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Comet Assay Studies on the Activation of Two Diaziridinylbenzoquinones in K562 Cells

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ABSTRACT. Two versions of the comet assay have been used to identify the difference in the modes of action of AZQ (2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone) and BZQ (2,5-diaziridinyl-3,6-bis(ethanolamino)-1,4-benzoquinone) in human leukaemia K562 cells and a K562-derived resistant cell line, BZQR. Using the standard alkaline assay, AZQ produced dose-dependent changes in the mean comet moments from K562 cells, consistent with the formation of strand breaks. This damage was repaired over a period of 6 hr after removal of the drug. The resistant cell line, BZQR, showed much smaller changes in comet moment under identical conditions. In contrast to AZQ, BZQ did not produce any measurable strand breaks in the K562 or BZQR cells. However, the comet radiation/crosslinking assay and a fluorescence-based assay revealed that BZQ extensively cross-links DNA in K562 cells. The extent of cross-linking is greatly reduced in the resistant cell line. BIOCHEM PHARMACOL 53;8:1115–1121, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. AZQ; BZQ; quinones; aziridinylquinones; resistance; comet; SCGE

The aziridinylbenzoquinones, AZQ[‖] and BZQ (Fig. 1) have undergone clinical trials as potential antitumour agents [1, 2]. We have previously shown that, although the two compounds have very similar structures, they can be activated by different mechanisms [3]. AZQ is a typical bioreductive alkylating agent, in that it is relatively inactive at physiologically relevant pH, but can be reduced by both one- and two-electron reducing enzymes to form semiquinones/hydroquinones that can alkylate DNA. Both interstrand cross-links and single-strand breaks have been detected in cells after exposure to AZQ [4–6]. In contrast, BZQ does not readily undergo bioreductive activation, but is much less stable than AZQ and can alkylate DNA in the absence of reduction to form cross-links [3, 7, 8].

The K562 human leukaemia cell line has been shown to be sensitive to diaziridinyl benzoquinones and readily produces semiquinones [9]. More recently, we developed two sublines of K562 that are resistant to AZQ (AZQR) and BZQ (BZQR). Surprisingly, these sublines were found to have similar mechanisms of resistance and showed cross-resistance to a number of other quinones and alkylating agents. These lines were shown to have decreased levels of the reducing enzymes, P450 reductase and DT-diaphorase, as well as increased levels of glutathione and superoxide dismutase [10].

The single cell gel electrophoresis (SCGE) assay or "comet assay" originally developed by Ostling and Johnson [11], is a method that can be used to estimate the extent of DNA damage and repair capacity within individual cells [12–14]. Essentially, treated cells are embedded in low-melting-point agarose and lysed in high salt/detergent to remove cytoplasmic and nuclear proteins. The nucleoid structures produced are then subjected to alkaline conditions to unfold the DNA, and then subjected to low-voltage electrophoresis. The damaged DNA (single-strand breaks, oxidized bases, and alkaline labile sites) migrates in the direction of the current, producing a characteristic "comet" shape. These images are viewed and quantified using a fluorescence microscope after staining with propidium iodide.

In view of the proposed contrasting mechanisms of AZQ and BZQ, we have used the alkaline version of the comet assay to quantify the initial DNA damage in K562 and the resistant subline, BZQR. Furthermore, we report on the use of a modified version of the assay that has allowed us to detect DNA cross-links in individual cells.

MATERIALS AND METHODS

Chemicals

AZQ was synthesised according to our published methods [15]. BZQ was synthesised according to our methods and those of Chou *et al.* [8, 16]. All other reagents were of the highest purity commercially available.

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‖ *Abbreviations:* AZQ, (2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone); BZQ, (2,5-diaziridinyl-3,6-bis(ethanolamino)-1,4-benzoquinone).

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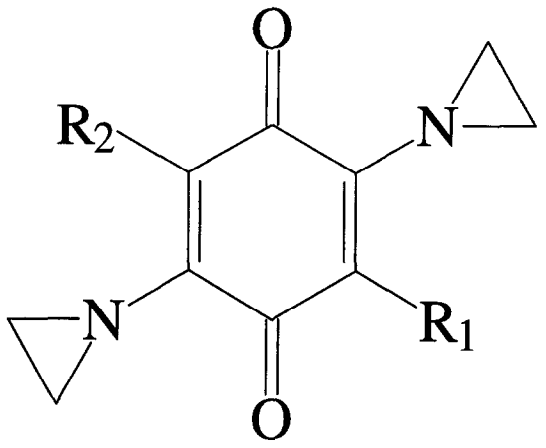

FIG. 1. Structures of diaziridinylbenzoquinones. $R_1 = R_2 = -NHCOOC_2H_5$ (AZQ); $R_1 = R_2 = -NHC_2H_4OH$ (BZQ).

