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Alkylation of DNA by the New Anticancer Agent 3,6-Diaziridinyl-2,5-bis(carboethoxyamino)-1,4-benzoquinone (AZQ)*

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Abstract—*The bifunctional cross-linking activity of 3,6-diaziridinyl-2,5-bis(carboethoxyamino)-1,4-benzoquinone (AZQ, NSC 182986) on isolated calf thymus DNA was studied, using ethidium bromide fluorescence assay. Between 1 and 350 μM AZQ produced a dose-dependent cross-linking effect in the presence of a 10-fold excess of sodium borohydride. No cross-linking was observed in the absence of the reducing agent. AZQ can also be activated by NADH and NADPH at pH 4. The AZQ cross-linking activity exhibited a strong pH dependency, highest at acidic pH, lower at alkaline pH and not seen under neutral conditions. It was also significantly inhibited under anaerobic conditions. At pH 5 the binding ratio was 1 molecule of AZQ per 191 bases at an AZQ dose of 300 μM. Our results suggest that reduced AZQ behaved like a bifunctional alkylating agent.*

INTRODUCTION

THE AZIRIDINYLBENZOQUINONE derivatives have shown a wide spectrum of antitumor activity, particularly against CNS neoplasma [1, 2]. Among them, AZQ is especially promising in that it is not only active against intraperitoneally implanted L1210 leukemia, P388 leukemia and B16 melanoma [1], but is also capable of eliciting long-term survivors from mice implanted with intracerebral L1210 leukemia or an ependymoblastoma [2, 3]. This class of compounds is known to induce cross-links in DNA [4]. In earlier studies, Iyer and Szybalski [5] showed that certain aziridinylquinones must be activated by reduction as alkylating agents. Lin *et al.* [6] subsequently proposed the concept of bioreductive alkylation for quinoid compounds. The antineoplastic effect of some aziridinylquinones was shown to correlate with their ability to cross-link DNA covalently [4]. Mitomycin C, an aziridinylquinone antibiotic, undergoes reduction to a hydroquinone before exerting its cytotoxic effect [7]. We have recently reported that AZQ caused single- and double-strand breaks in bacteriophage PM2 DNA after chemical reduction [8]. This investigation was undertaken to demonstrate that AZQ, like mitomycin C, must also be reduced before interacting with DNA.

MATERIALS AND METHODS

AZQ and radioactive AZQ uniformly labeled with ^{14}C in the benzoquinone moiety (sp. act.: 25 μCi/mg) were supplied by the Pharmaceutical Resources Branch, National Cancer Institute, Bethesda, MD.

Drug solutions were prepared fresh for each experiment by dissolving 1.1 mg AZQ in 1 ml 50% dimethyl sulfoxide (DMSO); the final concentration of DMSO in the incubation medium was less than 5%. Calf thymus DNA, NADH, NADPH and ethidium bromide were obtained from Sigma Chemical Co. (St. Louis, MO).

Binding of AZQ to DNA

Radioactive AZQ (100 μM) was incubated with calf thymus DNA (60 μg/ml) at 37°C for 30 min in the presence or absence of 1 mM $NaBH_4$ in 50 mM acetate buffer, pH 5. The reaction mixture was passed through a Sephadex G-50 column. The absorbance of the eluate was monitored at 260 nm. Fractions of 1 ml each were collected, and the

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radioactivity was measured with a Packard TRI-CARB scintillation counter after addition of 10 ml PCS, a toluene-based phase combining scintillation fluid available commercially. To measure the drug-to-nucleotide binding ratio, radioactive AZQ (1–350 μM) was incubated with calf thymus DNA as above. Samples were chilled at 4°C at the end of incubation, and 1 ml 20% cold trichloroacetic acid containing 2% sodium pyrophosphate was added. Acid-insoluble materials were collected on Whatman GF/C glass fiber filters. Binding ratio was calculated, assuming the average molecular weight of nucleotides to be 330. DNA (60 μg/ml) in 0.1 ml incubation mixture was equivalent to 18.18 nmol of nucleotide. All experiments were repeated twice in triplicates on different days.

