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THE ALKYLATION OF DNA *IN VITRO* BY 2,5-BIS(2-HYDROXYETHYLAMINO)-3,6-DIAZIRIDINYL-1,4-BENZOQUINONE (BZQ)—I

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Abstract—Cell toxicity by BZQ could not be explained by free radical formation and thus further work has been undertaken to elucidate a possible mechanism of cell killing. By using radiolabelled BZQ, *in vitro* DNA-drug binding has been investigated. The effect of salt, buffer and drug concentrations was determined in the pH range 4.0 to 8.0. The influence of *in situ* oxidation and reduction on BZQ binding was also studied as a function of pH. In an effort to ascertain any base specificity of BZQ binding the homopolymers, Poly[dG].Poly[dC] and Poly[dA].Poly[dT] were treated with radiolabelled BZQ in the pH range 4.0 to 8.0. A fluorescence assay was used to demonstrate the possible involvement of DNA cross-linking in cellular activity. From this work, it was concluded that BZQ functions as a bifunctional alkylating agent by an acid-assisted aziridine ring-opening mechanism and that other factors including oxidation or reduction are much less important.

The potential antitumour activity of aziridinylquinones has been recognized for over thirty years [1, 2] and in 1974, a detailed review of over fifteen hundred quinones tested for antitumour activity showed a relatively large number of active compounds possessing the aziridinyl moiety [3]. Two diaziridinylbenzoquinones, 2,5-bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone (AZQ) and 2,5-bis(2-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone (BZQ) have since undergone phase I/II trials [4, 5].

Several independent studies have reported on the feasibility of AZQ undergoing bioreductive activation [6, 7]. Related studies in our laboratory on the free radical properties of both AZQ and BZQ have confirmed that AZQ could easily be reduced to semiquinone radicals in a biological system under normal physiological conditions [8]. However, the main conclusion from this work was that BZQ is not so easily reduced and it was proposed that this drug may function by a different mechanism. Further work on the activation mechanisms of the two drugs essentially confirmed the respective reducability and also showed that the free radical production and cell killing do not always coincide. BZQ was found to be more toxic than AZQ towards the leukaemic cell lines tested [9].

BZQ has been shown to be much more labile than AZQ in slightly acidic solutions [10]. This is presumably because of the relative electron donating ability of the nitrogen atoms on the 2-hydroxyethylamino compared with the carboethoxyamino groups which will not only affect the reduction potential of the quinone moiety [8], but will also influence the state of protonation of the aziridine rings. The overall effect should be increased susceptibility to nucleophilic attack when acid-assisted ring-opening occurs in the presence of reactive cell components.

In this study we have investigated the efficiency of BZQ to alkylate DNA under a variety of conditions and have assessed the mechanisms of reactions.

MATERIALS AND METHODS

[1-^{3}H] Ethan-1-ol-2-amine hydrochloride (308 mCi/mg) was obtained from Amersham International. The microconcentrators were from Amicon (Centricon 30's, with 30,000 mol. wt cutoff were used except in one set of investigations where Centricon 10's were used with a cutoff of 10,000). Calf thymus DNA, Poly[dA].Poly[dT] and Poly[dG].Poly[dC] were obtained from Sigma. All other reagents were of the purest grade commercially available.

Synthesis of labelled drug. BZQ was synthesized according to the method of Chou *et al.* [11] with the exception that [1-^{3}H] ethan-1-ol-2-amine was included in the preparation. Briefly, the aqueous solvent from a 1 mCi sample was removed by passing a stream of warm air over the sample overnight. 2,5-Difluoro-3,6 diaziridinyl-1,4-benzoquinone (0.2 g) in tetrahydofuran (20 ml) was then added and the solution stirred for 2 hr, after which time unlabelled ethanolamine (0.1 ml) was added. The solution was stirred overnight, evaporated under vacuum and then filtered. The resulting solid was bulked up with crystalline, unlabelled BZQ and recrystallised from ethanol. The final product had an activity of 353 dpm/nmol and was shown by HPLC and TLC to be homogeneous. Aziridine ring-opened BZQ was prepared by incubating BZQ (0.1 mg) in dilute hydrochloric acid for 4 hr. The completion of the reaction was confirmed spectrophotometrically.

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Drug solutions were freshly prepared for each experiment by dissolving a small amount in dimethyl sulphoxide (0.2 ml). The final concentration of dimethyl sulphoxide in the incubation mixtures, even at the highest concentrations of drug, was less than 3%.