Cell Culture

Both sensitive and drug-resistant (K562 and BZQR) cells were maintained as suspension cultures in RPMI medium supplemented with 10% horse serum. Cells were maintained in log-phase growth by weekly serial dilution. Resistance was maintained in the resistant cell line by the addition of 1 μM BZQ to the culture medium at every fourth subculture. Cells were counted using a Coulter electronic counter and diluted to 5×10^4 cells per mL in fresh medium prior to drug treatment.

Growth Inhibition Studies

Cells were suspended at 5×10^4 per mL and treated for 1 hr at 37°C with either AZQ or BZQ at a concentration range between 1 μM and 50 μM. The cells were then centrifuged and resuspended in fresh medium and 1000 cells, in a total volume of 0.2 mL, from control and treated samples, were dispensed into each well of a 96-well round-bottomed microtitre plate (Bibby-Sterilin). After 5 days incubation at 37°C, the degree of growth inhibition was determined using the MTT assay as previously described [10]. The cytotoxicity of the cross-linking agent, formaldehyde, was similarly determined using this assay after a 5-day incubation with a constant challenge of between 0 and 5 μM.

Comet Assays

INITIAL DAMAGE. Cells were suspended at 5×10^4 per mL in fresh medium and treated for 1 hr at 37°C with either AZQ or BZQ at a concentration range between 5 and 20 μM. Additional samples were treated at 20 μM for 8 hr.

REPAIR STUDIES. K562-sensitive cells were treated for 1 hr at 37°C with AZQ at 5 and 20 μM, centrifuged, and washed, and then resuspended in fresh drug-free medium. Samples were taken at 0, 2, 4, and 6 hr post treatment and subjected to the comet assay.

CROSS-LINKING. Both K562 and BZQR cells were treated for 1 hr at 37°C with between 5 and 100 μM BZQ and then immediately chilled on ice. The samples were then subjected to 20 Gy irradiation from a Caesium-137 source. Samples were maintained on ice to prevent repair of DNA strand breaks prior to immediate processing in the comet assay. As a positive control, K562 cells were treated with the cross-linking agent, formaldehyde, at a range of concentrations between 1 and 5 μM, and then processed as for the BZQ treated samples (results not shown).

COMET ASSAY PROCEDURE. Glass microscope slides, frosted at one end, were precoated with 1% normal agarose in distilled water. These slides were allowed to air dry overnight prior to use. A 1% low-melting-point agarose (LMP) mixture in PBS was melted and held at 45°C; 1 mL of LMP was then added to 0.5 mL of cell suspension on ice and the resultant mixture was pipetted onto a precoated glass microscope slide and allowed to gel for 1–2 min before being transferred to an ice tray. The cooling on ice was to inhibit repair. The slides were then immersed in ice-cold lysing solution (100 mM EDTA, 10 mM Tris, 1% Triton X100, 1% DMSO, 2.5 M NaCl) for 1 hr. After lysis, the slides were washed by immersion in fresh double-distilled water for 15 min duration. This process was repeated 3 times.

The slides were then placed onto a flat bed electrophoresis tank and covered (5–6 mm) with alkali unwinding solution (50 mM NaOH, 1 mM EDTA, pH 12.5). The slides were left under subdued lighting for 45 min to allow the DNA to unwind before being subjected to electrophoresis at 0.6 V/cm for 25 min. Each slide was rinsed with 2 × 1 mL of 0.4M Tris-HCl, pH 8.0, and allowed to dry in air. The dried slides were then rehydrated for 20 min with double-distilled water, flooded with 2 × 1 mL of propidium iodide solution (2.5 μg/mL), and stained for 15 min. The slides were then immersed in 1 litre of double-distilled water for 1 hr to reduce excess background staining. The slides were cover slipped and then examined at 250 × magnification under an epifluorescent microscope (Zeiss-Jenamed) using green light from a 50-watt mercury source with a 580 nm reflector and a 590 nm barrier filter set. Images were captured using an attached Sony HAD-1 interline CCD camera and Image X software package. 25 images from each of 2 duplicate slides were captured and analysed and the individual "comet moments" as defined by Olive *et al.* [17], were calculated. Briefly, the total fluorescence of the image represents the amount of DNA present, and the length of the image, measured in pixels, represents the length of migration of the DNA. The head and tail areas of the image were identified and the intensity of each was determined. The tail moment is calculated by multiplying the fraction of DNA present in the tail by half the length of the tail.

