DNA Strand Scission and Cross-Linking by Diaziridinylbenzoquinone (Diaziouquone) in Human Cells and Relation to Cell Killing

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ABSTRACT

The effects of 3,6-diaziridinyl-2,5-bis(carboxethoxyamino)-1,4-benzoquinone (diaziouquone; AZQ) on various cell types were studied in relation to two chemical reactivities that this drug would be expected to have intracellularly. AZQ can undergo a reduction-oxidation cycle of the quinone function; this could generate free radicals which could produce DNA damage, especially DNA strand scission. The second reactivity, based on the two aziridine groups, could produce alkylation reactions that could produce DNA cross-links. DNA strand breakage and cross-linking were measured by alkaline elution and were compared with cell killing assayed by colony survival. Among the cell strains studied (human IMR-90, VA-13, and HT-29 and mouse L1210), marked differences were found in the magnitudes of DNA strand breakage and interstrand cross-linking produced by AZQ. Most striking, IMR-90 cells exhibited substantial strand breakage and little or no interstrand cross-linking, whereas the reverse was true for HT-29 cells. Cell killing correlated well with interstrand cross-linking but did not correlate with strand scission in these cell lines. It is concluded that AZQ produces DNA strand breaks and interstrand cross-links by different mechanisms which vary independently among different cell lines.

INTRODUCTION

AZO is an effective antitumor agent in experimental murine tumor systems (2, 12, 6) and has promising clinical activity against primary brain tumors (4, 7, 11, 18, 5) and lymphomas (1). The chemical structure of the drug suggests that it may have 2 types of chemical reactivities of possible biological significance: (a) it is a quinone and has been reported to undergo enzymatic reduction by microsomes to a free radical, probably a semiquinone (10). This intermediate can transfer an electron to molecular oxygen to form superoxide which could generate other radicals; (b) AZQ has 2 aziridine groups and therefore may be a bifunctional alkylating agent which may produce DNA cross-links. In the present work, we have measured the production of DNA strand breaks and interstrand cross-links by AZQ in several cell lines in order to determine which of these DNA lesions predominates. We find marked differences in the relative production of strand breaks and interstrand cross-links among different cell lines. Cell killing appeared to be correlated with interstrand cross-linking and not with DNA strand scission.

MATERIALS AND METHODS

Cell Culture. IMR-90, a strain of normal human embryo fibroblasts; VA-13, a cell line which originated by transformation of human embryo cell strain WI-38 with SV40; and mouse leukemia L1210 cells have been maintained in this laboratory for several years. HT-29 cells, derived from a human colon carcinoma, were obtained from Dr. E. Jensen and have also been maintained in this laboratory for several years. IMR-90 cells, VA-13 cells, and HT-29 cells were cultured in Eagle’s basal medium supplemented with 10% fetal calf serum, 1 μg/ml L-glutamine, gentamicin (50 μg/ml), and 0.02 μl-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer at 37°. L1210 cells were cultured in RPMI 1650 medium supplemented with 15% heat inactivated (60°, 45 min) fetal calf serum plus penicillin and streptomycin. For DNA damage studies, cells in exponential growth phase were labeled for 18 hr with 0.01 μCi of [2-14C] thymidine per ml (New England Nuclear, Boston, MA). The labeling period was followed by 20 to 24 hr of incubation in fresh medium.

Drug Treatment. AZQ was obtained through the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. The drug was dissolved in N,N-dimethylacetamide immediately before use and was diluted in water to 10 ml. The concentration of N,N-dimethylacetamide during treatment of cells did not exceed 0.15% and did not alter the DNA elution patterns. Cells (5 × 106) in 25-cm2 plastic flasks containing 10 ml of medium were treated for 1 hr at 37°. The cells were harvested and analyzed for DNA strand breaks and interstrand cross-links by means of alkaline elution assays (13, 16). In some experiments, when inhibition of DNA single strand break formation was studied, the cells were treated with the drug in the presence of dimethyl sulfoxide (Baker Chemical Company), glyceral, bovine catalase, or bovine superoxide dismutase (Sigma Chemical Company).

DNA Damage Measurements. DNA SSB, DNA interstrand cross-links, and DNA-protein cross-links were assayed by alkaline elution as described previously (8, 16). The assays for SSB and interstrand cross-links utilized 0.8-μm-pore-size polycarbonate filters (Nucleopore; pore size, 0.8 μm) and included proteinase K digestion to minimize protein adsorption to the filter. The assays for DNA-protein cross-links utilized polyvinyl chloride filters (pore size, 2 μm) and did not include proteinase K.