DNA alkylation. The standard incubation mixture consisted of DNA (0.25 mg), phosphate buffer (50 mM), sodium chloride (25 mM) and labelled BZQ (0.4 mM, added last) in a total volume of 1.0 ml. The contribution from each of these components was studied separately. The incubation mixtures were made up in the sample reservoir of the microconcentrator and maintained at 22 ± 1°. After the incubation time, the reaction was substantially inhibited by the addition of 1.0 ml phosphate buffer (0.4 M, pH 7.0) and centrifuged through the membrane at 3200 *g* for 30 min. The DNA was then washed extensively with distilled water and centrifuged until the filtrate gave only background counts. Removal of DNA from the membrane was effected by inverting the concentrator and centrifuging each time after washing with 3 × 0.2 ml of perchloric acid (10 mM). The combined washings were then evaporated overnight by a stream of warm air, redissolved in hydrochloric acid (10 mM, 0.4 ml) and the radioactivity was measured using a commercial scintillant.

Fluorescence assay. The fluorescence assay used to determine DNA crosslinking was similar to that previously reported [12] with some slight modifications. This technique relies on the fact that heat denaturing of DNA results in a loss of ethidium bromide fluorescence. If a drug can covalently crosslink DNA then heat denaturation is prevented. Essentially the DNA-drug incubation mixture (total vol. 0.2 ml) containing calf thymus DNA (0.1 mg), BZQ (0.2 mM) and phosphate buffer (30 mM, pH range 4.0–8.0) was incubated for 40 min at 22 ± 1°. On completion of the incubation time, the pH was adjusted to 7.0 with phosphate buffer (100 mM, pH 9.0 or 7.0). Blanks were set up which contained no BZQ. DNA (5.3 μg) was removed and added to the ethidium mixture (3 ml). The fluorescence solution contained ethidium bromide (0.5 μg/ml), tripotassium phosphate (20 mM, pH 11.8) and EDTA (0.5 mM) and was stored in a light-proof container at room temperature. The excitation wavelength was 525 nm while the emission was observed at 600 nm using 10 nm slit widths. The fluorescence readings were taken before and after heating to 96° for 3 min and cooling in ice for 5 min. The results were firstly calculated as the % fluorescence remaining and then by subtracting the blanks from the DNA-drug incubation readings and finally adjusted such that the value for pH 4.0 was equal to 100%. The results are an average of three determinations, each performed in duplicate.

RESULTS

Figure 1 shows the kinetics of the binding of labelled BZQ to DNA at pH 4.0 and 7.0. The binding is essentially complete within about 40 min at pH 4.0, while at pH 7.0 the reaction is substantially slower. Unfortunately it was not practical to follow the reaction at pH 7.0 to completion due to the instability of DNA solutions at ambient temperatures over long periods. If it is assumed that the average molecular weight of a nucleotide is 330 then the binding ratio of labelled BZQ to DNA nucleotide at pH 4 is 1:38. However, this ratio was observed to be dependent on the concentration of BZQ and this is illustrated in Fig. 2. The maximum is reached at about 1.5 mM BZQ and this corresponds to a ratio of 1:28. The pH dependence of the extent of binding after a set period of time (45 min) is shown in Fig. 3. The values obtained from using the finer membrane filter (Centricon 10) are included in Fig. 3, together with the extent of binding of the ring-opened BZQ.

The effects of varying the salt and buffer concentration on the binding of BZQ to DNA are shown in Fig. 4 in the form of a plot of the inverse of the concentration of bound BZQ versus the concentrations of salt or buffer.

In a previous study which employed a different technique to investigate the binding of AZQ to DNA, it was shown that even at pH 4, AZQ only binds significantly when it is reduced to the hydroquinone [13]. Thus, the extent of binding of BZQ when reduced by sodium dithionite was investigated. The results are shown in Fig. 5. Similarly, BZQ has been reported to interfere in the assay which

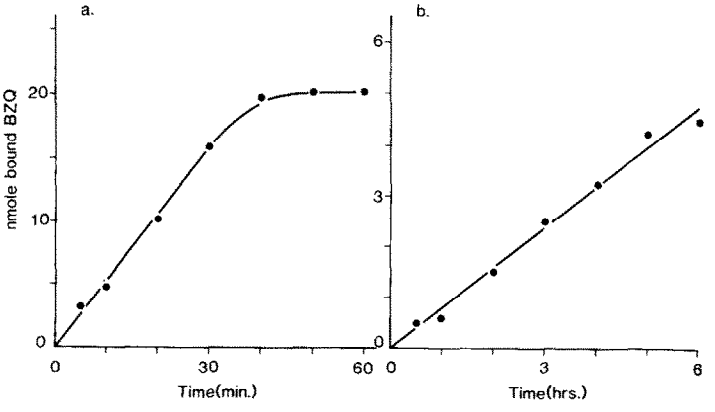

Fig. 1. The extent of binding of BZQ (0.4 mM) to DNA (0.25 mg) as a function of time at (a) pH 4.0 and (b) pH 7.0.