Fluorescence Cross-linking Studies

The cross-linking of BZQ in the K562 and BZQR cells was also determined by the ethidium bromide fluorescent tech-

nique of Garcia *et al.* [18]. Essentially, this method relies on the fact that heat denaturation of double-stranded DNA is inhibited when the DNA is cross-linked. The extent of denaturation is estimated by the fluorescence of bound ethidium bromide.

Cells (1.2×10^6) in 10 mL of RPMI medium were incubated with different concentrations of BZQ (0–50 μM) for 45 min at 37°C. After this time, the medium was removed by centrifugation at 1000 *g* and then washed 3 times with medium. Lysing solution (200 μM, 4 M NaCl, 50 mM KH_2PO_4, 10 mM EDTA, and 1% sarkosyl, pH 7.4) was then added together with 20 μL of heat-inactivated pancreatic RNAse (2 mg/mL). The samples were then incubated at 37°C for 16 hr. After this time, 3 mL of an ethidium bromide solution (5 μg/mL ethidium bromide, 20 mM KH_2PO_4, and 0.4 mM EDTA, pH 12) was added and the fluorescence was measured using a Shimadzu spectrofluorometer using 525 nm excitation and 600 nm emission wavelengths. The samples were then heat denatured in an oil bath at 95°C for 10 min and the measurements were retaken. The percentages of cross-linked DNA were determined from the difference in the fluorescence before and after denaturation using the formula:

$$C_t = (f_t - f_n)/(1 - f_n) \times 100$$

where C_t is the percentage crosslinks, f_t is the fluorescence after denaturation divided by the fluorescence before denaturation for the BZQ treated cells, and f_n is the fluorescence after denaturation divided by the fluorescence before denaturation for the control samples (no BZQ).

RESULTS

The histograms showing the effects of AZQ treatment on the K562 cells are shown in Fig. 2A–E and summarized in Fig. 3. Two typical images are shown in Fig. 4. It can be seen from this data that the control samples (no AZQ) have a population distribution of low comet moments, with 90% scoring below 5 and 100% scoring below 10. In contrast, the AZQ-treated samples show a small dose-dependent increase in DNA damage up to 10 μM and a significant increase at 20 μM, with over 50% of the population expressing high levels of damage (moments > 20). Prolonged exposure of up to 8 hr with 20 μM AZQ results in over 80% of the population having extensive DNA damage (Figs. 4A,B and 2E). This is consistent with AZQ being activated within the K562 cells and forming reactive oxygen species that produce DNA strand breaks [4, 9, 19]. The dose at which significant DNA damage can be measured is within the pharmacologically relevant dose range for this drug (Fig. 2A–E and Table 1). A similar correlation between cytotoxicity and comet moment has been reported for etoposide [20].

It has been shown that when TK6 lymphoblast cells are incubated with etoposide, the extent of DNA damage, as measured by the comet assay, is maximal at 8–10 hr after

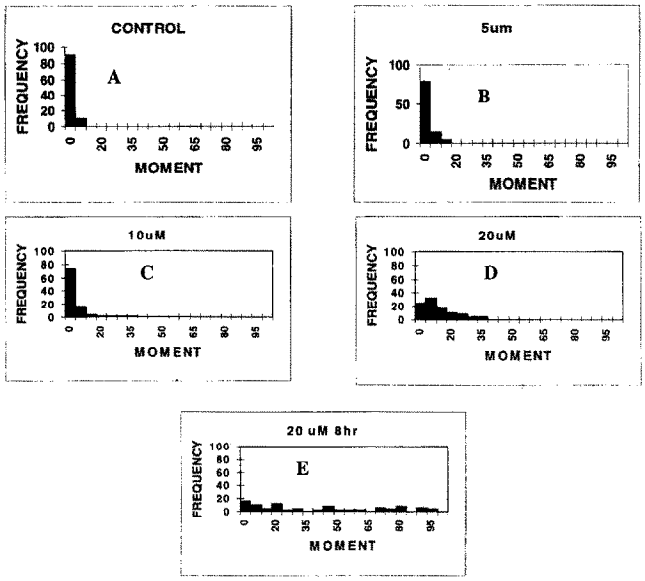

FIG. 2. The frequency of the mean comet moments for K562 cells treated with AZQ. (A) Control samples, no AZQ; (B) 5 μM AZQ; (C) 10 μM AZQ; (D) 20 μM AZQ, and (E) 20 μM for 8 hr.