Ethidium bromide fluorescence assay

The bifunctional cross-linking activity of AZQ was determined by the ethidium bromide assay [4, 9]. Calf thymus DNA (120 μg/ml) was incubated with AZQ in the presence or absence of 10-fold excess of reducing agents at 37°C for 30 min. A 20-μl portion of the reaction mixture was diluted with 2 ml of the assay solution (ethidium bromide, 0.5 μg/ml in 20 mM potassium phosphate buffer, pH 11.7, containing 0.4 mM EDTA). The fluorescence of the solution was measured at an excitation wavelength of 525 nm and an emission wavelength of 600 nm. The solution was heated at 96°C for 2 min and cooled at 22°C for 5 min. The fluorescence was again measured. The ratio of the fluorescence intensities before and after heating gave the extent of covalently linked complementary DNA (CLC-DNA) as an expression of the degree of cross-linking [9]. These experiments were also repeated twice in triplicate.

Statistical analysis

The results were expressed as mean ± S.D. Statistical significance was determined with the unpaired *t* test.

RESULTS

The binding of reduced AZQ to DNA is illustrated in Fig. 1. In the absence of $NaBH_4$, DNA eluted at fraction 4 and the AZQ peak appeared at fraction 10 (Fig. 1A). After incubation with 1 mM $NaBH_4$ at pH 5, a part of the AZQ co-eluted with DNA, as evidenced by the appearance of radioactivity in the DNA fractions (Fig. 1B). Figure 2 shows that the binding of AZQ to DNA increased with AZQ concentration and reached a maximum at about 300 μM AZQ. At this point the binding ratio was 1 molecule of AZQ per 191 bases, or 96 base pairs in the case of bifunctional cross-linking. In the absence of $NaBH_4$ reduction,

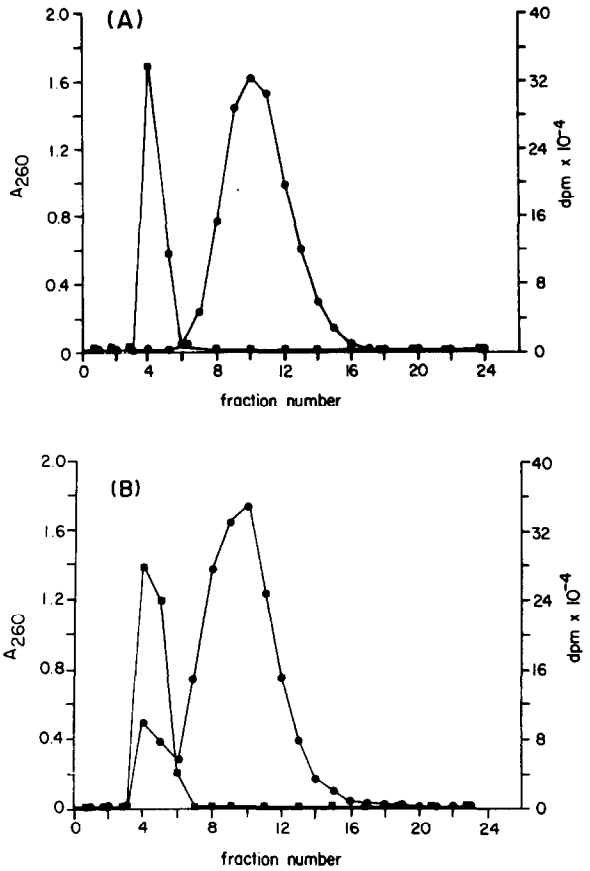

Fig. 1. Elution profile of gel filtration chromatography. Calf thymus DNA (60 μg/ml) was incubated with 300 μM of AZQ at pH 5 in the absence (A) or presence (B) of 3 mM $NaBH_4$ at 37°C for 30 min. The reaction mixture was applied to a Sephadex G-50 column and eluted with 1 mM potassium phosphate buffer, pH 7. Radioactivity (-●-●-) and absorbance at 260 nm (-■-■-) of the eluate were measured.

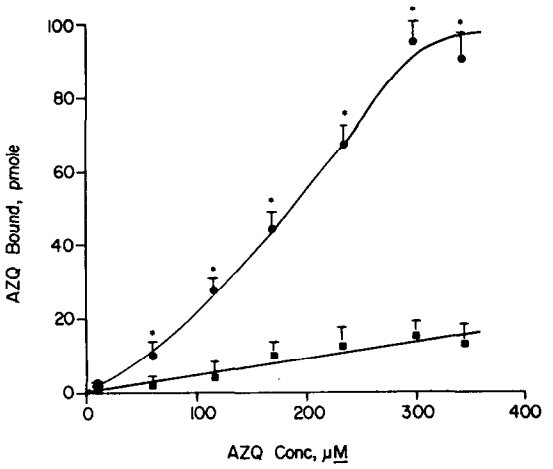

Fig. 2. Binding curve of AZQ to DNA. AZQ was incubated with 60 μg/ml DNA at pH 5 in the presence or absence of a 10-fold excess of $NaBH_4$. DNA was precipitated with trichloroacetic acid; the precipitate was collected and the radioactivity was counted. AZQ bound to DNA was determined in the presence (-●-●-) or absence (-■-■-) of $NaBH_4$. The radioactive AZQ concentrations were 1, 50, 100, 150, 200, 250, 300 and 350 μM.

