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CONTRASTING CYTOTOXIC MECHANISMS OF SIMILAR ANTITUMOUR DIAZIRIDINYLBENZOQUINONES

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The mechanisms of cytotoxicity of the antitumour diaziridinylbenzoquinones, AZQ and BZQ, have been investigated. HPLC analysis has been used to study the products as well as the rate of decomposition of acid-assisted ring-opening in aqueous medium as a function of pH. Microconcentrators with a molecular weight cutoff of 30 kDa were utilised to study the covalent binding of both compounds to calf thymus DNA. Radical production of both compounds in K562 cell incubations was followed using ESR and their uptake into K562 cells was monitored using radiolabelled compounds. The results show that these two diaziridinylbenzoquinones, although very similar in structure, have diverse mechanisms of cytotoxicity. The implications of these findings are discussed in the light of antitumour action.

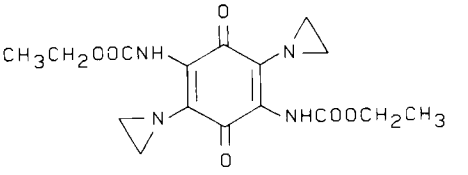
KEY WORDS: Diaziridinylbenzoquinones, AZQ, BZQ, cytotoxicity, mechanisms, ESR.

INTRODUCTION

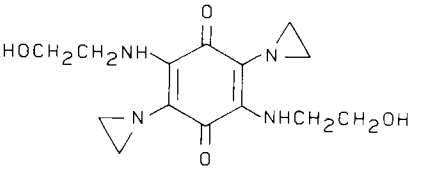
Vitamin K_1 and the coenzyme Q group are naturally occurring quinones which are essential to maintain certain biochemical processes. In the last few decades, many natural and synthetic quinones have found very useful applications as antitumour agents. The National Cancer Institute (NCI) published a lengthy report in 1974 on the response of a number of murine tumour cell lines to a series of quinone compounds.[1] This report clearly showed that a number of aziridinylquinones had significant activity against many of the tumour models. Since this time, relatively few aziridinylquinones have been synthesised and tested. Two notable exceptions, AZQ and BZQ (Figure 1), have undergone clinical trials as potential antitumour drugs in the USA and in the UK.

A considerable amount of research has gone into elucidating the molecular mechanisms of a range of antitumour quinones and it is now evident that a single mechanism cannot fully explain all of the observed cytotoxic effects. However, it is generally believed that AZQ in particular, can undergo bioreductive activation and alkylate DNA (eg.[2,3]). Aziridines have the potential to undergo ring-opening processes to form covalent adducts with available nucleophiles. Some aziridinylbenzoquinones have the added advantage in that the ring-opening process would be facilitated by the change of electron distribution when the non-aromatic quinone is reduced to an aromatic semiquinone or hydroquinone.

One of the main objectives of this work is to present data which show that even with two apparently similar aziridinylquinones, AZQ and BZQ, the overall mechanisms of



AZQ



BZQ

FIGURE 1 Structure of the diaziridinylbenzoquinones, AZQ and BZQ.

cytotoxicity can be quite different. The results presented include ESR studies of the diaziridinyl semiquinone radicals produced by incubation with cells as well as the cellular uptake of the radiolabelled compounds. We have also taken this opportunity to discuss data which questions some of the accepted mechanisms of bioreductive activation.

MATERIALS AND METHODS

AZQ was synthesised according to our published methods.[4] ^{14}C-AZQ was a generous gift from the Division of Drug Treatment, NCI, Maryland. ^{3}H-BZQ and BZQ were synthesised according to our methods[5] and those of Chou *et al.*[6] The drugs were first solubilized in DMSO and then diluted with the appropriate buffers (final concentration of DMSO $< 0.3\%$). All other reagents were of the highest purity available.

The ESR studies were conducted using a Varian E-9 X-band spectrometer with 100 kHz modulation. The procedure for determining the signals from the drug/cell incubations was as previously reported.[7] The ESR signals from the cell medium were observed by incubating the cells with AZQ, rapidly centrifuging the cells to a pellet (12,000 g for one minute) and aspirating off the medium.