removal of the drug. It was proposed that this damage is due to the onset of apoptosis [21]. In an attempt to see if a similar effect occurs when the K562 cells are treated with AZQ, the cells were exposed for 1 hr with 20 μM AZQ, washed, and then incubated in fresh medium for up to 6 hr. The results are summarized in Fig. 5, where it can be seen that the extent of damage, as determined by the mean comet moment, actually decreases over this period. This implies that, in the absence of drug, DNA repair is occurring over these longer time scales and significant apoptosis does not happen. The extensive damage observed on prolonged exposure of AZQ to the cells (Figs. 2E and 3) is, therefore, attributed to continued bioreduction of the compound to reactive species that compete with the repair.

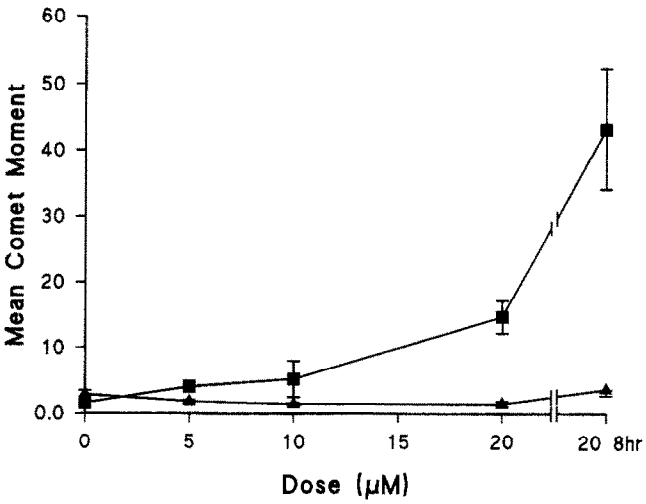

FIG. 3. Changes in mean comet moment with drug treatment; ■ AZQ; ▲ BZQ.

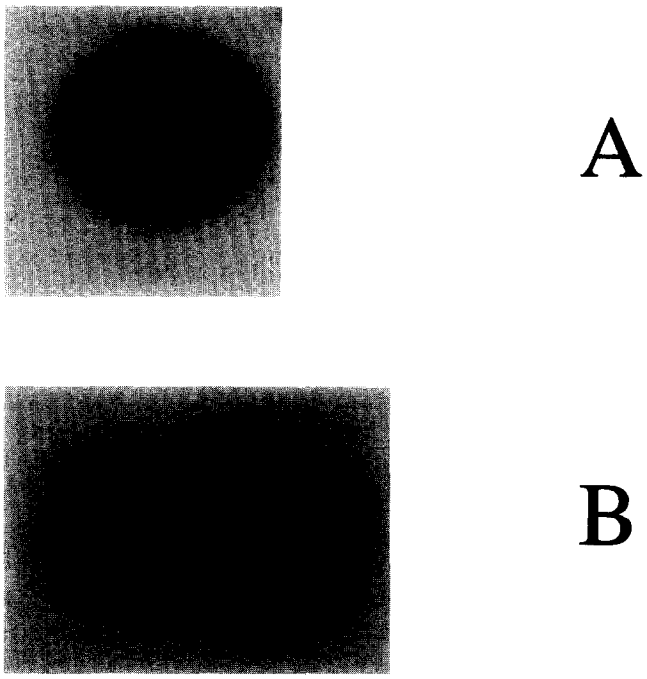

FIG. 4. Typical comet images. (A) K562 control (no AZQ); (B) K562 treated with 20 μM AZQ for 8 hr.

The mean comet moments for the cross-resistant BZQR cells were studied under identical conditions. The results, compared to those for K562, are summarised in Table 2. It can be seen from this table that the changes in mean comet moment are notably smaller than those in the parent cell line.