DNA Strand Breaks. Cells were harvested immediately after drug treatment and resuspended in ice-cold Eagle’s minimal essential medium. Aliquots of 2 to 5 × 106 [14C]thymidine-labeled cells were mixed with approximately the same number of 3H-labeled L1210 cells irradiated with 300 rads of γ-radiation as internal standards (see Ref. 16). After having been deposited on filters, cells were lysed with 2% SDS, 0.1 M glycerol, and 0.025 M EDTA, pH 10, and treated for 60 min with 0.5 mg of proteinase K per ml in the same lysis solution. Elution was then carried out at 2 ml/hr with tetrapropylammonium hydroxide-EDTA-0.1% SDS, pH 12.1. Samples were collected at 3-hr intervals and assayed for 14C and 3H radioactivity. The apparent DNA single-strand break frequency (PSSB) expressed in rad equivalents, was calculated by the formula

\[ P_{SSB} = \frac{\log (r_1/r_0)}{\log (P_{rad})} \]  

(1)
where $P_{\text{sm}} = 300$ rads and $R_1$, $R_0$, and $R_0$ are the retentions of $[\text{14C}]$DNA from drug-treated, untreated, and 300-rad-irradiated cells, respectively. The retention end points were taken at the point of elution when 50% of the $[\text{3H}]$DNA remained on the filter.

DNA Interstrand Cross-Linking. Interstrand cross-linking was usually measured after cells had been incubated for an additional 4 hr in drug-free medium. This allows time for the second-arm reaction of cross-link formation. Each sample was prepared as described in the case of DNA strand break assays and divided into 2 aliquots. One was used for SSB measurement, and the second was irradiated with 300 rads in the cold and eluted as before. If the nonirradiated sample exhibited no SSB, the following formula was used to calculate the apparent cross-link frequency ($P_c$), expressed in rad equivalents:

$$P_c = \left[ \sqrt{1 - R_0} \right] \left( 1 - R_1 \right) - 1 \quad P_{\text{sm}}$$  \hspace{1cm} (B)

where $P_{\text{sm}}$ is 300 rads and $R_1$ and $R_0$ are the fractions of $\text{14C}$-labeled DNA (drug-treated and untreated, respectively), which remained on the filter when 25% of $[\text{3H}]$DNA remained on the filter. When treated irradiated samples exhibited DNA strand breaks, the estimated drug-induced strand break frequency was used to correct the value of $R_0$ according to the formula

$$\log R_0 = \left( 1 + \frac{R_0}{P_{\text{sm}}} \right) \log R_0$$  \hspace{1cm} (C)

where $R_0$ is the corrected value which was then used in place of $R_0$ in Equation B (see Ref. 16 for details).

Cytotoxicity Studies. For colony formation assays, single-cell suspensions of IMR-90 cells, VA-13 cells, and HT-29 cells were prepared by treating monolayers as described previously (8). For controls, 100 cells were plated per 50-mm-diameter plastic dishes for IMR-90 cells and VA-13 cells or per 25 sq cm plastic flasks for HT-29 cells. The dishes or flasks were incubated for 24 hr to allow the cells to attach. For drug treatment, 100 to 6000 cells were used. The cells were exposed to the drug for 1 hr at $37^\circ$ in Eagle's MEM, washed, and placed in fresh medium. After 14 days (IMR-90 cells and VA-13 cells) or 10 days (HT-29 cells) of incubation, the colonies were fixed with methanol and stained with methylene blue, and colonies (>32 cells) were counted. Plating efficiencies were 11 to 27% for untreated IMR-90 and VA-13 cells and 62 to 88% for HT-29 cells. L1210 cells were treated in Eagle's medium or RPMI 1630 for 1 hr at $37^\circ$ and assayed for colony formation in soft agar in RPMI 1630 using the test tube method (3). Colony-forming efficiencies in untreated L1210 cells were 65 to 72% and were approximately the same in Eagle's MEM and RPMI 1630.

RESULTS

DNA Strand Scission. When VA-13 cells were treated with AZQ for 1 hr, there was a marked increase in DNA elution rate, depending on the AZQ concentration, as shown in Chart 1. In the semilogarithmic alkaline elution plots, linearity has been assumed to indicate the presence of randomly distributed DNA strand breaks, since linear patterns are usually obtained following X-irradiation (13, 16). The alkaline elution patterns obtained following treatment with AZQ deviated significantly from linearity (Chart 1), suggesting that there is a nonrandom aspect to the
AZQ-induced strand breakage, perhaps due to different break frequencies among different cells in the population. This is consistent with the concave shape or multicomponent character of the elution curves. [The curvature cannot be attributed here to DNA-protein cross-links, because DNA-protein cross-link effects were eliminated in these assays by the use of proteinase K and polycarbonate filters (16)]. The average DNA SSB frequency was estimated from the average slope of the elution curves up to the point at which 50% of the internal standard DNA had eluted (0.5 on the abscissa of Chart 1; see “Materials and Methods”).

