown residue contained residual N,N-dimethylacetamide, which was removed by trituration with hexane. Acetone (75 ml) was added to the semisolid residue, and the solution was kept at 5°C. Some unreacted AZQ (approximately 0.22 g) separated out and was removed by filtration. The filtrate was evaporated, and the residue was dissolved in methanol with a few drops of water and submitted to preparative thin-layer chromatography on Uniplate TM taper silica gel GF plates using the chloroform:methanol (10:1.35, v/v) solvent system. This solvent system resolved AZQ (Rf, 0.87), AZQMAA (Rf, 0.73), and AZQDAA (Rf, 0.57). Elution from the scraped bands with acetone (AZQMAA) or with acetone:methanol (3:2) (AZQDAA) and filtration and evaporation gave the two products.

AZQMAA was obtained as a dark brownish-red solid (0.63 g) and was crystallized from acetone-dry ether as clusters of tiny needles, m.p. 124–126°C (sintering at 105°C). Further purification was required as this material contained trace impurities tentatively identified on the basis of their molecular ion peaks as 2-(2-chloroethylamino)-5-(2-hydroxyethylamino)-3,6-bis(carbomethoxy)amine, 1,4-benzoquinone and 2-(2-acetoxyethylamino)-5-(2-hydroxyethylamino)-3,6-bis(carbomethoxy)amine. An HPLC separation was achieved using a Beckman Ultrasphere 5¿ C18 semipreparative column (10 x 250 mm) and an acetonitrile:H2O (15:85, v/v) isocratic mobile phase at a flow rate of 2.0 ml/min. A sample size of 500 µl in H2O:methanol (1:1) was used for injection. Collected fractions were lyophilized and evaporated to perform the HPLC-MS analysis.

AZQDAA was obtained as a dark-brown solid (0.31 g) and was crystallized from acetone-dry ether as clusters of tiny needles, m.p. 124–126°C (sintering at 105°C). Further purification was required as this material contained trace impurities tentatively identified on the basis of their molecular ion peaks as 2-(2-chloroethylamino)-5-(2-hydroxyethylamino)-3,6-bis(carbomethoxy)amine, 1,4-benzoquinone and 2-(2-acetoxyethylamino)-5-(2-hydroxyethylamino)-3,6-bis(carbomethoxy)amine. An HPLC separation was achieved using a Beckman Ultrasphere 5¿ C18 semipreparative column (10 x 250 mm) and an acetonitrile:H2O (15:85, v/v) isocratic mobile phase at a flow rate of 2.0 ml/min. A sample size of 500 µl in H2O:methanol (1:1) was used for injection. Collected fractions were lyophilized and evaporated to perform the HPLC-MS analysis.

**In Vitro Treatments**

AZQ was freshly dissolved in TFE and incubated with equal concentration of calf thymus DNA (1 µg/µl) in 500 µl of 20 mM sodium acetate, pH 4, at 37°C for 2 min to 24 h (TFE ≈ 9%). In another experiment, DNA (1 µg/µl) was incubated at 37°C for 4 h with varying concentrations (0.01 to 1.0 µg/µl) of AZQ. The mononucleotides dAp, dGp, dCp, and dTp (1 µg/µl) were incubated at 37°C for 24 h with an equal concentration of AZQ as described for DNA. The AZQ derivatives AZQMAA and AZQDAA (Fig. 1) were incubated for 4 h with calf thymus DNA as described for AZQ. Parallel incubations were also carried out for 4 h or 24 h by individually omitting DNA, nucleotide, AZQ, AZQ derivatives, and buffer to serve as controls. Unreacted AZQ or its derivative was removed by extracting 3 times with ethyl acetate, and the DNA was further purified by precipitation with ethanol. Concentration of DNA and nucleotides was estimated spectrophotometrically.

**3P Postlabeling DNA Adduct Analysis**

Adducts were analyzed by the 3P-postlabeling assay (16, 17) with alterations as follows. Control and AZQ-treated DNAs (10 µg) were enzymatically hydrolyzed to deoxyribonucleoside 3'-monophosphates (enzyme:substrate, 1:1; 37°C, 4 h), and the adducts were enriched by treatment with nuclease P1 (21) (enzyme:substrate, 1:3; pH 6; 37°C, 1 h). The enriched adducts were labeled with a molar excess of carrier-free [γ-3P]ATP (80 µCi; >3000 Ci/mmol) and T4 polynucleotide kinase (0.22 units/µl) as described (22), except that the incubation was at room temperature (23°C) for 45 min. Labeled adducts were separated by TLC on a C18 reversed-phase or PEI-cellulose layer as follows.