In contrast to AZQ, BZQ did not produce any significant DNA damage in the K562 cells when the assays were carried out under identical conditions. Indeed, there is an indication that the mean comet moment actually decreases after BZQ treatment (Fig. 3) and there is only a relatively small increase in moment after 8-hr treatment at 20 μM BZQ (Figs. 6E and 3). This latter result should be compared to that obtained with AZQ under similar conditions (Figs. 2E and 3). This lack of apparent reactivity is consistent with BZQ being unable to readily undergo bioreduction and, hence, DNA damaging reactive oxygen species are not formed [6, 19]. However, because BZQ can simply cross-link DNA in the absence of bioreduction, the efficiency of cross-linking was determined using the comet method.

Both K562 and the BZQR-resistant cells were treated with BZQ as described and subjected to the modified assay. The principle effect of cross-linking agents in this assay is to retard the migration of DNA from the cell nuclei. This migration is a result of strand breaks introduced by radiation postdrug treatment. The effectiveness of the cross-linking agent is measured as a % cross-linked DNA:

$$\% \text{ cross-linked DNA} = 100 - (100*x/y)$$

where x is the comet moment of the cell treated with BZQ and then irradiated to 20 Gy and y is the comet moment of the cells with no BZQ, but also irradiated to the same dose.

The results for the two cell lines are shown in Fig. 7. The powerful cross-linking agent, formaldehyde, was used as a positive control in these experiments. The K562 cells showed a BZQ dose-dependent increase in cross-linking up to about 20 μM and then a plateau was reached. In contrast, the BZQR cell line showed only a small effect over the same concentration range.

The ethidium bromide fluorescence method was used to confirm the differences in the ability of BZQ to cross-link DNA in K562 and BZQR cell lines. The results are shown in Fig. 8.

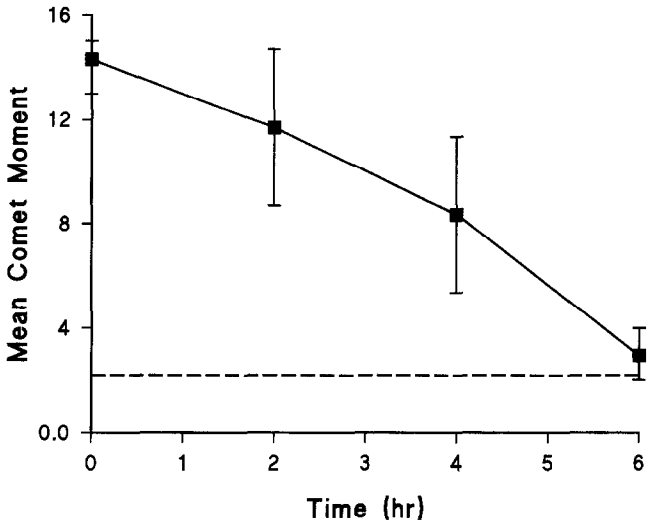

FIG. 5. Change in mean comet moment after removal of 20 μM AZQ. The dashed line shows the control (no AZQ) values.

DISCUSSION

The differences in reactivities of AZQ and BZQ can be explained by the electron distribution within the structures.

TABLE 1. IC_{50} values (μM) for the cell lines after 1 hr treatment with AZQ or BZQ, or constant challenge with formaldehyde. The averages are from three determinations.

	Cell lines	
Compounds	**K562**	**BZQR**
AZQ	5.2 ± 0.5	15.1 ± 2.2
BZQ	4.0 ± 0.3	7.5 ± 1.0
Formaldehyde	1.6 ± 0.4	3.0 ± 0.35

TABLE 2. Mean comet moments for AZQ-treated K562 and BZQR cells

Dose (μM)	K562	BZQR
0	1.55 ± 0.25	1.25 ± 0.25
5	4.0 ± 1.0	4.0 ± 2.1
10	5.0 ± 2.1	2.88 ± 0.4
20	14.7 ± 2.5	3.2 ± 0.6
20μM + 8 hr	43.1 ± 9.1	11.5 ± 4.2