1 molecule of AZQ bound to every 1212 bases. Significant binding of AZQ to DNA was observed only at 50 μM of AZQ or higher.

The cross-linking activity was strongly pH dependent, with both lower and higher pH favoring more cross-linking (Fig. 3). At pH 4 and 5 the mean percentages of CLC-DNA were 87 and 46%. At pH 10 CLC-DNA was 40%, but at pH 6–8 no significant cross-linking was observed. No AZQ-induced cross-linking was detected in the absence of $NaBH_4$ between pH 4 and 10. Figure 4 shows that in the presence of $NaBH_4$ at pH 4, the percentage of CLC-DNA increased with increasing AZQ concentrations, reaching a maximum of 85% CLC-DNA at 250 μM AZQ. Significant cross-links were observed at 50 μM of AZQ or higher.

AZQ could also be reductively activated by NADH and NADPH for cross-linking with DNA at pH 4 (Fig. 5). NADH and NADPH, each at 3 mM activated 300 μM AZQ, causing the formation of CLC-DNA of 62 and 68%. No significant cross-linking activity was detected without AZQ or a reducing agent.

The AZQ-induced cross-linking under anaerobic conditions was also investigated (Fig. 6). AZQ-induced DNA cross-links were reduced in the absence of oxygen. Under anaerobic conditions AZQ induced 17, 17 and 23% of CLC for 30, 100 and 300 μM of AZQ.

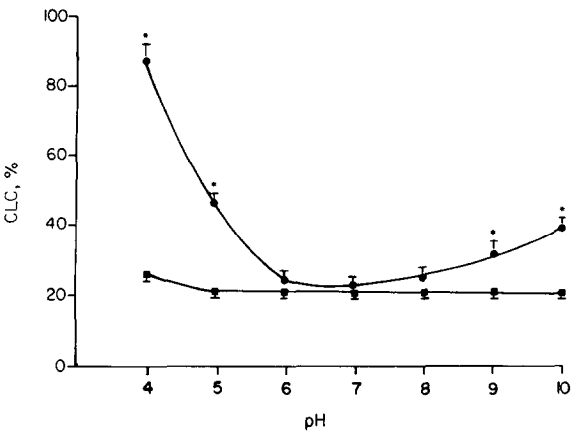

Fig. 3. pH dependency of AZQ-induced DNA cross-linking. AZQ (300 μM) was incubated with 120 μg/ml DNA in the presence of 3 mM $NaBH_4$ in various buffers, including 50 mM acetate (pH 4, 5), 50 mM trismaleate (pH 6, 7, 8), 50 mM Tris (pH 9) and 50 mM carbonate (pH 10). Portions of the mixture were taken out for the fluorescence assay as described in the text. The symbols are as in Fig. 2. Significant differences are indicated with stars.

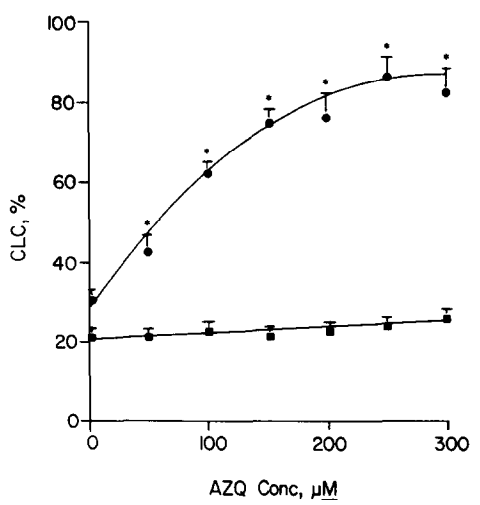

Fig. 4. Dose-response cross-linking activity of AZQ by the ethidium bromide fluorescence assay. Calf thymus DNA was treated with AZQ at 1, 50, 100, 150, 200, 250 and 300 μM in pH 4 acetate buffer. Symbols are as in Fig. 2.