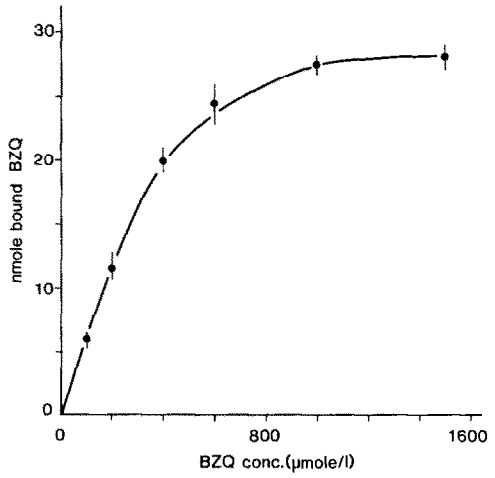

Fig. 2. The dependence of the extent of DNA binding on BZQ concentration.

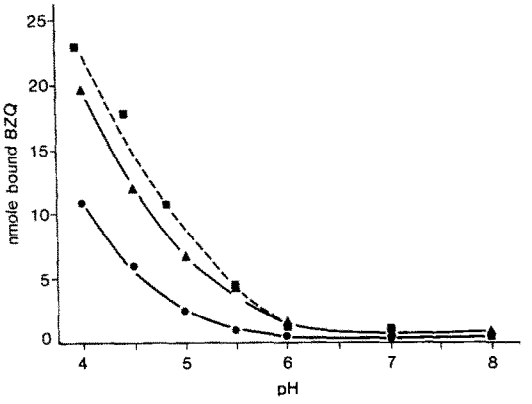

Fig. 5. pH dependence of BZQ binding to DNA: ● oxidation by $Fe(CN)_6^{3-}$ (1 mM), ■ reduction by sodium dithionite (2 mM), ▲ no addition (as Fig. 3).

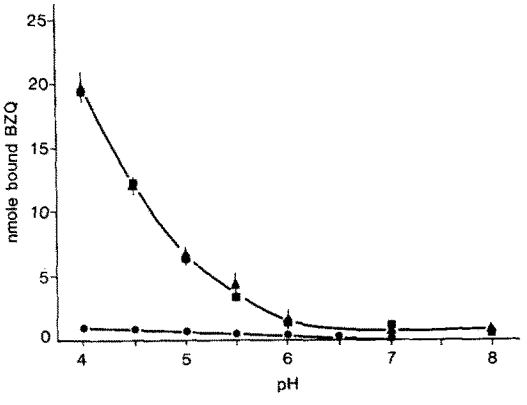

Fig. 3. pH dependence of the binding of BZQ to DNA using: ■ 30,000 mol. wt cutoff filter, ▲ 10,000 mol. wt cutoff filter, ● Aziridine ring-opened BZQ.

measures NADPH consumption using cytochrome *c* [9]. Subsequent experiments have shown that BZQ is readily oxidized by ferricytochrome *c* and a variety of other systems including peroxidases, tyrosinase and ferricyanide (Butler and Hoey, unpublished results). The effect of oxidized BZQ on the extent of binding was therefore investigated and the results are included in Fig. 5.

The pH dependence of the binding of BZQ to the double-stranded homopolymers is shown in Fig. 6.

A strong pH dependence was observed in the crosslinking activity of BZQ to DNA, comparable to the alkylating ability. The results are shown in Fig. 7.