**System 1.** Labeled DNA digest (0.01 to 1 µg) was applied to a water-washed PEI-cellulose thin layer (1.3 x cm long) and the sheet was developed overnight (about 20 h) with 3.2 M sodium phosphate, pH 5.5, about 2 cm onto a Whatman No. 17 chrome wick (or 10 cm onto a Whatman No. 1 wick) stapled to the top of the sheet. Alternatively, the labeled digest was applied to a 0.4 M ammonium formate (pH 6.2)-prewashed C18 thin layer (10 x 10 cm), and the development was overnight in 4 M ammonium formate, pH 6.2, onto a Whatman No. 1 wick protruding outside the tank. Prior to detection of adducts by intensifying screen-enhanced autoradiography, the PEI-cellulose chromatogram was briefly (5 seconds) washed in deionized water and dried or dried only in the case of a C18 chromatogram.

**System 2.** Complete separation of AZQ adducts was accomplished by a combination of the C18 reversed-phase and PEI-cellulose TLC. Labeled adducts (2 to 20 µg) were first purified by C18 TLC as described in System 1 and then eluted from the layer as follows. After it was dried with warm air for 15 to 20 min, the C18 layer containing adducts (about 1 - x 4-cm² area) was first wetted with water using a cotton swab, collected with a TLC scraper, and extracted 2 times (in 1.5-ml Eppendorf tubes) with 600 µl each of 2-butoxyethanol/water (4:6) at room temperature (23°C) for 20 min with continuous agitation. The combined eluate was dried under vacuum (SpeedVac concentrator; Savant Instruments, Farmingdale, NY) and reconstituted in 20 to 50 µl of water, and an aliquot (5 to 10 µl) was chromatographed on a 50 mm ammonium formate (pH 3.3)-prewashed PEI-cellulose thin layer (13 x 10 cm). Development was in 0.4 M ammonium formate, pH 3.3 (D1), and 3 M sodium phosphate, pH 5, 1 to 2 cm onto a Whatman No. 1 wick (D2). The chromatogram was dried with warm air prior to the D2
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development. Adducts were detected as described in System 1. To calculate adduct levels, total nucleotides (2 ng) were also labeled in parallel and analyzed by one-directional PEI-cellulose TLC (22). Adduct levels were evaluated by calculating relative adduct labeling, which was then translated into amol/μg of DNA (22).

RESULTS

Synthesis and Characterization of AZQMAA and AZQDAA. The syntheses of AZQMAA and AZQDAA relied on the reported ease of hydrolysis of AZQ to multiple products (23, 24). Under controlled hydrolysis conditions, AZQ reacted with N,N-dimethylacetamide to give both AZQMAA and AZQDAA. More extensive conditions induced considerable further degradation. These products were separated by preparative TLC and purified by HPLC. Purity analysis was performed by HPLC-MS which cleanly resolved AZQDAA, AZQMAA, and AZQ.

AZQMAA, which has not been previously reported in detail, possessed a similar NMR spectrum as AZQ with the exception of the loss of 4 aziridine protons and the appearance of a multiplet of 4 protons at δ3.66 to 3.78, representing the hydroxyethylamino protons and an additional one-proton singlet representing an NH proton. The mass spectrum indicated a molecular ion at m/z 382 with successive losses of 46 (C₂H₁₀OH), 31 (CH₂O), and 28 (CO) (Fig. 2, middle). These losses were similar to those reported for AZQMAA acetate (25) and, with the exception of the loss of 31, were observed with AZQ. Elemental analyses, UV, and IR spectra were consistent with the proposed structure.

AZQDAA had been previously reported with a m.p. of 182-184°C. Since our product had a m.p. of 215-217°C, we have reported its physical-chemical characteristics. The NMR, UV, and IR spectra are fully consistent with the proposed structure. The mass spectrum (Fig. 2, bottom) indicated successive losses of 46, 31, and 28 and also observed was an M + 2 ion with losses thereof. Other 1,4-benzoquinones including AZQ (Fig. 2 top) have been reported to exhibit M + 2 ions which result from hydroquinone formation due to the presence of water or other hydrogen radical sources in the mass spectrometer.

Analysis of Adducts. The standard 32P-postlabeling assay (16), with or without enrichment of adducts by butanol extraction (22) or nuclease P₁ treatment (21), has been used extensively to detect DNA adducts of polynuclear aromatics by multidirectional PEI-cellulose TLC. DNA adducts of carcinogens with one aromatic ring (such as toluidine and safrole), however, require purification of the labeled adducts by a low-salt (0.4 M) C₁₈ TLC prior to fractionation by PEI-cellulose TLC (17). Neither of the published chromatography procedures was directly applicable for AZQ adducts.