The stability of the two quinones were determined by incubating the drugs (20 μM) in 1.0 ml of 0.1 M sodium perchlorate/perchloric acid (pH 2.0–3.4) or 0.1 M sodium perchlorate/20 mM phosphate buffer (pH above 4.5). At the end of the incubation times, the reactions were stopped by the addition of 2.0 ml of 0.1 M phosphate buffer (pH 7.0). The samples were then analysed by HPLC using an analytical column of Spherisorb 5 ODSII. The eluting buffers were 50:50 methanol/0.01 M phosphate (pH 7.0) for AZQ and 70:30 methanol/0.01 M phosphate for BZQ. The flow rate was 1.0 ml/min and the absorbance was monitored at 344 and 384 nm for AZQ and BZQ, respectively.

The one-electron reduction potentials were found by using pulse radiolysis to generate semiquinone radicals which can then be set in equilibrium with standards of known potential. Full details of the method are given in earlier work.[8]

The covalent binding of the drugs to DNA was investigated using the methods reported previously,[5] using microconcentrators (mol wt cutoff of 30,000).

The kinetics of drug uptake into the cells were determined by incubating 50 μM labelled drug with the cells (2×10^5 cells/ml in RPMI medium supplemented with 10% horse serum) for selected periods of time at 37°C. At the end of the incubation times, the cells were rapidly centrifuged to a pellet which was then layered on top of an oil mixture (4:1 ratio of Dow Corning silicon oil: "3 in 1" mineral oil, E.R. Howard Ltd., Swindon, UK). The cells were centrifuged through the oil to remove any traces of medium, disrupted by sonication and then taken up into a commercial scintillant medium for ^{14}C or ^{3}H counting.

RESULTS AND DISCUSSION

1. Effect of pH on Drug Stability

The relevance of a detailed study of drug stability at low pH to possible chemotherapeutic effects is embodied in the fact that tumour cells exist under more acid conditions. To achieve selectivity over normal cells, it is necessary to exploit any factors which enhance differentiation.

All aziridines are unstable in aqueous medium due to their strained three-membered ring structure. Below neutral pH, the nitrogen on the aziridine ring becomes protonated and hence activated towards nucleophilic attack. The overall mechanism, which can be ascribed to two consecutive first order processes, is shown in Figure 2. Typical HPLC traces demonstrating the outcome of these changes for AZQ are shown in Figure 3. The chromatograms show that as AZQ disappears, two components begin to grow-in (Figures 3B & 3C) but eventually yield a single product. The identity of these species has been assigned to half- (second peak in Figure 3B) and fully ring-opened AZQ (Figure 3D). In the presence of sodium perchlorate and perchloric acid, the nucleophile comes from water since perchlorate itself reacts very weakly. However, several preliminary experiments have shown that, consistent with published observations,[9] the anions from other acids or buffers can act as nucleophiles

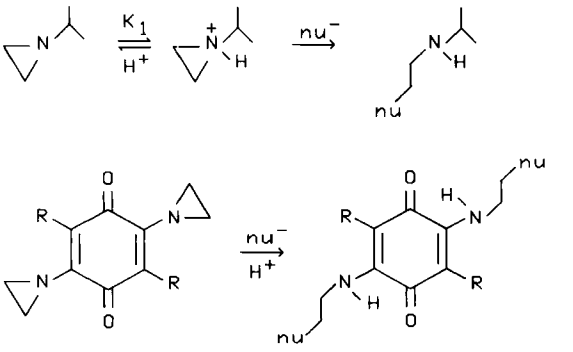


FIGURE 2 Mechanism of aziridine ring-opening and nucleophilic attack.

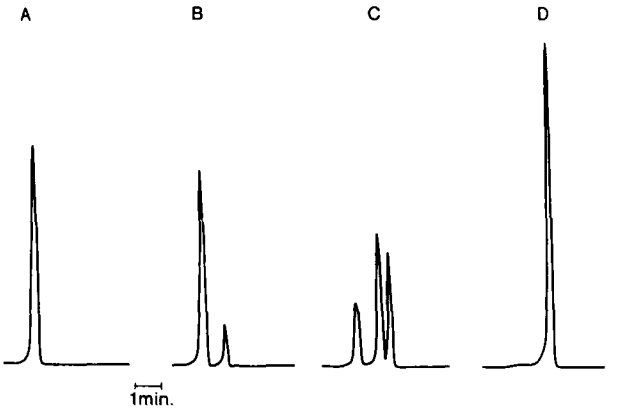


FIGURE 3 Typical HPLC traces showing the result of acid-assisted ring-opening of AZQ to half ring-opened and fully ring-opened forms at pH 3.0: A = zero time, B = 5 min, C = 50 min, D = 200 min.

in these reactions. The rate constants for the decomposition of AZQ and BZQ as a function of pH are shown in Figure 4.

