Induction of DNA Strand Breaks and Cross-Links by 2,5-Diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone in Chinese Hamster Ovary Cells

Cheung Lam King, Walter N. Hittelman, and Ti Li Loo

ABSTRACT

The DNA-damaging effects of 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone (AZQ) in Chinese hamster ovary cells were investigated. As determined by alkaline elution, DNA strand breaks were observed in cells treated with 50 μM AZQ for 2 hr. The single-strand break frequency was 31.3 ± 5.3 (S.D.) rad equivalents. Strand breaks could also be detected at lower drug concentrations if proteinase K treatment was included before strand breaks were observed in cells treated with 50 μM AZQ, 25, and 50 μM AZQ, respectively. Both DNA-DNA and DNA-protein cross-links in AZQ-treated cells were revealed by the proteinase K assay. The DNA strand breaks induced by AZQ were rapidly rejoined within 1 hr after drug removal. DNA interstrand cross-links increased within the first 6 hr of postincubation and then slightly decreased by 12 hr, and most of the cross-links disappeared after cells were allowed to recover for 24 hr. DNA-protein cross-links were immediately formed during the drug treatment period and were gradually decreased after drug removal.

INTRODUCTION

The aziridinylbenzoquinone derivatives have shown a wide spectrum of antitumor activity (3). They are rationally designed to be lipid soluble and not ionizable at physiological pH, thus allowing them to cross the blood-brain barrier with relative ease. Among them, AZQ (NSC-182986) is especially promising in that it is not only active against i.p. implanted L1210 leukemia, P388 leukemia, and B16 melanoma (3) but is also capable of increasing the life spans of mice implanted with an ependymoblastoma (3, 4). AZQ is currently undergoing phase I and II clinical trials. The mechanism of its antitumor action is not known, although its structure suggests that it may be a bifunctional cross-linking agent (1, 12). It has been reported that AZQ stimulated oxygen consumption by rat liver microsomes with the formation of semiquinone free radicals (6). We have reported recently that reduced AZQ was rapidly rejoined within 1 hr after drug removal. DNA interstrand cross-links increased within the first 6 hr of postincubation and then slightly decreased by 12 hr, and most of the cross-links disappeared after cells were allowed to recover for 24 hr. DNA-protein cross-links were immediately formed during the drug treatment period and were gradually decreased after drug removal.

MATERIALS AND METHODS

AZQ was supplied by the Pharmaceutical Resources Branch of the National Cancer Institute. The drug solution was prepared fresh for each experiment by dissolving AZQ in 10% dimethyl sulfoxide and further diluted with 0.9% NaCl solution. [methyl-3H]Thymidine (specific activity, 40 Ci/mmol) and [2-14C]thymidine (specific activity, 40 mCi/mmol) were obtained from ICN, Irvine, CA. Proteinase K was purchased from Sigma Chemical Co., St. Louis, MO, and tetracyprylammonium hydroxide was from RSA Corporation, Ardsley, NY.

Cell Culture. CHO-K1 cells were obtained from Dr. P. N. Rao (M. D. Anderson Hospital, Houston, TX). They were grown in monolayers in 25-cm² tissue flasks in 10 ml of McCoy’s Medium 5A supplemented with 10% fetal calf serum. The doubling time of these cells was 12 to 14 hr. For alkaline elution studies, cells in exponential growth phase were treated for 24 hr with either [3H]thymidine (10 nCi/ml) or [14C]thymidine (0 nCi/ml). The radioactivity was chased into high-molecular-weight DNA by a 7 hr incubation period in 10 ml fresh medium before drug treatment.

Alkaline Elution. Essentially, the procedure of alkaline elution of Kohn et al. was adopted in our studies (10). [14C]Thymidine-labeled CHO cells were treated with 0.1 ml of AZQ solutions of varying concentrations for 2 hr at 37°C. Control cells received the vehicle only. In some experiments, cells were further incubated in the absence of the drug for 0.5 to 24 hr. [3H]Thymidine-labeled cells that had been irradiated with 300 rads of γ-ray from a 137Cs source were included in the elution assay as an internal standard. Portions of the incubation mixture containing 5 x 10⁶ 14C-labeled and 3H-labeled cells were loaded onto polyvinyl chloride filters of 2.5-μm pore size (BSWP-02500; Millipore Corp., Bedford, MA) and lysed with 5 ml of 0.2% sodium dodecylsarcosine-2 M NaCl-0.04 M EDTA (pH 10). The solution was drained by gravity, and the filters were gently washed with 5 ml of 0.02 M EDTA, pH 10. DNA was eluted with tetracyproplylammonium hydroxide-0.02 M EDTA (pH 12.1) at a flow rate of 0.035 ml/min. For the proteinase digestion assay, cell lysates were treated with 5 ml lysing solution containing proteinase K (0.5 mg/ml) for 1 hr, and the DNA was eluted with the tetracyproplylammonium hydroxide solution containing 1% of sodium dodecyl sulfate. Fractions were collected at 1.5-hr intervals for 15 hr. The eluates were mixed with 10 ml of PCS (Phase Combining System, Amersham, Arlington Heights, IL) containing 0.7% glacial acetic acid and counted in a Packard Model 2650 Tri-Carb scintillation spectrometer. The filters were treated with 0.4 ml of 1 N HCl at 70°C for 1 hr, followed by 2.5 ml 0.4 N NaOH. Radioactivity on the filter was measured as above. The fraction of [14C]DNA remaining on the filter was plotted against that of [3H]DNA remaining on the filter on a double logarithmic scale. The apparent single-strand break frequency (P0) in (rad-equivalents) produced by AZQ was estimated by the equation