DNA Adducts in Vitro. When enzymatically hydrolyzed control and AZQ-DNAs were enriched, 32P labeled, and analyzed by C₁₈ TLC in a relatively high-salt solution (4 M ammonium formate, pH 6.2), a block of adduct radioactivity was detected (Fig. 3A). Higher ammonium formate concentration up to 8 M still did not retain the entire adduct radioactivity at the origin, and the salt concentration lower than 1 M resulted in up to 50% adduct losses. Because of the large size of the C₁₈ adduct area, we were unable to transfer adducts from the C₁₈ to a PEI-cellulose layer (17) for subsequent separation. Separation of the labeled digest by PEI-cellulose TLC in a high (3.2 M) concentration of sodium phosphate, pH 5, showed several adducts (Fig. 3B). However, removal of the salt by washing the chromatogram in water (for 5 min) resulted in substantial adduct losses. Thus, neither the C₁₈ nor PEI-cellulose TLC system provided adequate adduct resolution, although both the chromatography systems are simple and rapid for detecting AZQ adducts.

Elution of the C₁₈-retained AZQ-DNA adducts and subsequent 2-directional PEI-cellulose TLC using low-salt (0.4 M ammonium formate, pH 3.3) and high-salt (3 M sodium phosphate, pH 5) solutions resulted in satisfactory separation of...
AZQ adducts. Fig. 4b shows two major and at least eight minor adduct spots. Measurement of the adduct radioactivity revealed that the major adducts comprised over 60% of the total binding (Spot 1 = 22%; spot 2 = 40%), and 15 to 20% of the total adduct radioactivity remained at the origin of the chromatogram; control samples prepared by individually omitting DNA, AZQ, or buffer which was processed in parallel showed no spots (Fig. 4a). Although retention of the radioactive contaminants on the C18 layer somewhat varied, it did not interfere in subsequent adduct analysis. Hydrolysis of the DNA using higher enzyme:DNA ratios or by use of a larger amount of [γ-32P]ATP (200 μCi) and polynucleotide kinase (0.4 unit/µl) did not result in increased adduct recoveries as compared with the values obtained under the conditions specified in “Materials and Methods,” suggesting that the assay conditions were optimal. The solvent in which AZQ was dissolved had a marked influence on the extent of DNA binding. For instance, a 2- to 3-fold drop in the binding levels was observed when N,N-dimethylacetamide was substituted for TFE, and up to a 10-fold reduction occurred when dimethyl sulfoxide was substituted for TFE (data not shown), but the spectrum of adducts was similar. The higher reactivity of AZQ in the solvent TFE may be related to its greater solubility and stability. TFE as a solvent has also been used for other alkylating agents.* The pH at which AZQ was reacted with DNA had no effect in terms of the types of adducts; however, the magnitude of the adducts was much greater at pH 4 than observed at pH 6 and pH 7.2 (data not shown), and therefore all reactions were conducted at the lower pH.

The detection limit of this procedure was determined by serially diluting the AZQ-modified DNA (1514 ± 259 fmol/µg of DNA) by 10, 100, 1,000, and 10,000-fold with (unadducted) calf thymus DNA. The 32P-postlabeling analysis (System 2) of these DNA samples showed the method was capable of detecting 3 to 10 amol of adduct/µg of DNA (>1 adduct/10⁹ nucleotides), although quantitation of <1 adduct/3 × 10⁸ was not very reliable due to interference of the chromatographic background radioactivity.

To assess the recovery of AZQ-DNA adducts after the nuclease P₁ enrichment, the DNA digest (0.2 µg) was labeled with AZQ for 2 min and ½, 2, 4, 8, and 24 h showed the maximal DNA binding at about 2 h (1926 ± 378 fmol/µg of DNA) with the binding levels remaining practically unchanged through 4 h (1514 ± 259 fmol/µg of DNA). However, the binding declined to about 43% after 24 h, indicating that adducts were unstable (Fig. 5). Dose-dependent formation of AZQ adducts was examined by keeping the DNA concentration (1 µg/µl) constant and varying AZQ concentrations (0.1 to 1 µg/µl). A linear dose-response relation was observed between 0.1- and 0.3-µg/µl concentrations of AZQ, and then the binding levels plateaued (Fig. 5). A substantial DNA binding (49 ± 14 fmol/µg of DNA) was observed even at a DNA:AZQ ratio of 100:1, indicating that this alkylating agent is highly reactive.