The lone pair of electrons on each of the ethanolamine groups in BZQ can be donated into the quinone ring system, making the aziridine rings more susceptible to protonation and ultimately nucleophilic attack. This is in contrast to the urethane groups in AZQ which tend to withdraw electrons from the quinone ring, leaving less electron density available to the aziridine rings, which results in more difficult protonation. Hence, as shown in Figure 4, BZQ is much more unstable than AZQ.

2. *Reduction*

The electron donation/withdrawal properties of the different side groups on AZQ and BZQ also affect the one-electron reduction potentials. $E_7^1(Q/Q^{\cdot -})$ for AZQ has been determined as -70 ± 10 mV,[8] although more recent determinations from our lab-

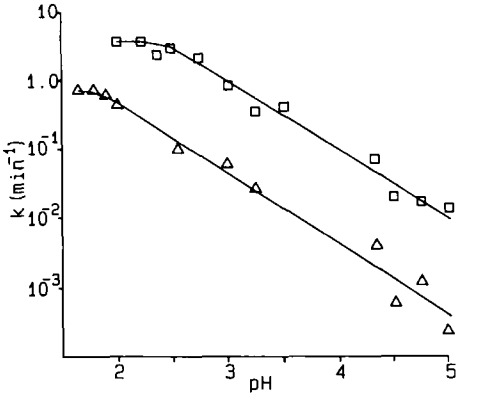


FIGURE 4 The rate of decomposition of AZQ and BZQ as a function of pH: (△) AZQ, (□) BZQ.

oratory, with a larger range of standards, would suggest the value is slightly higher, -61 ± 15 mV (Hoey and Butler, unpublished results). The corresponding value for BZQ is -376 ± 15 mV. Thus, AZQ can readily be reduced by several enzyme systems including cytochrome P-450 reductases and xanthine oxidase whereas BZQ is much more difficult to reduce. In the case of AZQ, the redistribution of electron density away from the central ring, results in a system which allows an electron to be added with comparative ease. Conversely the BZQ side groups transfer electron density into the ring, making the addition of an extra electron less favourable.

The relative ease of reduction of AZQ can also be observed when the drug is incubated with cells. ESR studies clearly show that AZQ is reduced to a semiquinone radical by many cell systems, even in the presence of oxygen.[7,10] A typical scan is shown in Figure 5A which is the usual 5-line signal observed for diaziridinylbenzoquinones. However, despite several attempts using different conditions, we have not been able to observe an ESR signal from BZQ (Figure 5C).

3. DNA Alkylation

The differences in the acid stability and the reduction potentials of the quinones also affects the reactions with DNA. The results are summarised in Figure 6. BZQ is

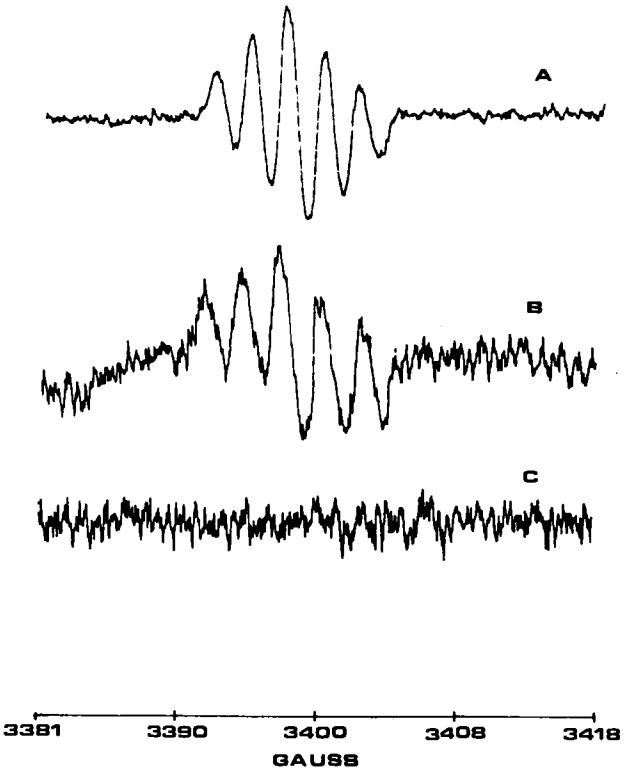


FIGURE 5 ESR signals observed from: A) K562 cells incubated with AZQ, B) Medium alone from the above incubation mixture and, C) K562 cells incubated with BZQ.