DISCUSSION

Aliphatic aziridines are known to readily undergo acid assisted ring-opening processes to form addition products in the presence of suitable nucleophiles. The extent and rate of a particular reaction is dependent upon the state of protonation of the aziridine ring and the strength of the nucleophile (e.g. 14 and

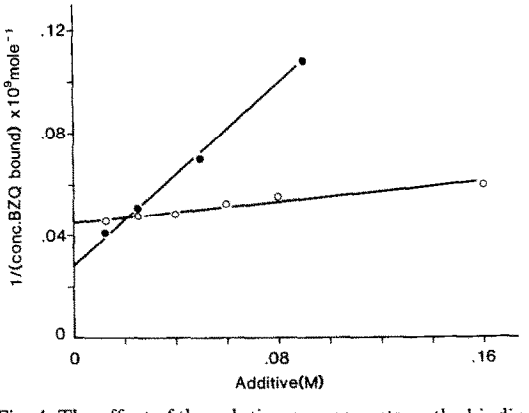

Fig. 4. The effect of the solution components on the binding of BZQ to DNA at pH 4.0: ● NaCl, ○ phosphate buffer.

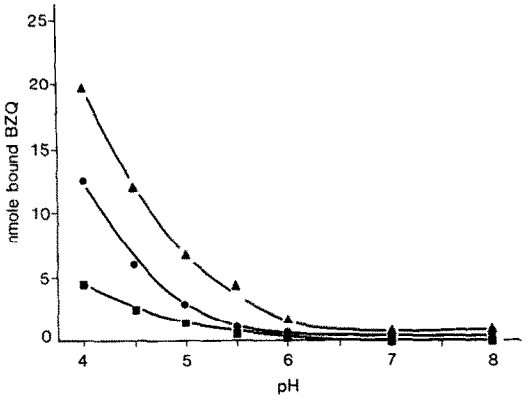

Fig. 6. pH dependence of BZQ binding to the double stranded polymers: ▲ DNA, ● Poly[dG].Poly[dC], ■ Poly[dA].Poly[dT].

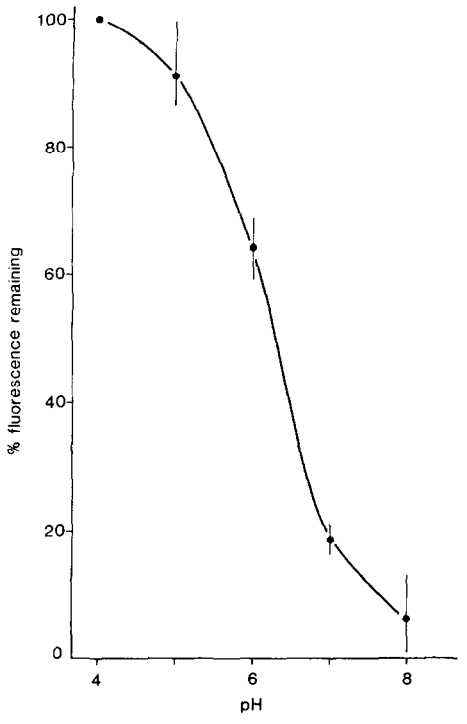

Fig. 7. pH dependence of the crosslinking activity of BZQ.

references therein). The reactions of BZQ are very similar to those of the simple aliphatic aziridines and it would appear that the quinone moiety does not play a predominant role in its reactions. The minimum scheme that will explain the present results is illustrated in Fig. 8.

It has been demonstrated that RSU-1069, an imidazole aziridine reacts efficiently with inorganic phosphates and nucleotides [15]. It was therefore suggested that the DNA phosphate backbone may be a potential target for this drug and could be partially responsible for the production of the strand breaks [16]. However, under our conditions phosphate appears to be a poor nucleophile for BZQ and is much less efficient than chloride or DNA (Fig. 4). Similarly, there is little evidence for extensive strand breakage by reaction of BZQ with the phosphate groups in DNA as the same amount of bound BZQ–DNA adducts were retained by both the 30,000 and the 10,000 cutoff filters (Fig. 3). The difference in the extent of phosphorylation of the two compounds could be due to the reactivities of the acid and neutral forms of the phosphate and the pK's of the aziridines. The RSU-1069 aziridine has a higher pK than BZQ [17] and it has been shown that the HPO_4^{2-} form of the buffer is about 40 times more reactive than the $H_2PO_4^-$ form [15]. Thus, in more acid pHs, although the aziridine rings of BZQ are protonated and hence activated towards nucleophilic attack, the phosphate will be in the less reactive form. Conversely, although the more reactive form of the phosphate will be present at neutral pH, the aziridine rings of BZQ will be relatively unreactive.