AZQ-reactive Sites and Distinction between Monoadducts and Possible Cross-Link Adducts. Aziridine moieties in AZQ have been considered sites responsible for the antitumor activity of this drug. To establish that the aziridine moieties were primarily involved in interaction with DNA, AZQ was converted to AZQMAA and AZQDDA derivatives. The 32P-postlabeling analysis of DNA incubated with these derivatives indicated no detectable DNA binding with AZQDDA, but AZQMAA was found to bind to the DNA as efficiently as did AZQ (Fig. 7). These results suggest that the DNA binding is mediated via the aziridine site(s). A comparison of 2-directional profiles of the AZQ- and AZQMAA-DNA adducts indicated that adducts 1 to 10 were also produced by AZQMAA (not shown), suggesting

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* K. Hemminki, personal communication.

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Fig. 4. 32P fingerprints of AZQ-DNA adducts. Following purification on a C18 layer, the eluted labeled adducts (~7.06 µg of DNA) were resolved by 2-directional PEI-cellulose TLC and detected by exposure to X-ray film at room temperature for 30 min.
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Fig. 5. Time-dependent formation of AZQ-DNA binding. Calf thymus DNA (1 µg/µl), reacted in vitro at 37°C with an equal concentration of AZQ, was analyzed by the 32P-postlabeling. Points, mean; bars, SD.

Fig. 6. Dose-dependent formation of AZQ-DNA adducts. Calf thymus DNA (1 µg/µl) was reacted in vitro with varying concentrations of AZQ (0.01 to 1 µg/µl) by incubation at 37°C for 4 h, and adducts were analyzed by the 32P-postlabeling.

that they are monoadducts. The adduct radioactivity remaining at the chromatogram origin (Fig. 4b) may contain cross-link adduct(s), although we cannot rule out that part or whole of the origin adduct radioactivity may have also resulted due to depurination of some of the labile AZQ adducts.

Identification of AZQ Adducts. To identify the nature of DNA bases involved in adduct formation, dAp, dGp, dCp, and dTp were reacted with AZQ, and the adducts were analyzed by 32P-postlabeling in parallel with AZQ-DNA adducts. The binding in fmol/µg of nucleotides was in the descending order as follows: dAp (5623 ± 684), dGp (2077 ± 176), dCp (1881 ± 375), and dTp (121 ± 11), indicating all the nucleotides reacted extensively, except dTp which reacted only slightly. Control nucleotides processed in parallel showed no adducts. Cochromatography of the nucleotide and DNA adducts using the solvents indicated in “Materials and Methods” showed (Fig. 8) that Spots 5 to 8 were clearly guanine derived, but other adducts cochromatographed with more than one nucleotide, i.e., Spot 1 (C, G); Spot 2 (A, G); Spot 3 (C, G); Spot 4 (A, G); Spot 9 (C, G); and Spot 10 (A, G). When 0.6 M Tris-HCl, pH 8, was substituted in Direction 2, DNA Adduct 2 separated from dAp adduct (not shown), indicating that it was guanine derived. However, the second major adduct 1 still coigrated with both dGp and dCp in many other solvents (0.6 M ammonium formate, pH 2.2, and pH 3.8; 0.5 M Tris-HCl, pH 9.5; methanol:4 N ammonia, 1:1; and 0.4 M lithium chloride, 0.05 M sodium phosphate, pH 6). These results suggest that the predominant adduct 2 and several minor adducts (nos. 5 to 8) are all guanine derived. In attempts to further characterize their chemical nature, a 5'-32P-labeled 171-base pair Haelll/HindIII restriction fragment of rat liver DNA (18) was treated in vitro with AZQ and then exposed to vigorous alkaline conditions (1 M pyrimidine; 90°C; 30 min) in order to obtain strand cleavage at the labile adduct sites (26). When this digest was electrophoresed alongside base-specific chemically cleaved size markers on a 12% polyacrylamide/7 M urea sequencing gel, discrete radioactive bands were obtained at almost all guanine residues in the sequence (data not shown). These results suggest that AZQ alkylates guanine at the N7 position.