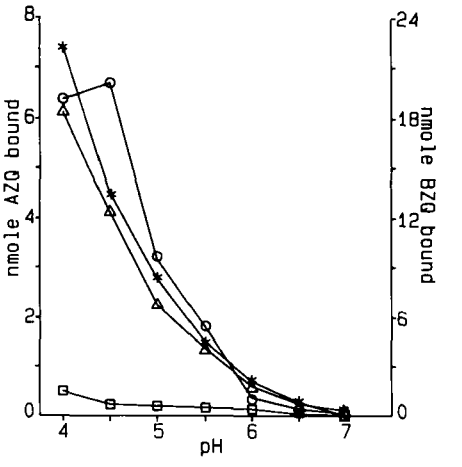


FIGURE 6 Binding of calf thymus DNA to AZQ and BZQ in the absence or presence of 2.0 mM sodium dithionite. □ AZQ ○ AZQ + dithionite △ BZQ * BZQ + dithionite.

unstable with respect to acid-assisted ring-opening and hence is more active in reactions with DNA at low pH. Reduction of the quinone to a hydroquinone increases the reactivity by only a small extent. This is in contrast to AZQ which, as the quinone, does not react significantly with DNA but on reduction to the more responsive hydroquinone, shows a very substantial increase in DNA binding.

There has been a great deal of interest for several years in the development of hypoxic bioreductive alkylating agents for use in the treatment of solid tumours. It is generally believed that in order to achieve selective damage in hypoxic tumour cells over the fully oxygenated normal cells, the reduction potential of such compounds has to be more negative than the one electron reduction potential of the $O_2/O_2^{\cdot-}$ couple i.e., -155 mV.[11] Under such conditions the equilibrium:

$$Q^{\cdot-} + O_2 \rightleftharpoons Q + O_2^{\cdot-} \quad (1)$$

favours $O_2^{\cdot-}$ production in the presence of oxygen. Mitomycin C is a well known hypoxic bioreductive alkylating agent which has a potential of -310 mV[12] and hence meets this criterion. It would be expected by this simple principle that BZQ, with its more negative reduction potential, could be a hypoxic bioreductive agent whereas AZQ, being of much more positive potential, could not. However, BZQ has a potential which is even more negative than mitomycin C and indeed may be so negative that the cellular reducing enzyme systems cannot efficiently reduce it. The relative rates of reduction of some antitumour quinones by xanthine oxidase have been reported,[8] and show that BZQ reduction is approximately a factor of three slower than mitomycin C. In any case, it can be seen from Figure 6 that the increase in DNA alkylation by BZQ following reduction is almost insignificant. If BZQ showed selective toxicity towards hypoxic tumour cells, this could be explained by the fact that these types of cells are simply more acidic than the normal cells due to anaerobic glycolysis. Intracellular pH as low as 5.9 has been measured in certain anaerobic cells.[13]

By contrast, AZQ has a more positive potential than that of the $O_2/O_2^{\cdot-}$ couple and

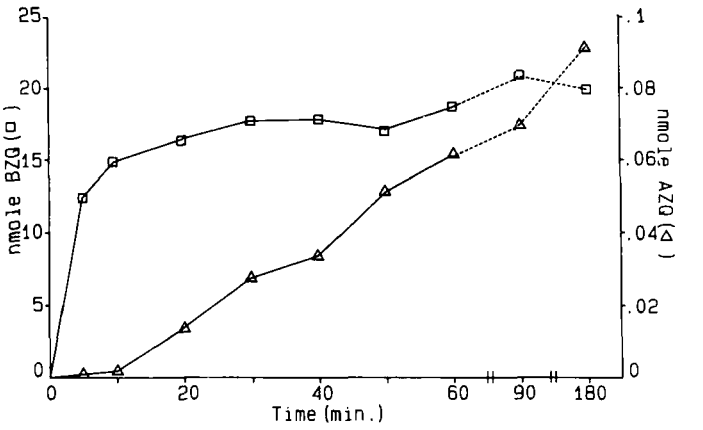


FIGURE 7 The kinetics of cell uptake for (△) AZQ and (□) BZQ in K562 cells.