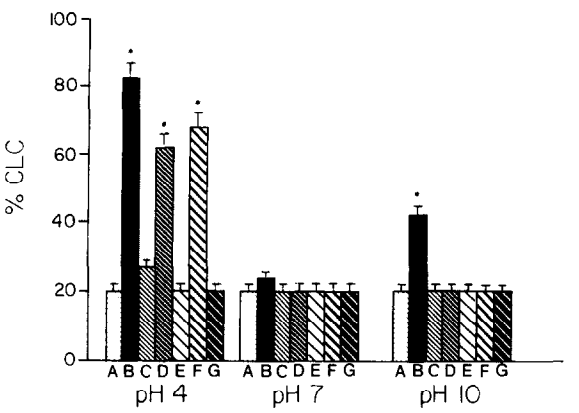

Fig. 5. Reductive activation of AZQ for cross-linking at pH 4, 7 and 10. A, DNA alone; B, AZQ + $NaBH_4$; C, +$NaBH_4$; D, AZQ + NADH; E, +NADH; F, AZQ + NADPH; G, +NADPH.

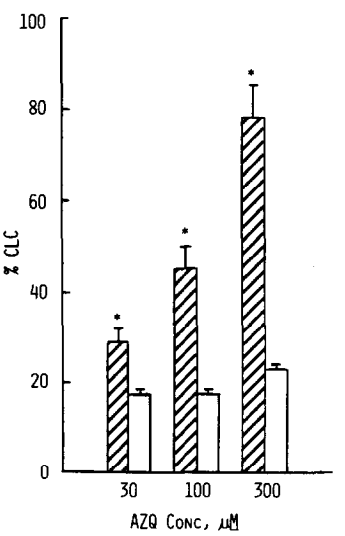

Fig. 6. Oxygen requirement in AZQ-induced DNA cross-linking. Calf thymus DNA was incubated with various amounts of AZQ and $NaBH_4$ in 50 mM acetate buffer, pH 4, under anaerobic and aerobic conditions. Hatched bars and open bars represented cross-linking activities in aerobic and anaerobic conditions respectively. Significant differences are indicated by stars.

DISCUSSION

Previously, Akhtar *et al.* [4] demonstrated that the antineoplastic activities of bifunctional aziridinylquinones were correlated to their covalent cross-linking activities. Our studies suggest that AZQ produces covalently cross-linked DNA only in the presence of a reducing agent, $NaBH_4$, NADH or NADPH (Fig. 5). The binding ratio was 191 bases per molecule of AZQ at a dose of 300 μM, being higher than that of streptonigrin (250 bases/mol of streptonigrin at 800 μM) [10] but lower than that of mitomycin C (20 bases/mol of mitomycin C at 300 μM) [9]. The binding of AZQ was additionally demonstrated by the ethidium fluorescence assay. However, even at the highest concentration of AZQ (300 μM), the percentage (87%) of CLC-DNA was less than 100% after renaturation. This suggests that AZQ may induce breakage in calf thymus DNA, as it does in bacteriophage PM2 DNA [8]. As a result, some of the DNA fragments are separated from the cross-linked DNA and fail to return to the duplex form.

The AZQ-induced DNA cross-linking was strongly pH dependent, with lower pH favoring more cross-linking (Fig. 2). In an acidic environment the aziridinyl moiety is probably active in a manner similar to mitomycin C activation [9]. We also tested the AZQ-induced cross-linking effect under anaerobic conditions. Paradoxically, cross-linking activity was markedly decreased in an anaerobic environment (Fig. 6). We speculate that the following reactions may have taken place to generate semiquinone free radicals for cross-linking:

$$AZQ \xrightarrow{\text{reduction}} AZQH_2$$

$$AZQH_2 + O_2 \longrightarrow AZQH\cdot + HO_2^{\cdot}.$$

AZQ was first reduced to the hydroquinone ($AZQH_2$), which would form the semiquinone free radical ($AZQH\cdot$) upon reoxidation: a reaction sequence modified from that proposed for mitomycin C [9]. Elimination of oxygen may therefore inhibit the formation of the semiquinone radicals. Recently, we found that AZQ induced similar DNA strand breaks and cross-links in Chinese hamster ovary cells (data not shown). How much do these effects contribute, if at all, to the antitumor action of AZQ remains to be elucidated.

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