\[ P0 = P_{\infty} \frac{\log(\eta/\eta_0)}{\log(\eta_0/\eta)} \]

where \( P_{\infty} \) equals 300 rads of γ-ray and \( \eta, \eta_0, \) and \( \eta_0 \) represent the...
fraction of $^{14}$C-labeled DNA retained on the filter for the control, AZQ-treated, and irradiated cells when 50% tritiated DNA is retained on the filter (5). In addition, the apparent cross-linking frequency was calculated by the formula

$$P_c = \left( \frac{[(1-R_0)/(1-R_1)]^{1/\alpha} - 1} \right) \times (P_{bo} + P_{we}) \quad (10)$$

where $R_1$ represents the fraction of $[^{14}$C]DNA in the AZQ-treated irradiated cells. The strand break frequency and cross-linking frequency were estimated both in the presence and absence of proteinase K digestion. Each experiment was repeated 3 times, and the results were expressed as the mean ± S.D.

RESULTS

AZQ-induced DNA Strand Breaks. The possibility of induction of DNA strand breakage by AZQ was ascertained by treating CHO cells with AZQ for 2 hr and analyzing by alkaline elution. As shown in Chart 1, DNA strand breaks appeared only in cells treated with 50 μM AZQ. The single-strand break frequency without proteinase K in rad-equivalents was 31.3 ± 5.3 (S.D.). DNA strand break could also be detected in cells treated with 25 μM AZQ if proteinase K was included before elution. The single-strand frequencies were then 35.7 ± 6.2 and 68.8 ± 7.5 for 25 and 50 μM AZQ.

Detection of DNA-DNA and DNA-Protein Cross-Links. To determine whether AZQ could induce DNA-DNA and DNA-protein cross-links, cells were irradiated with 300 rads of γ-ray from a 137Cs source after a 2-hr treatment with AZQ. Formation of either DNA-DNA or DNA-protein cross-links increases the retention of DNA as compared with that of irradiated control DNA. As shown in Chart 2, the rate of DNA elution induced by γ-irradiation was decreased in cells receiving as low as 6.25 μM AZQ, and the degree of cross-linking increased with AZQ concentrations. Since DNA-protein cross-links could be removed by protease (5), increases in DNA elution rate after protease treatment would indicate the presence of DNA-protein cross-links. Additionally, DNA retention was reduced by proteolytic digestion with proteinase K. Thus, in irradiated cells treated with 25 μM AZQ, proteinase K reduced cross-link frequency from 230.3 ± 31.7 to 139.1 ± 16.7 rad equivalents. However, some of the DNA cross-links resisted proteinase K digestion even after prolonged treatment, suggesting the continued presence of DNA-DNA cross-links. The data are summarized in Table 1.

![Chart 1](image1.png)

Chart 1. Induction of DNA strand breaks in CHO cells treated with AZQ. The cells were treated with AZQ: ○, 12.5 μM; ●, 25 μM; ■, 50 μM. An additional cell-sample (▲) received 300 rads of γ-irradiation only. Δ, control cells without any treatment. Right panel and left panel represent DNA eluted with or without proteinase K treatment, respectively.

![Chart 2](image2.png)

Chart 2. DNA cross-links in CHO cells treated with AZQ: O, 6.25 μM; □, 12.5 μM; ■, 25 μM; ■, 50 μM. Δ, cells that did not receive drug treatment. All cells received 300 rads of γ-irradiation before alkaline elution. Proteinase K treatment included in some experiments is shown in the right panel.