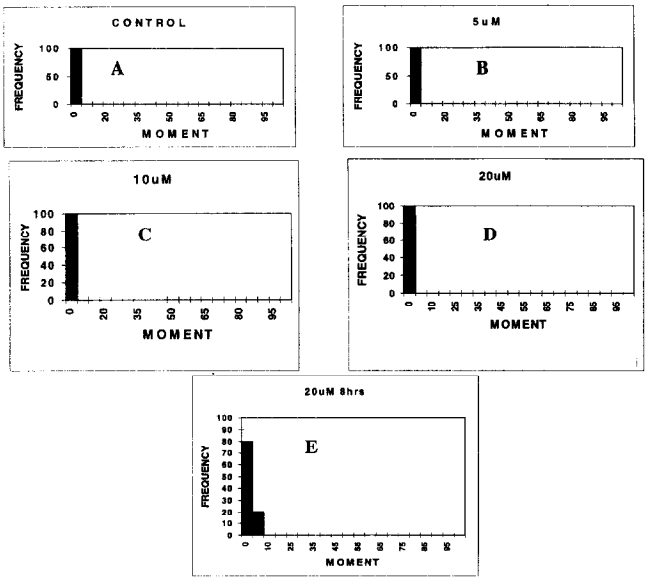

FIG. 6. The frequency of the mean comet moments for K562 cells treated with BZQ. (A) Control samples, no BZQ; (B) 5 μM BZQ; (C) 10 μM BZQ; (D) 20 μM BZQ, and (E) 20 μM BZQ after 8 hr.

The nitrogens of the ethanolamine group in BZQ are essentially electron-donating such that the quinone ring in BZQ is more difficult to reduce than the quinone of AZQ, which has electron-withdrawing urethane side groups. Hence, whereas AZQ can be reduced by the one-electron reducing enzymes, such as cytochrome P450 reductase and xanthine oxidase [19, 22], or the two-electron reducing DT-diaphorase [6, 7], BZQ is a much poorer substrate for these enzymes [3, 6, 19]. Hence, although both quinones are capable of alkylating DNA by virtue of the reactive aziridines [e.g. 3, 4, 7], only AZQ can readily form semiquinone radicals. These radicals can form reactive oxygen species that can result in DNA strand breaks [23].

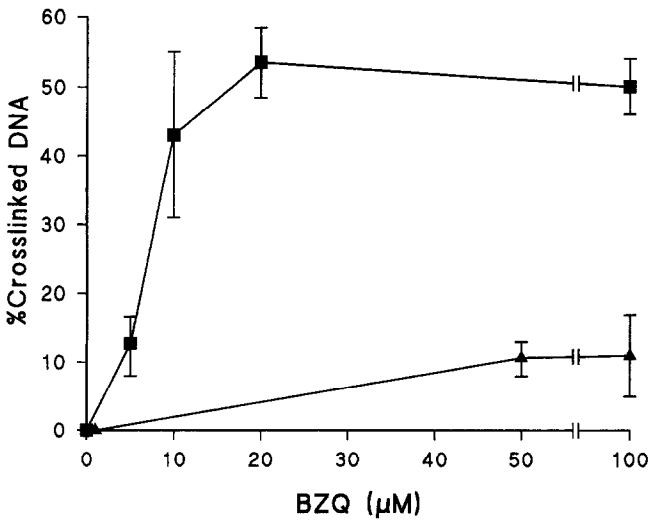

FIG. 7. Formation of cross-links by BZQ as measured in the comet assay; ■ K562, ▲ BZQR.

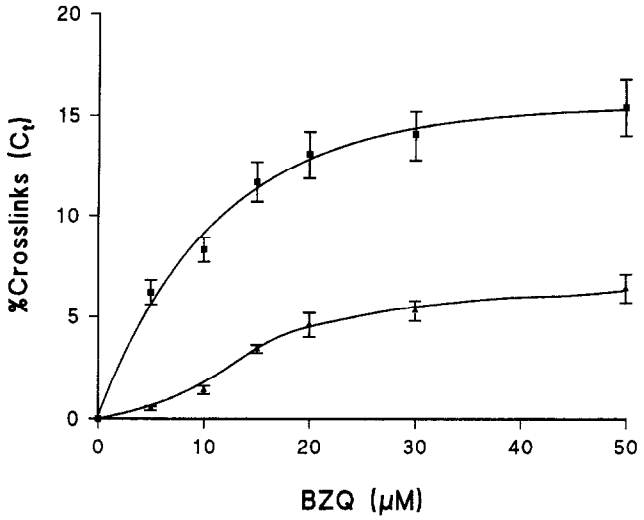

FIG. 8. Formation of cross-links by BZQ as measured in fluorescence assay; ■ K562; ▲ BZQR.