When different cell types were compared, marked differences were observed in AZQ-induced strand break frequencies (Chart 2). The normal and transformed human embryo cell strains (IMR-90 and VA-13, respectively), exhibited high and approximately equal extents of strand breakage, whereas the human colon carcinoma cell line, HT-29, showed little or no strand breakage. Mouse leukemia L1210 cells, when treated in the same type of Eagle’s MEM used for the growth and treatment of the human cells, exhibited low but significant strand break frequencies (Chart 2). When L1210 cells were treated in RPMI 1630 (the type of medium in which they were grown), however, the strand break frequencies were approximately 4-fold higher (Chart 2).

When treated cells were incubated in the absence of drug for 4 hr, the DNA strand breaks disappeared, presumably due to a repair process (Chart 3A).

Effects of Free Radical Scavengers. Because of the possibility that the AZQ-induced DNA strand breakage may stem from the reduction of the quinone group and consequent production of hydroxyl radicals, we tested the effects of free radical scavengers. The AZQ-induced DNA strand breakage in VA-13 cells was found to be partially inhibited by several types of free radical scavengers (Table 1). Glycerol (1 M) produced approximately 50% inhibition, and a lesser degree of inhibition was obtained with 1.3 M dimethyl sulfoxide. These compounds did not by themselves produce DNA strand breaks. Catalase and, to a lesser degree, superoxide dismutase also produced partial inhibitions. The combination of catalase plus superoxide dismutase had an effect similar to that of catalase alone. The effectiveness of the enzymes may be limited by their poor penetration into cells.

**DNA Interstrand and DNA-Protein Cross-Linking.** The formation of interstrand and DNA-protein cross-links by AZQ in VA-13 cells is illustrated in Chart 3. Both types of cross-links reduce the elution rate of DNA from filters; for the assays, an appropriate frequency of DNA strand breaks was introduced by exposing the cells to X-rays so as to generate a suitable DNA elution rate, the reduction of which is taken as a measure of cross-linking. The effect of DNA-protein cross-linking can be eliminated by the use of proteinase K and nonadsorbent filters; any remaining reduction in elution rate is then taken to be due to interstrand cross-linking. Chart 3B shows the total cross-linking effect (mainly DNA-protein cross-links in these experiments), whereas Chart 3A shows the effect of interstrand cross-links remaining after the elimination of DNA-protein cross-links.

As has been observed previously with several other cross-linking agents (14, 15), including melphalan, chloroethylnitrosoureas, and cis-dichlorodiammineplatinum (II) complexes, AZQ-induced interstrand cross-links accumulate slowly during incu-
bation after the drug has been removed. The delayed cross-linking is attributed to a slow second alkylation reaction in which drug-DNA monoadducts react with the opposite strand. In the case of AZQ, interstrand cross-linking appeared to be maximal after 4 to 6 hr of posttreatment incubation at 23° (data not shown). Some of the early rise in interstrand cross-linking may, however, really represent the disappearance of strand breaks. Most of our measurements were made after 4 hr of posttreatment incubation, at which time the SSB were almost completely repaired (Chart 3A).

Marked differences in interstrand cross-linking were observed among different cell lines (Chart 4). IMR-90 cells did not produce detectable interstrand cross-links, and L1210 cells produced barely measurable quantities. In VA-13 cells, significantly more interstrand cross-linking was seen. HT-29 cells, however, showed by far the greatest interstrand cross-linking.

**Cytotoxicity.** AZQ produced different degrees of cytotoxicity in the cell lines studied (Chart 5). The most sensitive were HT-29 cells, in which a 3-log cell kill was found at concentration of 30 μM AZQ. The 3 other cell lines were much less sensitive, but the differences between them were statistically significant. Ten % survival was found for HT-29, VA-13, IMR-90, and L1210 cells at the following approximate drug concentrations: 18, 45, 82, 100 μM, respectively. When L1210 cells were treated with the drug in RPMI 1630 medium instead of Eagle’s MEM, the cytotoxic effect of AZQ increased, and the cell sensitivity was similar to that exhibited by VA-13 cells.

**DISCUSSION**

As anticipated, we find that AZQ can produce both DNA cross-links and DNA strand breaks in mammalian cells. The cross-links presumably arise through bifunctional alkylations by the 2 aziridine groups. As has generally been the case for a variety of bifunctional alkylating agents (14, 15), we find that AZQ produces DNA-protein cross-links, as well as DNA interstrand cross-links. It is possible that intrastrand cross-links also are formed, but our methodology would not detect these lesions.