![Table 1](image3.png)

Table 1

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>$P_{bo}$</th>
<th>$P_r$ (total)</th>
<th>$P_{bo}$ (proteinase K-treated)</th>
<th>$P_r$ (proteinase K-resistant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>0</td>
<td>39.7 ± 4.7</td>
<td>0</td>
<td>19.8 ± 3.2</td>
</tr>
<tr>
<td>12.5</td>
<td>0</td>
<td>124.3 ± 19.5</td>
<td>0</td>
<td>63.8 ± 7.3</td>
</tr>
<tr>
<td>25.0</td>
<td>0</td>
<td>230.3 ± 31.7</td>
<td>35.7 ± 6.2</td>
<td>138.1 ± 16.7</td>
</tr>
<tr>
<td>50.0</td>
<td>0</td>
<td>313 ± 5.3</td>
<td>625.1 ± 63.2</td>
<td>455.9 ± 50.5</td>
</tr>
</tbody>
</table>

*Mean ± S.D.*

Repair of DNA Lesions. The repair of the AZQ-induced strand breaks and cross-links was also studied. CHO cells were treated with 25 μM AZQ for 2 hr, reincubated in drug-free medium for various intervals, and analyzed by alkaline elution with and without proteinase K treatment. The results are shown in Charts 3 and 4. The apparent strand break frequency decreased rapidly after drug removal, and practically no strand breaks could be detected by 4 hr (Chart 3). Chart 4 shows that the formation of DNA-DNA cross-links increased within 6 hr after drug removal and then slightly decreased by 12 hr. Most of the cross-links were removed in cells incubated in drug-free medium for 24 hr. These data also suggest that DNA-protein cross-links appeared...
AZQ-INDUCED DNA DAMAGE

The means of total and proteinase K resistant cross-links. Following drug removal. •, total cross-links; •, proteinase K resistant cross-links.

Degree of DNA-protein cross-links (A) was obtained from the difference between the means of total and proteinase K resistant cross-links.

immediately after drug treatment and gradually decreased upon drug removal.

DISCUSSION

In our studies, we have shown that, in cultured CHO cells, AZQ not only induced DNA strand breaks (Chart 1) but also DNA-DNA and DNA-protein cross-links (Chart 2). DNA cross-links were detected after a 2-hr incubation with 6.25 μM AZQ. Single strand breaks occurred in cells treated with higher drug concentrations. The single-strand break frequencies in these studies were probably underestimated, because AZQ may produce many cross-links (both DNA-DNA and DNA-protein) that interfere with the detection of strand breaks by alkaline elution. In cells treated with 25 μM AZQ, strand breaks could be detected only after the removal of DNA-cross-links by proteinase K treatment (Chart 1). Thus, AZQ is similar to some intercalating agents that likewise produce protein-associated DNA strand breaks not detectable before proteolytic digestion (14, 15). However, in another series of experiments (data not included), we failed to demonstrate intercalation by AZQ by viscometric measurements, although such intercalation activities by quinoid drugs have been suggested (11).

In the present study, the strand break frequency decreased 90% within 1 hr of postincubation. This may be accounted for partly by the rejoining of the DNA strand breaks and partly by the gradual formation of DNA cross-links. We thought that AZQ-induced strand breaks are repaired soon after drug removal as are strand breaks caused by other drugs or radiation (7, 18). To calculate the cross-link frequency in the DNA repair experiments, we assumed that DNA strand breaks have been completely removed during the repair period.

We found that very few DNA interstrand cross-links were removed in AZQ-treated cells, even after a 12-hr recovery period. Significant repair was not observed until 18 hr after postincubation. The conversion of AZQ-DNA monoadducts to bifunctional DNA-DNA cross-links occurred within 6 hr of postincubation, which was rapid as compared with other cross-linking agents (5, 11). The cross-linking characteristic of AZQ is reminiscent of that of cisplatin. Both agents produce significant amounts of cross-links within a relatively brief drug treatment period, and the maximum cross-linking activity appears several hr after drug removal. In addition, most of the DNA-DNA cross-links are removed after 24 hr of postincubation (9).

The formation of AZQ cross-links may be mediated through the aziridinyl rather than the carboethoxyamino moieties, since the aziridine rings are essential for the antitumor effects, while the carboethoxyamino groups are not (3). Furthermore, nitrogen mustard alkylates DNA through the formation of active aziridinyl functions (9). Gutierrez et al. (6) found that rat liver cell nuclei could be used instead of NADPH:cytochrome P-450 reductase to activate and stimulate AZQ for O2 consumption. The Km values of AZQ for both systems were the same. Recently, Szmygiero (16) reported that AZQ induced DNA interstrand cross-links and single strand breaks in L1210 cell nuclei; these actions required NADPH. We speculate that AZQ may have been reduced in the nucleus by a reductase ultimately to generate free radicals that caused DNA breakage and cross-links. We have shown previously that AZQ causes DNA scissions and interstrand cross-links only after reduction, reminiscent of earlier reports that some quinoid antibiotics likewise require prior reductive activation for antitumor effects (2, 13).

Akhtar et al. (1) reported that the antitumor effect of certain aziridinyl benzoquinones could be correlated with their abilities to form covalent cross-links with isolated λ DNA. Using cisplatin and its trans-isomer, Zwelling et al. (17) suggested that the interstrand cross-links but not the DNA protein cross-links correlated with cytotoxicity. Our study illustrates that AZQ caused DNA strand breaks as well as DNA-DNA and DNA-protein cross-links in cultured CHO cells, but the significance of these lesions in the AZQ-induced cytotoxicity remains to be determined.

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REFERENCES

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