hence the above equilibrium is normally over to the left hand side. The semiquinone radical in simple aqueous solutions is stable in the presence of oxygen. However, the above equilibrium would be rapidly shifted in the presence of superoxide dismutase (SOD) due to removal of the $O_2^{\cdot -}$ radical.[14] The semiquinone of AZQ is therefore less stable in oxygenated cellular systems than the above simple equilibrium would predict. A similar argument applies to the stability of the hydroquinone of AZQ as the semiquinone and hydroquinone of AZQ are believed to be in equilibrium.[10]

4. *Cell Uptake/Efflux*

The kinetics of the cell uptake for AZQ and BZQ are shown in Figure 7. It can be seen that whereas the concentration of BZQ in the cells and in the medium rapidly comes into equilibrium, AZQ does not achieve equilibrium even after several hours. In a previous study on the relative cytotoxicity of AZQ and BZQ, we demonstrated that there was a lack of correlation between the AZQ semiquinone radical production and the cytotoxicity of AZQ.[7] However in that study, AZQ was incubated with the different cell lines for only one hour. During this period, the AZQ semiquinone radical signal could be observed by ESR but AZQ was not very toxic to the cells. By contrast BZQ, after a one hour incubation, showed considerable toxicity towards the cell lines tested but no ESR signal was observed. More recent studies have shown that by using a constant cell challenge protocol, (i.e., where drug and cells are incubated for extended periods) AZQ and some novel AZQ analogues can be extremely toxic to a variety of cell types.[15] In K562 cells for example, the ID_{50} values change from 5.6 μM for a one hour incubation to 0.1–0.13 μM for a constant challenge protocol using AZQ.

Why is BZQ rapidly taken up into the cells and AZQ not? The present study (Figure 5A) and other work[7,10] clearly show that AZQ is reduced by many different cell lines to its semiquinone radical. However, in order to have been able to measure these signals by standard ESR techniques, the concentration of radicals has to be more than about 1.0 μM. This concentration is much higher than the total concentration of AZQ found in the cells after a few hours (see Figure 7) and yet the ESR signals can be

observed after only a few minutes incubation. It is evident that the majority of the ESR signal must be coming from radicals in the medium. The ESR signal from the medium alone, separated from the cells after incubation with AZQ, is shown in Figure 5B.

The concentration of semiquinone within cells reaches a steady state when the rate of reduction of the quinone by the reducing enzymes equals the rate of decay of the semiquinone by reactions with other cell components, including oxygen, SOD (see above), or dismutation to form an equilibrium with its hydroquinone. It is therefore not possible to accurately quantify the concentration of radicals after rapid removal of the cells. However, Figure 5B clearly demonstrates that the medium does contain a significant amount of the semiquinone radicals. It is possible that by reducing the quinone to a semiquinone or hydroquinone, the cells can more efficiently lower the intracellular concentration of drug by an efflux mechanism. This could partially explain why BZQ, which is not easily reduced, can rapidly come into equilibrium within the cells.

5. *Modified Structures*

There have been hundreds of aziridinylquinones synthesised since the late 1940's but only a minority of these have been screened as antitumour agents. The differences in the properties of AZQ and BZQ highlighted above, clearly show the consequences of simple modifications to the structure of diaziridinylbenzoquinones. Our laboratory is currently synthesising a set of novel diaziridinylquinones in an attempt to investigate their structure/activity relationships. One series of compounds, for example, is based on the AZQ structure (Figure 1, $CH_2CH_3^-$ replaced by CH_3^-; *n*- and *i*-$C_3H_5^-$; *n*-, *i*- and sec-$C_4H_9^-$ and mixtures wherein $R_1 \neq R_2$). The reduction potentials of these compounds vary from -40 to -160 mV and it has been shown that the cytotoxicity in K562 and L1210 cell lines, the intensity of the ESR signals and the DNA cross-linking ability of these compounds, as measured by a fluorescence technique, can be directly correlated with the ease of reduction of the compounds.[15]

One of the main conclusions from these investigations is that there is tremendous scope for subtly modifying the structures of these types of compounds in order to achieve many different types of cytotoxic mechanisms.

It is hoped that future work will include the production of AZQ analogues with specific oligonucleotide-targetted sequences attached, the development of AZQ-analogues which fit the criteria necessary to qualify as hypoxic chemotherapeutic agents and the production of AZQ analogues which will direct the drug to sites within the cell other than DNA, giving further information about the metabolism of these quinones.

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