The strand breaks probably arise by a free radical mechanism stemming from intracellular reduction of the AZQ quinone func-

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**Table 1**

<table>
<thead>
<tr>
<th>Agent added</th>
<th>Concentration</th>
<th>SSB frequency (% of control)</th>
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<tbody>
<tr>
<td>Glycerol</td>
<td>1.0 M</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>1.0 M</td>
<td>69 ± 8</td>
</tr>
<tr>
<td>Catalase</td>
<td>100 μg/ml</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>50 μg/ml</td>
<td>52 ± 6</td>
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<tr>
<td></td>
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<td>84 ± 6</td>
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*Mean ± S.D. for 3 or more determinations, with the exception of catalase (100 μg/ml), for which data from 2 independent experiments are shown.
tion. Thus, the free radical quenchers, glycerol and dimethyl sulfoxide, were found to diminish the AZQ-induced strand breakage (Table 1). The strand breakage was also diminished by catalase, suggesting that hydrogen peroxide plays a role in the reaction mechanism. Superoxide dismutase, on the other hand, produced only slight inhibition of strand breakage. Nevertheless, it will be seen in the following paper (19) that superoxide probably is involved in the strand breakage. The reason that the effect of superoxide dismutase was so small in the experiments with intact cells may be that the enzyme does not penetrate into the cell and that superoxide is too short-lived to equilibrate between intracellular and extracellular compartments. Catalase perhaps also fails to enter cells, but it produces a larger effect because hydrogen peroxide, being more stable, perhaps does equilibrate inside and outside the cell; hence, its destruction outside could reduce its concentration inside.

The major finding in the current work is that human cell strains can differ markedly in the relative production by AZQ of DNA strand breaks and interstrand cross-links. The most striking differences were between the normal human embryo cell strain, IMR-90, and the human colon carcinoma cell line, HT-29. IMR-90 cells responded to AZQ with the formation of a high frequency of DNA strand breaks (Chart 2) but almost no detectable interstrand cross-links (Chart 4). These cells survived AZQ treatment relatively well in colony-forming assays (Chart 5). By contrast, HT-29 cells exhibited almost no detectable signal in the strand break assay (Chart 2), whereas they exhibited a large amount of interstrand cross-linking (Chart 4) and showed a high sensitivity to killing by AZQ (Chart 5). It is of interest to note that HT-29 cells tend to be resistant to some other DNA cross-linking agents, including chloroethylnitrosoureas (9) and cisplatin (17). Among the cell strains studied, sensitivity to killing by AZQ appeared to correlate with interstrand cross-linking but not with DNA strand breakage.

The validity of these conclusions is subject to the following methodological considerations. In the alkaline elution assays, the presence of a high frequency of DNA strand breaks may obscure the assay for interstrand cross-links and, conversely, the presence of a high frequency of interstrand cross-links can diminish the assay for strand breaks (16). The magnitudes of these interfering effects have been estimated by reconstruction experiments in which known frequencies of X-ray-induced strand breaks were combined with known frequencies of nitrogen mustard-induced interstrand cross-links in cells that were then subjected to standard alkaline elution assays. These considerations were applied to our data to test the soundness of the conclusions regarding the possible roles of strand breaks and interstrand cross-links in cytotoxicity. HT-29 cells showed high interstrand cross-linking but no increase in elution rate in the strand break assay; the question is how much strand breakage might have been hidden because of the interstrand cross-links. On the basis of the reconstruction experiments, the strand break frequency in the HT-29 cells could have been, at most, 100 rad equivalents at 75 \( \mu \text{M} \) AZQ. This is small compared with at least 400 rad equivalents of strand breaks produced by this concentration of AZQ in IMR-90 and VA-13 cells. Thus, the possible effect of interstrand cross-links on the strand break assay does not alter the marked divergence between strand breakage and cytotoxicity in these cells.

Conversely, VA-13 cells showed high strand break frequencies and low values for interstrand cross-links; the question is the degree to which the cross-linking might have been underestimated because of the presence of strand breaks. Based on the results of the reconstruction experiments, the interstrand cross-linking might have been underestimated by as much as a factor of 3. Taking this into account, the interstrand cross-link frequencies in VA-13 cells were still significantly less than in HT-29 cells and were, in fact, quantitatively consistent with the cytotoxicity difference seen in Chart 5.

We conclude that, within the limited selection of cell types studied in the current work, cell killing by AZQ correlated very well with the production of interstrand cross-links and did not correlate with the production of strand breaks. This suggests that AZQ would be particularly effective against tumors consisting largely of cells that form many interstrand cross-links in response to this drug.

When intracellular conditions are such that, in addition to reduction of AZQ, there is also a prominent cycle of reoxidation, then free radical reactions leading to DNA strand breakage may predominate. Our results show that the conditions favoring this reaction in cells are independent of the conditions that favor interstrand cross-linking. The formation of DNA strand breaks may not contribute to the ability of AZQ to selectively kill tumor cells but might produce deleterious effects in a chemotherapeutic setting.

REFERENCES


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