In a previous study on the binding of AZQ to DNA, it was observed that the binding only became significant, even at pH 4, when the quinone was reduced [14]. This could be as a consequence of lower pK values for the protonation of the aziridines of AZQ compared to BZQ. It is expected that whereas the electron withdrawing urethane side groups of AZQ will facilitate the reduction of the quinone moiety [8], they should also reduce the ease of protonation of the aziridines. However, the pK's should increase on reduction to the hydroquinone such that the aziridines are more easily protonated. BZQ is much more difficult to reduce than AZQ, $E_7(Q/Q^-) = -376$ mV, cf. -70 mV for AZQ [8] and it is evident from Fig. 5 that whereas reduction by sodium dithionite leads to a slight increase in alkylation, this increase is not as dramatic as that observed with AZQ.

Several preliminary experiments have shown that BZQ is easily oxidized. Interestingly, a quinone of very negative reduction potential, Mitoxantrone $\{E_7(Q/Q^-) = -527$ mV [18]$\}$, has recently been shown to be readily oxidized by horseradish peroxidase/H_2O_2 [19]. In instances where electron donation occurs to yield a quinone of negative

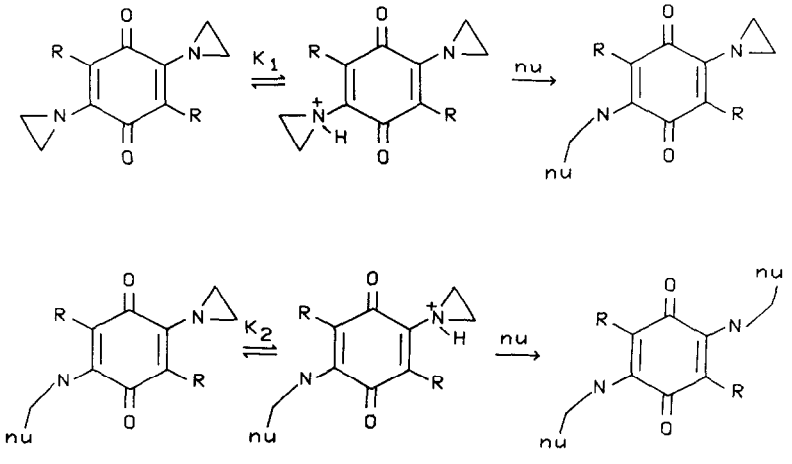

Fig. 8. The minimum scheme which will account for the reactions of BZQ with nucleophiles.

reduction potential, it is very probable that similar electron migration would also facilitate quinone oxidation. However, it is observed in Fig. 5 that the oxidation reaction of BZQ prevents alkylation.

The reaction of alkylating agents with specific sites in DNA is governed by a variety of considerations including steric factors. It is therefore not possible at this stage to accurately quantify the selectivity of BZQ towards the individual bases in DNA. However, it would appear from Fig. 6 that the GC homopolymers are more susceptible towards alkylation than the AT homopolymers. From a detailed study on the alkylation of DNA by another aziridinyl quinone, mitomycin C, it was shown that the major covalent link occurs at the N^2 position of guanine. Both monofunctional and bifunctional N^2 adducts are formed [20]. The diaziridinyl benzoquinones were designed originally to be bifunctional alkylating agents via the two aziridine groups. Using the alkaline elution method, AZQ has been shown to crosslink DNA in human tumour cell lines and although the cell killing correlated with DNA–DNA crosslinks, it did not correlate with the DNA–protein cross links [21]. It is evident from Fig. 7 that BZQ can act as a bifunctional alkylating agent since it also covalently crosslinks DNA. Work is in progress to identify the structures of these adducts.

The development of tumour-selective agents for cancer treatment requires the exploitation of certain cellular properties that specifically distinguish cancer cells from the normal population. It is now well established that some tumours have a relatively lower interstitial pH due to anaerobic glycolysis (e.g. 22 and references therein). However, even the most acidic human tumours have a pH of only about 5.9. It can be seen from the crosslinking studies (Fig. 7) that approximately three times more crosslinked DNA is produced at pH 6 than at 7. This difference could be of significance in the selective toxicity. Work is in progress to study the further effects on BZQ toxicity in leukaemic cell lines by using agent which lower interstitial pH. Similarly, BZQ should not efficiently function as a bioreductive alkylating agent unlike mitomycin C or AZQ. One of the main findings from this study is that BZQ can readily undergo facile ring-opening processes by reactions with less important nucleophiles or by oxidation. These processes, if they occur as readily *in vivo*, will detoxify the quinone. It is widely believed that the hypoxic tumour cell has a greater capacity for reductive activation than the normal oxygenated cell. If this is true then the reverse might also be true, i.e. the normal cell should have a greater capacity for oxygenated activation in the case of BZQ and thus the normal cell could have a greater detoxifying capacity.

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