DISCUSSION

AZQ is a symmetrical molecule having at least two types of reactive moieties, each with potential to damage DNA (Fig. 1). (a) The quinone can undergo a reduction-oxidation cycle generating free radicals which could produce DNA damage, especially DNA strand scissions (8), and (b) under physiological conditions without exogenous activation, the two aziridine rings can alkylate DNA, producing DNA-DNA and DNA-protein

Fig. 7. One-directional TLC separation on PEI-cellulose using 3.2 M sodium phosphate, pH 5, of 32P-labeled adducts of vehicle only (1), AZQ (2), AZQMAA (3), and AZQDAA (4) reacted in vitro with calf thymus DNA.
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a. DNA  b. dGp  c. a+b  d. dAp  e. a+d  f. dCp  g. a+f

Fig. 8. Cochromatography of adducts of DNA and indicated mononucleotides reacted in vitro with AZQ. Adducts were mapped by PEI-cellulose TLC as described in the text.

cross-links (8, 9). Since only the aziridine groups appear essential for the antitumor activity of AZQ, the aziridine-DNA interaction could be important for chemotherapeutic response.

In this report we have used a modification of the standard $^{32}$P-postlabeling assay to detect relatively polar adducts of the antitumor agent AZQ. Like many other alkylating agents (27), AZQ showed a spectrum of multiple adducts interacting predominantly with guanine, although several minor adducts could be related to adenine and cytosine. The aziridine moieties of AZQ were found to be responsible for inducing DNA adducts as evidenced by complete loss of DNA reactivity when both the aziridine groups were hydrolyzed to their diaminoalcohol derivatives. No noticeable effect was, however, seen on the extent of DNA binding when only one aziridine group was hydrolyzed, indicating that only one aziridine group is required for producing monoadducts. Consistent with this finding is the conclusion of Egorin et al. (28), who showed that when the aziridine rings of AZQ were hydrolyzed, the resulting molecule was not accumulated by L1210 cells and did not form free radicals when added to L1210 cells. AZQ was a much less effective inhibitor of $[H]$thymidine incorporation by L1210 cells than was AZQ.

Previously described chromatography procedures for detecting polynuclear carcinogen adducts (16, 17, 19) or adducts with one aromatic ring (such as alkenylbenzenes; 17) were not applicable due to the polar nature of AZQ adducts. Unlike the published procedure in which a low-salt (0.4 M ammonium formate) C$\text{r}$reversed-phase TLC permitted separation of the labeled adducts from the bulk of the nonadduct radioactivity, the present method required an unexpectedly much higher ammonium formate concentration (4 M) to retain most or all labeled AZQ adducts on the C$\text{r}$layer. Furthermore, subsequent separation on a PEI-cellulose layer required a unique combination of non-urea salt solutions of extreme ionic strengths, such as relatively low salt (0.4 M ammonium formate, pH 3.3) in the first direction and a rather high salt (3 M sodium phosphate, pH 5) in the second direction. Although this method involves an additional step as compared with the procedures published for aromatic carcinogen adducts (16, 17, 19), this chromatography strategy should be applicable for sensitive detection of relatively polar adducts.

The in vitro reaction of AZQ with individual nucleotides (dAp, dGp, dCp, and dTp) indicated that while dAp and dCp showed higher binding than dGp, the principal adduct detected at the DNA level is a guanine derivative. Since our sequencing data suggest that labile AZQ adducts, most probably $N^2$ of guanine, are present in the DNA, and the $^{32}$P-postlabeling assay probably does not detect products formed by interaction with other alkylating agents (e.g., 29), it is possible that the presumably $N^2$-guanine adduct was also not detected in our analysis and, in that case, AZQ-dGp binding levels would be greater than obtained in this study.

With this highly sensitive and improved methodology requiring only microgram amounts of DNA, it should be possible to quantify DNA adducts in the peripheral blood lymphocytes and biopsies of target tissues of patients receiving AZQ chemotherapy. Such studies in humans may help establish relationships between the DNA modifications and another genotoxic endpoint, such as sister chromatid exchange. Kligerman et al. (11) have shown that AZQ is a potent sister chromatid exchange inducer in patients receiving AZQ chemotherapy, more potent than in in vivo-exposed mice. Therefore, it should be possible to make direct comparisons for DNA adduct-forming capability of AZQ between rodents and humans exposed in vivo under controlled conditions. Finally, since large interindividual vari-
ations have been reported in drug uptake and metabolism and in repair capabilities of the induced DNA lesions (30, 31), quantitation of AZQ-DNA adducts in relation to dose could help determine a biologically effective dose range in individual patients and minimize exposure to excessive doses. Some of these studies are currently being pursued in our laboratories. Poirier et al. (32) have already demonstrated a correlation between the ability of patients receiving the anticancer drug cisplatin to form high levels of DNA adducts and the frequency of tumor remission. Adduct distribution in peripheral blood cell DNA and tumor tissue was also shown to be similar by these investigators (32).

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