A dose- and time-dependent increase in DNA damage in the K562 cells after AZQ treatment has been observed (Figs. 2 and 3). These results are consistent with the formation of strand breaks. Prolonged exposure of the cells to AZQ results in extensive damage (Fig. 2E), but this is not due to apoptosis because when the drug was removed, the damage was subsequently repaired. The most highly damaged cells were observed to have repaired most of their strand breaks within about 6 hr (Fig. 5). In addition, the tail moments of the cells exposed to 20 μM for 8 hr have a mean comet moment of only 43 and do not show the characteristic shape of apoptotic cells [24].

It can be seen in Table 1 that a 1 hr incubation of AZQ with the K562 cells gives an IC_{50} value of 5.2 μM. However, the above experiments showed that extensive repair occurs even at 20 μM. Hence, the implications from these results are that the single-strand breaks, as measured by the comet assay, are not responsible for the overall toxicity of AZQ. Studies are in progress using the comet technique to assess the double-strand breaks produced in these cells. However, it is equally likely that the overall cytotoxicity of AZQ is due to the formation of covalent cross-links *via* bioreductive production of the hydroquinone/semiquinone [e.g. 7, 9]. In the comet assay, these cross-links will lead to a reduction in the apparent degree of DNA migration and effectively lower the comet tail moment (as for BZQ, see below). However, it was not possible to measure any cross-linking in the AZQ-treated cells because the effects were masked by the strand breaks.

Interestingly, the strand-break assay shows that even at a dose of 20 μM AZQ for 8 hr, about 15–20% of the cells appear to be undamaged (Fig. 2E). This could be due to several reasons that include the presence of a small amount of cross-links, a distribution in the different rates of repair, and/or a resistant subpopulation.

The BZQR cell line showed much less evidence of DNA

damage compared to the parent cell line (Table 2). We have previously reported that this subline is cross-resistant to AZQ, other quinones, and different alkylating agents [10]. Furthermore, the levels of the one- and two-electron reducing enzymes are lowered in this cell line compared to K562. The subsequent lack of DNA strand breaks in these cell lines was, therefore, predictable and is verified by the results presented here.

In direct contrast to AZQ, the effects of BZQ on K562 in the alkaline comet assay are markedly different. The frequency distribution of the comet moments in the BZQ-treated cells show little heterogeneity (Figs. 6A–D and 3) and, indeed, there is only a small change in the moments after prolonged (8-hr) exposure (Fig. 6E). Hence, BZQ does not readily produce strand breaks. Previous studies have shown that this compound can form cross-linked adducts with DNA, mainly at the N7 position of guanine [7], and the activation of the aziridines in BZQ can occur simply as a consequence of acid-assisted ring opening [8].

The radiation/drug treatment of Olive *et al.* [24, 25] was therefore used to determine the cross-linking efficiency of this compound. In the case of BZQ, the irradiated cells showed a dose-dependent reduction in migration, but the effect reached a plateau at around 20 μM (Fig. 7). Significantly, a similar rise to a plateau was observed from the ethidium bromide fluorescent assay (Fig. 8) even though, due to problems with sensitivity with the latter assay, the investigations had to be carried out under slightly different conditions. Essentially, both of these results indicate the limitations of the two assays that rely on the unwinding of double-stranded DNA. The effect of one molecule cross-linking DNA is not greatly enhanced by the addition of more cross-links in close proximity and, so, a plateau is reached. However, both assays show that the cross-linking in the BZQR cell line is greatly reduced compared to that of the parent line (Figs. 7 and 8), and this difference is consistent with the different cytotoxicity values (Table 1). It is also evident from these studies that, even though the BZQR cell line has lower levels of the main reducing enzymes compared to K562 [10], the characteristic formation of radical-induced DNA strand breaks does not play a significant role in the cytotoxicity of BZQ. The other possible reasons for the resistance of BZQR cells to BZQ and the other alkylating agents are currently under investigation.

In conclusion, we have been able to demonstrate that the comet assay is a powerful tool that can be used to study the main underlying mechanisms of DNA-damaging agents and these studies can be used to investigate both repair and resistance. It is interesting to note that, although BZQR is not dramatically resistant to BZQ or AZQ compared to the parent K562 cells (Table 1), both comet assays were sensitive enough to be able to show differences in the extent of DNA damage. Furthermore, because only very small numbers of cells are required for the assays, these techniques can be used to study the effects of novel potential antitumour drugs on clinical material. This work is in progress.

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